Apresentação de subsídios ao exame técnico

Número do Processo: BR 11 2019 022972 8

Dados do Interessado

Interessado 1 de 4

Nome ou Razão Social: ASSOCIAÇÃO BRASILEIRA INTERDISCIPLINAR DE AIDS
Tipo de Pessoa: Pessoa Jurídica
CPF/CNPJ: 29263068000145
Nacionalidade: Brasileira
Qualificação Jurídica: Associação com intuito não econômico
Endereço: Avenida Presidente Vargas, 446 - 13° andar
Cidade: Rio de Janeiro
Estado: RJ
CEP: 20071907
País: Brasil
Telefone: (21) 2223-1040
Fax:
Email: carolinne@abiaids.org.br

Interessado 2 de 4

Nome ou Razão Social: FEDERAÇÃO NACIONAL DOS FARMACÊUTICOS - FENAFAR

Tipo de Pessoa: Pessoa Jurídica

CPF/CNPJ: 00679357000148

Nacionalidade: Brasileira

Qualificação Jurídica: Associação com intuito não econômico

Endereço: Rua Barão de Itapetininga, 255, 11º andar, conjunto 1105, Centro

Cidade: São Paulo

Estado: SP

CEP:

País: BRASIL

Telefone:

Fax:

Email:

Interessado 3 de 4

Nome ou Razão Social: FÓRUM ONG AIDS RS - FOARS

Tipo de Pessoa: Pessoa Jurídica

CPF/CNPJ: 07959716000160

Nacionalidade: Brasileira

Qualificação Jurídica: Associação com intuito não econômico

Endereço: Rua dos Andradas, 1560, 6º andar, Centro Histórico

Cidade: Porto Alegre

Estado: RS

CEP:

País: BRASIL

Telefone:

Fax:

Email:

Interessado 4 de 4

Nome ou Razão Social: ASSOCIAÇÃO DE GAYS E AMIGOS DE NOVA IGUAÇU - AGANI

Tipo de Pessoa: Pessoa Jurídica

CPF/CNPJ: 00790968000169

Nacionalidade: Brasileira

Qualificação Jurídica: Associação com intuito não econômico

Endereço: Rua Marcial, 42, Juscelino

Cidade: Mesquita

Estado: RJ

CEP:

País: BRASIL

Telefone:

Fax:

Email:

Referência Petição

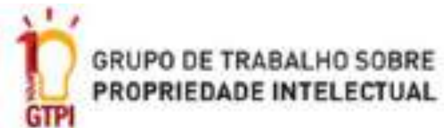
Pedido : BR112019022972-8

Documentos anexados

Tipo Anexo	Nome
Esclarecimento	Subsidio BR112019022972.pdf
Anterioridades D1-D5	Anterioridades D1-D5.pdf
Procuração	Anexos D6-D8.pdf
Procuração	Anexos D9-D11.pdf
Procuração	Anexos D12-D17.pdf

Declaração de veracidade

☑ Declaro, sob as penas da lei, que todas as informações acima prestadas são completas e verdadeiras.



AO ILUSTRÍSSIMO SENHOR DIRETOR DE PATENTES DO INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL

Número do pedido: **BR112019022972-8 - WO2018204368 - PCT US2018030459**
Data de depósito: **01/05/2018**
Prioridade unionista: **US62/500,238 02/05/2017**
Depositante: **MERCK SHARP & DOHME LLC (US)**
Título: **FOMULAÇÕES ESTÁVEIS DE ANTICORPOS DE RECEPTOR DE MORTE PROGRAMADA 1 (PD-1) E MÉTODOS DE USO DAS MESMAS**

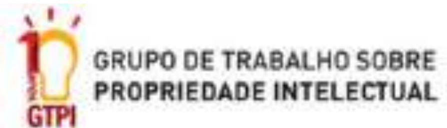
ASSOCIAÇÃO BRASILEIRA INTERDISCIPLINAR DE AIDS - ABIA, pessoa jurídica de direito privado, sem fins lucrativos, inscrita no CNPJ sob o nº 29.263.068/0001-45, com sede à Avenida Presidente Vargas, 446, 13º andar, Centro - Rio de Janeiro - RJ, CEP 20071-907, na pessoa de seu representante legal nos termos de seu Estatuto Social (anexos 6 e 7), por sua advogada (anexo 8);

FEDERAÇÃO NACIONAL DOS FARMACÊUTICOS - FENAFAR, pessoa jurídica de direito privado, sem fins lucrativos, inscrita no CNPJ sob o nº 00.679.357/0001-48, com sede à Rua Barão de Itapetininga, 255, 11º andar, Conjunto 1105, Centro, São Paulo - SP, na pessoa de seu representante legal nos termos de seu Estatuto Social (anexos 9 e 10), por sua advogada (anexo 11);

FÓRUM ONG AIDS RS - FOARS, pessoa jurídica de direito privado, sem fins lucrativos, inscrita no CNPJ sob o nº 07.959.716/0001-60, com sede à Rua dos Andradas, 1560, 6º andar, Centro Histórico, Porto Alegre - RS, na pessoa de seu representante legal nos termos de seu Estatuto Social (anexos 12 e 13), por sua advogada (anexo 14);

ASSOCIAÇÃO DE GAYS E AMIGOS DE NOVA IGUAÇU - AGANI, pessoa jurídica de direito privado, sem fins lucrativos, inscrita no CNPJ sob o nº 00.790.968/0001-69, com sede à Rua Marcial, 42, Juscelino, Mesquita - RJ, na pessoa de seu representante legal nos termos de seu Estatuto Social (anexos 15 e 16), por sua advogada (anexo 17);

vem respeitosamente à presença de Vossa Senhoria, com fulcro no artigo 31 da Lei nº 9.279/1996 - Lei da Propriedade Industrial (LPI), apresentar o presente



SUBSÍDIO AO EXAME TÉCNICO

do pedido de patente **BR112019022972-8**, com base nos fatos e fundamentos a seguir.

1. DA LEGITIMIDADE DAS ORGANIZAÇÕES PROPONENTES, DA MOTIVAÇÃO E DA TEMPESTIVIDADE DO PRESENTE SUBSÍDIO

A concessão de uma patente confere um privilégio temporário de exploração com exclusividade do seu objeto, durante o qual o titular da patente é o único autorizado a explorar a invenção. Essa situação de monopólio legal é excepcional e só deve existir nos casos em que o objeto da patente cumpra todos os requisitos e condições estabelecidos em lei para sua concessão. O objeto do presente pedido de patente, como será detalhado abaixo, é de extrema importância para saúde pública brasileira, uma vez que se trata de uma **formulação de pembrolizumabe**, um anticorpo monoclonal utilizado no tratamento de diversos tipos de câncer.

A legitimidade das organizações que apresentam o presente subsídio ao exame técnico, nos termos do artigo 31 da LPI, verifica-se diante de suas históricas e respeitadas trajetórias na defesa dos direitos humanos, com ênfase para o direito à saúde, acesso a tratamento e assistência farmacêutica de qualidade, além de ativa atuação no campo da implementação de políticas públicas na área de propriedade intelectual, com vistas à primazia do interesse público.

A **Associação Brasileira Interdisciplinar de Aids (Abia)** é uma associação civil, de natureza filantrópica, sem fins lucrativos. A Abia foi fundada em 12 de março de 1987 e é uma das mais antigas ONG dedicadas ao combate da epidemia de HIV no Brasil e à garantia de direitos às pessoas vivendo com HIV, tendo como um dos seus fundadores o sociólogo Herbert de Souza (o "Betinho"), figura de reconhecida importância na vida pública brasileira. A Abia segue como uma das mais conceituadas e reconhecidas entidades sobre a matéria no Brasil e com amplo reconhecimento entre seus pares no âmbito nacional e internacional, e conta, na sua composição, com pesquisadores, profissionais e ativistas de notório saber nessa temática, considerados referências em seus campos de atuação no Brasil. Mais informações em: www.abiaids.org.br.

A Abia coordena o **Grupo de Trabalho sobre Propriedade Intelectual (GTPI) da Rede Brasileira pela Integração dos Povos (Rebrip)**. A Rebrip congrega organizações da sociedade civil brasileira para acompanhar e monitorar os acordos comerciais nos quais o governo brasileiro está envolvido, a fim de avaliar e minimizar potenciais impactos no cotidiano da população e em políticas públicas que visam

assegurar a efetivação dos direitos humanos no Brasil. Mais informações sobre a Rebrip estão disponíveis em www.rebrip.org.br. Um dos temas relevantes no âmbito da discussão sobre comércio e direitos humanos refere-se à propriedade intelectual, motivo pelo qual a Rebrip constituiu um grupo de trabalho para encaminhar as reivindicações da sociedade civil sobre esta questão, fundado em 2003. O GTPI reúne diversas entidades da sociedade civil e busca discutir, acompanhar e incidir no tema da propriedade intelectual e, sobretudo, mitigar o impacto dos efeitos negativos do atual sistema de patente no acesso aos medicamentos essenciais da população brasileira. Mais informações sobre o GTPI/Rebrip podem ser consultadas em www.deolhonaspatentes.org.

A **Federação Nacional dos Farmacêuticos (Fenafar)** é uma entidade representativa da categoria farmacêutica a nível nacional. Fundada em 25 de outubro de 1974, possui hoje 22 sindicatos filiados. A Fenafar teve papel decisivo no processo de debate que culminou na constituição do Sistema Único de Saúde e na construção da Política Nacional de Assistência Farmacêutica. Nesses 49 anos, a Fenafar construiu uma história de lutas na promoção de ações que envolvem o medicamento, desde a sua produção até a orientação correta para o usuário sobre o seu uso racional. Essa luta sempre esteve vinculada à concepção da Saúde como direito de todos para a construção de um país menos desigual, mais soberano e desenvolvido. Mais informações em: www.fenafar.org.br.

O **Fórum Ong Aids RS (Foars)** foi fundado em 1999, reúne 48 organizações gaúchas voltadas à prevenção e conscientização acerca da epidemia de HIV. Com sede em Porto Alegre, atua em diferentes regiões do estado pela melhoria da qualidade de vida e pelos direitos das pessoas soropositivas. Algumas das suas ações são: articular a força das diversas ONGs que integram o Fórum para otimizar os resultados; conscientização acerca da prevenção; incentivo à aceitação dos portadores de si mesmos; desconstrução de tabus e preconceitos sobre a doença; melhora da qualidade de vida para pessoas vivendo com HIV; participar da formulação de políticas públicas inclusivas de prevenção e controle da aids; denunciar todas as formas de omissão, transgressão e violação dos direitos humanos, civis, políticos e sociais das pessoas que vivem com HIV. Mais informações em: https://www.forumongaidsrs.org/.

A **Associação de Gays e Amigos de Nova Iguaçu (Agani)**, conhecida pelo nome fantasia de Associação de Gays e Amigos de Nova Iguaçu, Mesquita e Rio de Janeiro (Aganim) é uma organização não governamental sem fins lucrativos, criada em 17 de dezembro de 1988 no bairro Juscelino, até então município de Nova Iguaçu. Com atuação extensiva a vários municípios da baixada fluminense, o grupo procura manter um equilíbrio entre suas atividades nas áreas de prevenção das DST/Aids e garantir a construção de uma cultura em defesa dos direitos e do respeito às

diversidades, afirmando a heterogeneidade e a pluralidade como valores da nossa sociedade.

O artigo 31 da Lei nº. 9.279/1996 (LPI)[1] estabelece que terceiros interessados podem enviar informações para subsidiar o exame de pedidos de patentes.

> Art. 31. Publicado o pedido de patente e até o final do exame, será facultada a apresentação, pelos interessados, de documentos e informações para subsidiarem o exame. Parágrafo único. O exame não será iniciado antes de decorridos 60 (sessenta) dias da publicação do pedido.

A organização proponente possui amplo interesse e legitimidade para atuar como interessada no pedido de patente **BR112019022972-8**, ora em análise, diante de suas trajetórias e missões sociais apresentadas acima.

A presente petição é motivada pela apresentação do pedido nacional de invenção, protocolado sob n° 870190111452 em 31/10/2019, assim como pelo cumprimento de exigência do pedido apresentado em 29/08/2022 por meio da petição de n° 870220077996.

A presente subsidiante, verificando que o teor do pedido **BR112019022972-8** (doravante mencionado como BR2972) não é passível de patenteabilidade, utiliza-se da permissão dada pelo artigo 31 da LPI e vem perante V. Sªs. apresentar apontamentos como forma de subsidiar o exame técnico do referido pedido de patente.

Ademais, a presente petição é tempestiva nos termos do aludido artigo 31 da LPI e artigo 32 da Instrução Normativa n° 30/2013[2].

2. DO PROCESSO ADMINISTRATIVO

Os principais eventos já ocorridos no processo administrativo do pedido BR2972 estão resumidos abaixo:

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[1] Brasil. Lei nº 9.279, de 14 de maio de 1996. Regula direitos e obrigações relativos à propriedade industrial. Brasília, DF: Presidência da República, 15 maio 1996.

[2] Instituto Nacional da Propriedade Industrial - INPI. Instrução Normativa nº 30, de 4 de dezembro de 2013. Estabelecimento de normas gerais de procedimentos para explicitar e cumprir dispositivos da Lei de Propriedade Industrial - Lei nº 9279, de 14 de maio de 1996, no que se refere às especificações dos pedidos de patente.

1. Em 01/05/2018, o pedido de patente BR2972 foi depositado pela Merck Sharp & Dohme LLC, doravante denominada como MSD ou simplesmente depositante, por meio da entrada em fase nacional do pedido PCT de número PCT US2018030459, cuja data de prioridade mais antiga é 02/05/2017, referente ao pedido de patente dos Estados Unidos de número US62/500,238. O quadro reivindicatório (QR) apresentado na solicitação de entrada na fase nacional, em 31/10/2019, continha 57 reivindicações, sendo cinco independentes: duas de formulação, duas de método de tratamento e uma de uso.
2. Em 22/04/2021, por meio da petição de n° 870210036806, o QR foi modificado, passando a ter 22 reivindicações, sendo quatro independentes: três de formulação e duas de uso. Também consta uma reivindicação genérica de "Invenção de produto, processo, sistema, kit ou uso".
3. O pedido de exame foi peticionado em 26/04/2021, por meio do protocolo n° 800210133871.
4. Em 31/05/2022, por meio da RPI 2682, o INPI publicou uma exigência preliminar para o pedido, que foi respondida tempestivamente 29/08/2022 por meio da petição n° 870220077996. O QR foi novamente modificado, passando a conter 20 reivindicações, sendo seis independentes: três de formulação, duas de uso e uma reivindicação genérica de "Invenção de produto, processo, sistema, kit ou uso".

3. PEMBROLIZUMABE, PEDIDOS DE PATENTE, PREÇO E ACESSO

O pembrolizumabe (MK-3475) é um anticorpo monoclonal humanizado que atua como inibidor da proteína *Programmed Death* 1 (PD-1), uma proteína de superfície celular que atua como um ponto de verificação imunossupressor. Ele age bloqueando a interação do receptor PD-1 com seus ligantes PD-L1 e PD-L2, o que permite a reativação da resposta imune antitumoral por linfócitos T.

O Keytruda®, nome do produto comercial contendo solução injetável de pembrolizumabe 100 mg/4 mL (25 mg/mL), adicionado dos excipientes histidina, cloridrato de histidina monoidratado, sacarose, polissorbato 80 e água para injetáveis, possui registro sanitário ativo na Anvisa pela farmacêutica Merck Sharp & Dohme (MSD)[3]. De acordo com a bula do medicamento, o pembrolizumabe é indicado para o tratamento de uma ampla gama de tumores sólidos, como melanoma, câncer de

[3] Disponível em: https://consultas.anvisa.gov.br/#/medicamentos/2737640?substancia=25930&substanciaDescricao=pembrolizumabe

pulmão de células não pequenas, carcinoma urotelial, câncer gástrico, carcinoma de células renais, câncer de cabeça e pescoço, câncer esofágico, câncer endometrial, câncer de mama triplo-negativo, câncer cervical e carcinoma cutâneo de células escamosas. Ele também é utilizado em linfoma de Hodgkin e neoplasias com alta carga mutacional ou deficiência no reparo do DNA, independentemente do local primário do tumor.

A posologia do pembrolizumabe e seu regime terapêutico (monoterapia ou combinação) variam conforme a indicação. De forma geral, para adultos, a dose recomendada é 200 mg administrados por via intravenosa em infusão de 30 minutos a cada 3 semanas (Q3W), ou 400 mg a cada 6 semanas (Q6W) também por via intravenosa.

O preço Máximo de Venda ao Governo (PMVG), estabelecido pela Câmara de Regulação do Mercado de Medicamentos (Cmed) em 11/03/2025, varia de R$ 12.785,30 a 17.233,78 (ICMS 0% a 23%)[4]. O preço de comercialização do Keytruda® (pembrolizumabe) 100 mg/4 mL no Brasil varia conforme a farmácia e as condições de pagamento. Com base em pesquisa realizada em maio de 2025, os valores encontrados variam de R$ 19.700,00 a 28.800,00[5].

Em 2020, a Comissão Nacional de Incorporação de Tecnologias no SUS (Conitec) recomendou a incorporação do pembrolizumabe para o tratamento de melanoma avançado não cirúrgico e metastático, condicionando a decisão à negociação de preços com a fabricante[6]. Durante o processo de avaliação, a Conitec considerou que um valor razoável para a incorporação seria de aproximadamente R$ 12 mil por paciente por mês (equivalente a 3 PIB per capita) e o valor efetivamente estabelecido para reembolso via Autorização de Procedimentos de Alta Complexidade (APAC) é inferior, fixado em R$ 7.500 mensais por paciente[7] - já que o medicamento não é de compra centralizada pelo Ministério da Saúde. Esse montante é significativamente menor do que o custo real do tratamento mensal de um paciente,

[4] Anvisa. Câmara de Regulação do Mercado de Medicamentos (Cmed). Preços máximos de medicamentos por princípio ativo, para compras públicas. Disponível em: file:///C:/Users/carol/Downloads/pdf_conformidade_gov_20250311_173150789.pdf

[5] Consulta realizada em: https://www.oncologmedicamentos.com.br/keytruda-100-mg-4-ml-sol-inj-ct-fa-4-ml/p e https://www.oncomg.com.br/keytruda-100mgml-pembrolizumabe-frasco-4ml

[6] Ministério da Saúde. Terapia-alvo (vemurafenibe, dabrafenibe, cobimetinibe, trametinibe) e imunoterapia (ipilimumabe, nivolumabe, pembrolizumabe) para o tratamento de primeira linha do melanoma avançado não-cirúrgico e metastático. Relatório de recomendação n° 541, 2020. Disponível em: https://www.gov.br/conitec/pt-br/midias/relatorios/2020/relatorio_541_terapiaalvo_melanoma_final_2020.pdf

[7] Ministério da Saúde. Portaria GM/MS nº 638, de 28 de março de 2022. Altera atributos de procedimentos e exclui procedimento da Tabela de Procedimentos, Medicamentos, Órteses, Próteses e Materiais Especiais do Sistema Único de Saúde - SUS. Disponível em: https://bvsms.saude.gov.br/bvs/saudelegis/gm/2022/prt0638_29_03_2022.html

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estimado em cerca de R$ 22.727,00 por mês[8], o que tem dificultado sua ampla disponibilização no SUS.

Para outras indicações, como câncer de células renais[9], câncer de pulmão de células não pequenas[10], câncer de cólon e reto metastático com alta instabilidade de microssatélites[11] e carcinoma espinocelular de cabeça e pescoço recidivado ou metastático[12], o pembrolizumabe não foi incorporado ao SUS. Essas decisões foram baseadas em análises da Conitec, que consideraram que as razões de custo-efetividade e impacto orçamentário foram desfavoráveis ao SUS.

É importante destacar que, mesmo para o melanoma, onde o pembrolizumabe foi incorporado no SUS, houve relatos de atrasos na disponibilização efetiva do medicamento aos pacientes. Segundo informações da CNN Brasil[13], apesar da incorporação em 2020, pacientes enfrentaram dificuldades no acesso ao tratamento devido a entraves administrativos e logísticos ocasionados pelos altos preços do medicamento.

Uma busca simples por jurisprudência revela o elevado número de ações judiciais envolvendo o pembrolizumabe (Keytruda®), evidenciando o grau de judicialização desse medicamento no país. O volume de processos é um indicativo da incapacidade do sistema público de garantir acesso regular a essa tecnologia, levando pacientes a recorrerem ao Judiciário como última alternativa.

O caso do pembrolizumabe é emblemático para compreender como a combinação entre preços abusivos e a ausência de incorporação ampla no SUS transforma o acesso ao tratamento em uma disputa judicial constante, que

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[8] Ministério da Saúde. Regimes de tratamento com cetuximabe ou pembrolizumabe para carcinoma espinocelular de cabeça e pescoço recidivado ou metastático. Relatório de recomendação n° 919, 2024. Disponível em: https://www.gov.br/conitec/pt-br/midias/relatorios/2024/relatorio-de-recomendacao-no-919-regimes-de-tratamento-com-cetuximabe-ou-pembrolizumabe

[9] Ministério da Saúde. Pembrolizumabe, axitinibe, ipilimumabe e nivolumabe para tratamento de primeira linha de câncer de células renais. Relatório de recomendação n° 660, 2021. Disponível em: https://www.gov.br/conitec/pt-br/midias/relatorios/2021/20210830_relatorio_660_pembrolizumabe_axitinibe_ipilimumabe_nivolumabe_ccr_1_linha_final.pdf

[10] Ministério da Saúde. Pembrolizumabe (em monoterapia ou associado à quimioterapia) para pacientes com câncer de pulmão de células não pequenas avançado ou metastático (PD-L1 positivo) em primeira linha de tratamento. Relatório de recomendação n° 859, 2023. Disponível em: https://www.gov.br/conitec/pt-br/midias/relatorios/2023/20231227_pembrolizumabe_859_cancer_pulmao_cnp.pdf

[11] Ministério da Saúde. Pembrolizumabe em primeira linha de tratamento em indivíduos com câncer de cólon ou reto metastático. Relatório de recomendação n° 863, 2023. Disponível em: https://www.gov.br/conitec/pt-br/midias/relatorios/2023/20231229_relatorio_863_pembrolizumabe_cancer_colon_reto.pdf

[12] Ministério da Saúde. Regimes de tratamento com cetuximabe ou pembrolizumabe para carcinoma espinocelular de cabeça e pescoço recidivado ou metastático. Relatório de recomendação n° 919, 2024. Disponível em: https://www.gov.br/conitec/pt-br/midias/relatorios/2024/relatorio-de-recomendacao-no-919-regimes-de-tratamento-com-cetuximabe-ou-pembrolizumabe

[13] Disponível em: https://www.cnnbrasil.com.br/saude/na-lista-do-sus-desde-novembro-de-2020-remedios-contra-cancer-ainda-nao-sao-fornecidos/

sobrecarrega o sistema de justiça e pressiona os orçamentos públicos. Embora o medicamento tenha sido incorporado ao SUS para uso restrito, inúmeros pacientes com outros tipos de cânceres seguem recorrendo à Justiça para obter o tratamento.

Com um custo que ultrapassa R$ 17 mil por frasco, o pembrolizumabe impõe barreiras significativas ao acesso e representa um desafio orçamentário grave para estados e para a União. A frequência das decisões judiciais revela um paradoxo: de um lado, o Judiciário atua para proteger o direito à vida e à saúde frente à omissão do poder público; de outro, o crescente número de ações envolvendo medicamentos de alto custo como o pembrolizumabe contribui para o descontrole orçamentário e para a ampliação das desigualdades no acesso, beneficiando quem tem recursos - jurídicos, financeiros ou de conhecimento - para acionar a Justiça, em detrimento de outras pessoas com risco de vida.

A situação de monopólio de comercialização, viabilizada pela proteção da propriedade intelectual, é um dos fatores que impactam nos altos preços e restrição do acesso. Em pesquisa realizada, em bases de dados privadas e públicas, e análise do conteúdo dos pedidos de patente identificados, foi possível identificar 32 pedidos de patentes relacionados ao pembrolizumabe (Quadro 1), dois dos quais foram concedidos (PI0812913-4 e BR112017003108-6). A sobreposição de pedidos de patente (*evergreening*) gera expectativa de direito para os objetos reivindicados. Caso todos os pedidos sejam concedidos, o pembrolizumabe pode ficar sob monopólio patentário pelo menos até o ano 2042.

Quadro 1: Pedidos de patente relacionados ao pembrolizumabe no Brasil.

PEDIDO DE PATENTE	TÍTULO	DEPOSITANTE
PI0812913-4 WO2008156712 PCT US2008007463	Anticorpos monoclonais ou fragmento de anticorpo para o receptor de morte programada humano PD-1, polinucleotideo, método para produzir os referidos anticorpos ou fragmentos de anticorpos, composição que os compreende e uso dos mesmos	Merck Sharp & Dohme B.V. (NL)
BR112013022832-6 WO2012120500 PCT IL2012000109	Linhagens de células de baixo teor de fucose e usos das mesmas	Merck Serono S.A. (CH)
BR112016017256-6 WO2015119930 PCT US2015014212	Combinação de um antagonista de pd-1 e um inibidor de vegfr para o tratamento de câncer	Pfizer Inc. (US) / Merck Sharp & Dohme LLC (US)
BR112016017700-2 WO2015119944	Usos de um antagonista de uma proteína de morte programada 1 e de um inibidor de ido1, e, kit	Incyte Corporation (US) / Merck Sharp & Dohme Corp. (US)

PCT US2015014247		
BR112017003108-6 WO2016028656 PCT US2015045447	Anticorpo ou fragmento de ligação a antígeno do mesmo, molécula de ácido nucleico isolado, vetor de expressão,microorganismo transgênico, composição, e, método para produção de um anticorpo ou fragmento de ligação a antígeno	Merck Sharp & Dohme LLC (US)
BR112017016434-5 WO2016137850 PCT US2016018843	Cristal de um anticorpo monoclonal anti-pd-1, métodos para produzir cristais de um anticorpo monoclonal anti-pd-1 e para cristalizar um anticorpo monoclonal anti-pd-1, bem como e composição farmacêutica e seu uso	Merck Sharp & Dohme LLC (US)
BR112019022488-2 WO2018201028 PCT US2018029915	Biomarcadores para terapêutica do câncer	Merck Sharp & Dohme Corp. (US) / Amgen Inc. (US)
BR112019022695-8 WO2018204343 PCT US2018030420	Formulações estáveis de anticorpos anti-ctla4 isolados e em combinação com anticorpos do receptor de morte programada 1 (pd-1) e métodos de uso das mesmas	Merck Sharp & Dohme LLC (US)
BR112019022972-8 WO2018204368 PCT US2018030459	Fomulações estáveis de anticorpos de receptor de morte programada 1 (pd-1) e métodos de uso das mesmas	Merck Sharp & Dohme LLC (US)
BR112019023591-4 WO2018208968 PCT US2018031876	Terapias de combinação para tratar câncer	Tesaro, Inc. (US) / Merck Sharp & Dohme B.V. (NL)
BR112019023909-0 WO2018213260 PCT US2018032692	Uso de um imunoconjugado que se liga a folr1, e, uso de um imunoconjugado que se liga a folr1 e pembrolizumab	Immunogen, Inc. (US) / Merck Sharp & Dohme LLC (US)
BR112020007203-6 WO2019075366 PCT US2018055667	Composições e métodos para tratamento de linfoma difuso de células B grandes	Amgen Inc. (US) / Merck Sharp & Dohme LLC (US)
BR112020007494-2 WO2019084418 PCT US2018057731	Composições e métodos para tratamento do câncer de fígado	Merck Sharp & Dohme Corp. (US) / Amgen Inc. (US)
BR112020009759-4 WO2019099597 PCT US2018061165	Anticorpos específicos para transcrito 3 semelhante à imunoglobulina (ILT3) e usos dos mesmos	Merck Sharp & Dohme LLC (US)
BR112020016331-7	Métodos para tratar câncer com anticorpos anti-pd-1	Merck Sharp & Dohme LLC (US)

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WO2019160751 PCT IB2018060181		
BR112020015915-8 WO2019160755 PCT US2019017188	Usos de um anticorpo anti-pd-1 e um anticorpo anti-ctla4 ou fragmentos de ligação ao antígeno dos mesmos, bem como kit para tratamento de um paciente com câncer	Merck Sharp & Dohme LLC (US)
BR112020021134-6 WO2019201894 PCT EP2019059755	Aditivos para formulações de proteínas para aprimorar estabilidade térmica	Merck Patent GMBH (DE)
BR112020022642-4 WO2019217455 PCT US2019031166	Método para tratar câncer em um indivíduo, e, estojo	MSD International GMBH (CH) / Genmab A/S (DK)
BR112021007318-3 WO2020079692 PCT IL2019051129	Tratamento de adenocarcinoma pancreático metastático	Biolinerx Ltd. (IL) / Merck Sharp & Dohme Corp. (US)
BR112021008122-4 WO2020092233 PCT US2019058339	Cristais de anticorpo anti-pd-1 humano e métodos de uso dos mesmos	Merck Sharp & Dohme LLC (US)
BR112021008582-3 WO2020096915 PCT US2019058339	Cristais de anticorpo anti-pd-1 humano e métodos de uso dos mesmos	Merck Sharp & Dohme LLC (US)
BR112021008679-0 WO2020096917 PCT US2019059583	Métodos para tratar câncer, e composição farmacêutica	Merck Sharp & Dohme LLC (US)
BR112021008873-3 WO2020097139 PCT US2019059954	Formulação	Merck Sharp & Dohme LLC (US)
BR112021017892-9 WO2020185722 PCT US2020021783	Terapias de combinação anti-cancer compreendendo agentes bloqueadores de CTLA-4 e PD-1	Merck Sharp & Dohme LLC (US)
BR112022007971-0 WO2021086909 PCT US2020057650	Combinação de antagonista do PD-1, inibidor da tirosina quinase VEGFR/FGFR/RET e inibidor da CBP/Beta-Catenina para o tratamento do câncer	Eisai R&D Management Co., Ltd. (JP) / MSD International GmbH (CH) / Merck Sharp & Dohme LLC (US)
BR112022008233-9	Regime de dosagem de anticorpos anti-CD27 para tratamento de câncer	Merck Sharp & Dohme LLC (US)

WO2021087016 PCT US2020057817		
BR112022012081-8 WO2021126906 PCT US2020065208	Método para tratar câncer, kit, e, uso de uma combinação terapêutica	Merck Sharp & Dohme LLC (US)
BR112022022304-8 WO2021225851 PCT US2021029777	Métodos para tratar câncer usando uma combinação de um antagonista PD-1, uma quimiorradioterapia e um inibidor de PARP	Merck Sharp & Dohme LLC (US)
BR112022026086-5 WO2021262562 PCT US2021038171	Métodos para tratar câncer ou doença de Von-Hippel Lindau e para tratar carcinoma de célula renal, kit, e, uso de uma combinação terapêutica	Merck Sharp & Dohme LLC (US) / Eisai R&D Management Co., Ltd. (LP)
BR112023004781-1 WO2022060678 PCT US2021050143	Terapia de combinação de um antagonista de pd-1, antagonista de lag3 e lenvatinibe, ou um sal farmaceuticamente aceitável do mesmo, para tratamento de pacientes com câncer	Merck Sharp & Dohme LLC (US) / Eisai R&D Management Co., Ltd. (JP)
BR112023004981-4 WO2022060767 PCT US2021050365	Regimes de dosagem do anticorpo anti-ILT4 ou sua combinação com anticorpo anti-PD-1 para o tratamento de câncer	Merck Sharp & Dohme LLC (US)
BR112023020867-0 WO2022216580 PCT US2022023250	Métodos para tratamento de câncer com administração subcutânea de anticorpos anti-PD1	Merck Sharp & Dohme LLC (US)

O pembrolizumabe é um produto estratégico para o Complexo Econômico-Industrial da Saúde (Ceis), estando listado desde 2017[14] na lista de produtos estratégicos para o SUS[15], elegível para projetos de Parcerias para o Desenvolvimento Produtivo (PDP). Na rodada de PDP de 2017/2018, foi submetida uma proposta de projeto de PDP, que não foi aprovada, por não ser um produto de aquisição centralizada[16] - um dos critérios utilizados para aprovação ou continuidade

[14] Brasil. Ministério da Saúde. Portaria nº 252, de 26 de janeiro de 2017. Define a lista de produtos estratégicos para o Sistema Único de Saúde (SUS), nos termos dos anexos a esta Portaria. Diário Oficial da União. 30 Jan 2017. Disponível em: https://pesquisa.in.gov.br/imprensa/jsp/visualiza/index.jsp?data=30/01/2017&jornal=1&pagina=77&totalArquivos=192
[15] Posteriormente chamada de matriz de desafios produtivos e tecnológicos em saúde.
[16] Disponível em: https://www.gov.br/saude/pt-br/composicao/sectics/pdp/medicamentos-vacinas-e-hemoderivados/medicamento-vacina-e-hemoderivados-propostas-de-projeto-de-pdp-nao-aprovadas-de-2017-2018/view

desse arranjo de transferência de tecnologia. Todavia, o produto continuou listado na matriz de desafios produtivos e tecnológicos em saúde, publicada em 2023[17].

4. DA MATÉRIA REIVINDICADA NO PEDIDO BR112019022972-8

O QR em exame apresenta as 20 reivindicações, transcritas a seguir para pronta referência:

1. Formulação de anticorpo anti-PD-1 humano, **caracterizada** pelo fato de que compreende:
   a) 100 mg/mL a 200 mg/mL de um anticorpo anti-PD-1 humano ou fragmento de ligação ao antígeno do mesmo;
   b) 5 mM a 20 mM de tampão de histidina;
   c) um estabilizador selecionado a partir do grupo que consiste em:
      i) 6% a 8% peso/volume (p/v) de sacarose, trealose ou (2-hidroxipropil)-β-ciclodextrina;
      ii) 3% a 5% p/v de manitol, sorbitol, L-arginina, um sal farmacêuticamente aceitável de L-arginina, L-prolina ou um sal farmaceuticamente aceitável de L-prolina;
      e iii) 1,8% a 2,2% p/v de glicina ou um sal farmaceuticamente aceitável da mesma;
   d) 0,01% a 0,10% de tensoativo não iônico; e
   e) 1 mM a 20 mM de antioxidante,

em que o anticorpo anti-PD-1 humano ou fragmento de ligação ao antígeno do mesmo compreende três CDRs de cadeia leve compreendendo CDRL1 de SEQ ID NO: 1, CDRL2 de SEQ ID NO: 2 e CDRL3 de SEQ ID NO: 3 e três CDRs de cadeia pesada de CDRH1 de SEQ ID NO: 6, CDRH2 de SEQ ID NO: 7 e CDRH3 de SEQ ID NO: 8.

2. Formulação de anticorpo anti-PD-1 humano de acordo com a reivindicação 1, **caracterizada** pelo fato de que a formulação tem um pH entre 5,0 e 6,0.

3. Formulação de anticorpo anti-PD-1 humano de acordo com a reivindicação 1 ou 2, **caracterizada** pelo fato de que o estabilizador é selecionado a partir do grupo que consiste em:
   i) 6% a 8% p/v de sacarose, trealose ou (2-hidroxipropil)-β-ciclodextrina;
   ii) 3% a 5% de manitol, sorbitol ou L-prolina ou um sal farmaceuticamente aceitável de L-prolina;
   e iii) 1,8% a 2,2% p/v de glicina ou um sal farmaceuticamente aceitável da mesma.

4. Formulação de anticorpo anti-PD-1 humano de acordo com qualquer uma das reivindicações 1 a 3, caracterizada pelo fato de que o tampão é um tampão de histidina, o estabilizador é sacarose, o tensoativo não iônico é polissorbato 80 e o antioxidante é L-metionina ou um sal farmaceuticamente aceitável da mesma, em que a formulação compreende:
   a) 100 mg/mL a 200 mg/mL de um anticorpo anti-PD-1 humano ou fragmento de ligação ao antígeno do mesmo;
   b) 5 mM a 20 mM de tampão de histidina;
   c) 6% a 8% p/v de sacarose;
   d) 0,01% a 0,04% p/v de polissorbato 80; e
   e) 1 mM a 20 mM de L-metionina ou um sal farmaceuticamente aceitável da mesma.

5. Formulação de anticorpo anti-PD-1 humano de acordo com a reivindicação 1, caracterizada pelo fato de que o tampão é um tampão de histidina, o estabilizador é sacarose, o tensoativo não iônico

[17] Brasil. Ministério da Saúde. Portaria nº 2.261, de 08 de dezembro de 2023. Estabelece a Matriz de Desafios Produtivos e Tecnológicos em Saúde. Diário Oficial da União. 08 Dez 2023. Disponível em: https://bvsms.saude.gov.br/bvs/saudelegis/gm/2023/prt2261_08_12_2023.html

é polissorbato 80 e o antioxidante é L-metionina ou um sal farmaceuticamente aceitável da mesma, em que a formulação compreende:

a) 100 mg/mL a 200 mg/mL de um anticorpo anti-PD-1 humano ou fragmento de ligação ao antígeno do mesmo;
b) 8 mM a 12 mM de tampão de histidina;
c) 5 mM a 10 mM de L-metionina ou um sal farmaceuticamente aceitável da mesma;
d) 6% a 8% p/v de sacarose; e
e) 0,01% a 0,04% p/v de polissorbato 80.

6. Formulação de anticorpo anti-PD-1 humano de acordo com a reivindicação 5, caracterizada pelo fato de que compreende:

a) 125 mg/mL a 200 mg/mL de um anticorpo anti-PD-1 humano ou fragmento de ligação ao antígeno do mesmo;
b) 10 mM de tampão de histidina;
c) 10 mM de L-metionina ou um sal farmaceuticamente aceitável da mesma;
d) 7% p/v de sacarose; e
e) 0,02% p/v de polissorbato 80.

7. Formulação de anticorpo anti-PD-1 humano de acordo com a reivindicação 6, caracterizada pelo fato de que o antioxidante é HCl de Lmetionina.

8. Formulação de anticorpo anti-PD-1 humano de acordo com qualquer uma das reivindicações 5 a 7, caracterizada pelo fato de que compreende adicionalmente de 1,25% a 2,5% p/v de L-arginina ou um sal farmacêuticamente aceitável da mesma.

9. Formulação de anticorpo anti-PD-1 humano de acordo com qualquer uma das reivindicações 1 a 8, caracterizada pelo fato de ser uma solução reconstituída a partir de uma formulação liofilizada.

10. Formulação de anticorpo anti-PD-1 humano de acordo com qualquer uma das reivindicações 1 a 9, caracterizada pelo fato de que o anticorpo anti-PD-1 humano ou fragmento de ligação ao antígeno do mesmo compreende uma região VL que compreende a sequência de aminoácidos estabelecida na SEQ ID NO: 4 e uma região VH que compreende a sequência de aminoácidos estabelecida na SEQ ID NO: 9.

11. Formulação de anticorpo anti-PD-1 humano de acordo com qualquer uma das reivindicações 1 a 10, caracterizada pelo fato de que a formulação compreende uma cadeia leve compreendendo ou consistindo em uma sequência de aminoácidos conforme estabelecida na SEQ ID NO: 5 e uma cadeia pesada compreendendo ou consistindo em uma sequência de aminoácidos conforme estabelecida na SEQ ID NO: 10.

12. Formulação de anticorpo anti-PD-1 humano de acordo com qualquer uma das reivindicações 1 a 11, caracterizada pelo fato de que a formulação compreende um anticorpo anti-PD-1 humano que é pembrolizumabe.

13. Formulação de anticorpo anti-PD-1 humano, caracterizada pelo fato de que compreende:

a) 130 mg/mL de um anticorpo anti-PD-1 humano;
b) 10 mM de tampão de histidina;
c) 7% p/v de sacarose;
d) 0,02% de polissorbato 80; e
e) 10 mM de L-metionina ou um sal farmaceuticamente aceitável da mesma;

em que o anticorpo anti-PD-1 humano compreende uma cadeia leve compreendendo uma sequência de aminoácidos conforme estabelecida na SEQ ID NO: 5 e uma cadeia pesada compreendendo uma sequência de aminoácidos conforme estabelecida na SEQ ID NO: 10.

14. Formulação de anticorpo anti-PD-1 humano de acordo com a reivindicação 13, caracterizada pelo fato de que o anticorpo anti-PD-1 humano é pembrolizumabe.

15. Formulação de anticorpo anti-PD-1 humano, caracterizada pelo fato de que compreende:

a) 165 mg/mL de um anticorpo anti-PD-1 humano;

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b) 10 mM de tampão de histidina;
c) 7% p/v de sacarose;
d) 0,02% de polissorbato 80; e
e) 10 mM de L-metionina ou um sal farmaceuticamente aceitável da mesma;
em que o anticorpo anti-PD-1 humano compreende uma cadeia leve compreendendo ou consistindo em uma sequência de aminoácidos conforme estabelecida na SEQ ID NO: 5 e uma cadeia pesada compreendendo ou consistindo em uma sequência de aminoácidos conforme estabelecida na SEQ ID NO: 10.

16. Formulação de anticorpo anti-PD-1 humano de acordo com a reivindicação 15, caracterizada pelo fato de que o anticorpo anti-PD-1 humano é pembrolizumabe.

17. Uso da formulação de anticorpo anti-PD-1 humano definida em qualquer uma das reivindicações 1 a 16, caracterizado pelo fato de ser para a preparação de um medicamento para tratar infecção crônica em um paciente humano.

18. Uso da formulação de anticorpo anti-PD-1 humano definida em qualquer uma das reivindicações 1 a 16, caracterizado pelo fato de ser para a preparação de um medicamento para tratar câncer em um paciente humano.

19. Uso da formulação de anticorpo anti-PD-1 humano de acordo com a reivindicação 17 ou 18, caracterizado pelo fato de que a formulação é para administração subcutânea.

20. Invenção de produto, processo, sistema, kit ou uso, caracterizada pelo fato de que compreende um ou mais elementos descritos no presente pedido de patente.

O Quadro 2 apresenta um resumo dos tipos de reivindicações do pedido de patente BR2972.

Quadro 2: Resumo dos tipos de reivindicações do pedido de patente BR2972.

TIPO DE PROTEÇÃO	REIVINDICAÇÕES	DETALHES
Formulação	1 a 16	Formulação de anticorpo anti-PD-1 humano, tampão de histidina, um estabilizador, tensoativo não iônico e antioxidante.
Uso	17 a 19	Uso da formulação para fabricação de medicamento.
Invenção	20	“produto, processo, sistema, kit ou uso, caracterizada pelo fato de que compreende um ou mais elementos descritos no presente pedido de patente.”

5. DA FALTA DE CLAREZA E PRECISÃO

O artigo 24 da Lei da Propriedade Industrial (LPI) dispõe:

> Art. 24. O relatório deverá descrever clara e suficientemente o objeto, de modo a possibilitar sua realização por técnico no assunto e indicar, quando for o caso, a melhor forma de execução.

As formulações do pedido BR2972 contêm **um** anticorpo anti-PD-1 humano **ou fragmento de ligação ao antígeno** do mesmo.

A partir do parágrafo [0118] do relatório descritivo (RD) do pedido BR2972 (página 32) são descritas inúmeras sequências de regiões determinantes de complementaridade (CDR) que podem compor anticorpos anti-PD-1 humanos sem, no entanto, descrever com clareza anticorpos ou quaisquer fragmentos. Apenas a partir do parágrafo [0130] (RD, página 34) são identificados anticorpos por suas cadeias leves e pesadas, as quais incluem porções constantes (Fc), porções variáveis (Fab) e regiões determinantes de complementaridade (CDR). Contudo, nenhum fragmento foi descrito.

Apesar da descrição de anticorpos apenas por porções, como as CDR, ser uma das formas de definir anticorpos aceitas pelo INPI (Instrução Normativa n° 118/2020[18], parágrafo 165), tal reivindicação não define de forma clara e precisa a matéria objeto de proteção do presente pedido de patente, que reivindica uma formulação. Acontece que, conforme explicação da própria depositante, na **formulação de produtos contendo proteínas** existem questões estruturais intrínsecas de cada uma delas que demandam individualização das estratégias utilizadas para compor as formulações:

> "A identificação de **fatores intrínsecos e extrínsecos que contribuem para a estabilização de proteínas termofílicas** fornece informações valiosas para estabilizar produtos farmacêuticos proteicos e para projetar proteínas mutantes mais estáveis. No entanto, **as diferenças estruturais entre as diferentes proteínas são tão significativas que a generalização das estratégias de estabilização universal não foi bem-sucedida**. Muitas vezes, **as proteínas precisam ser avaliadas individualmente**. (Petição 870220077996, de 29/08/2022, pág. 11/259) (grifo nosso)

> "não há um caminho único a seguir no desenvolvimento de formulações comerciais e **as proteínas têm que ser avaliadas caso a caso**" (Petição 870220077996, de 29/08/2022, pág. 11/259) (grifo nosso)

Dessa forma, o técnico no assunto não teria como prever, diante de todas as alternativas do relatório descritivo, qual a anticorpo ou qual o fragmento é de fato objeto da composição do pedido de patente que foi, conforme afirmações da própria depositante, obtida após avaliação específica para um anticorpo.

Portanto, o pedido BR2972 **não atende às disposições do artigo 24 da LPI por não descrever clara e suficientemente o objeto, de modo a possibilitar sua realização por técnico no assunto**.

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[18] Instituto Nacional da Propriedade Industrial - INPI. Instrução Normativa nº 118, de 12 de novembro de 2020. Institui a nova versão das Diretrizes de Exame de Pedidos de Patente na Área de Biotecnologia.

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Complementarmente, o artigo 25 da LPI dispõe:

> Art. 25. As reivindicações deverão ser fundamentadas no relatório descritivo, caracterizando as particularidades do pedido e definindo, de modo claro e preciso, a matéria objeto da proteção.

As reivindicações do pedido BR2972 falham em definir de modo claro e preciso, a matéria objeto da proteção.

As reivindicações 1 a 9, referentes a formulações do pedido BR2972, compreendem **um** anticorpo anti-PD-1 humano ou fragmento de ligação ao antígeno do mesmo, o qual é definido como aquele que "*compreende três CDRs de cadeia leve compreendendo CDRL1 de SEQ ID NO: 1, CDRL2 de SEQ ID NO: 2 e CDRL3 de SEQ ID NO: 3 e três CDRs de cadeia pesada de CDRH1 de SEQ ID NO: 6, CDRH2 de SEQ ID NO: 7 e CDRH3 de SEQ ID NO: 8*."

Conforme afirmações da própria depositante a composição pleiteada foi obtida após avaliação **específica para um <u>único</u> anticorpo**. No entanto, nas reivindicações 1 a 9, o anticorpo é descrito apenas pela sequência dos seus CDR e, na reivindicação 10, o anticorpo é descrito apenas pela sequência da região variável, restando inúmeras possibilidades de sequências da porção constante (Fc) que poderiam vir a compor o anticorpo completo. Desta forma, fica prejudicada a devida clareza e precisão da matéria pleiteada. Adicionalmente, as reivindicações 10 a 16 definem ainda outros dois anticorpos (definidos pelas partes do anticorpo), tornando o quadro reivindicatório ainda mais impreciso.

Ainda, a reivindicação 20, nada define e sequer pode ser diferenciado se trata-se de uma reivindicação de produto ou processo.

Portanto, as reivindicações 1 a 16 e 20 do pedido BR2972 **não atendem às disposições do artigo 25 da LPI por falharem em definir de modo claro e preciso, a matéria objeto da proteção.**

6. DA PATENTEABILIDADE DO PEDIDO BR112019022972-8

6.1 Do estado da técnica do pedido BR112019022972-8

No presente subsídio ao exame técnico, são citados os documentos do estado da técnica descritos, todos publicados antes da data de prioridade mais antiga do pedido BR2972-8 (Quadro 3).

Quadro 3: Documentos do estado da técnica utilizados neste subsídio.

DENOMINAÇÃO	REFERÊNCIA	DATA DE PUBLICAÇÃO
D1	US2014234296. Stable formulations of antibodies to human programmed death receptor PD-1 and related treatments.	21/08/2014
D2	Manning MC, Chou DK, Murphy BM, Payne RW, Katayama DS. Stability of protein pharmaceuticals: an update. Pharm Res, 27(4):544-75, 2010.	09/02/2010
D3	Lam XM, Yang JY, Cleland JL. Antioxidants for prevention of methionine oxidation in recombinant monoclonal antibody HER2. J Pharm Sci, 86(11):1250-5, 1997.	1997
D4	Ji JA, Zhang B, Cheng W, Wang YJ. Methionine, tryptophan, and histidine oxidation in a model protein, PTH: mechanisms and stabilization. J Pharm Sci,98(12):4485-500, 2009.	2009
D5	Hada S, Kim NA, Lim DG, Lim JY, Kim KH, Adhikary P, Jeong SH. Evaluation of antioxidants in protein formulation against oxidative stress using various biophysical methods. Int J Biol Macromol, 82:192-200, 2016.	21/10/2015

D1 descreve formulações estáveis de anticorpos que inibem o receptor de morte programada PD-1 para uso no tratamento de câncer ou infecções**.**

D2 é um artigo científico que descreve as principais causas de instabilidade em proteínas e as estratégias de formulação que podem aumentar a estabilidade, dentre as quais destaca-se a L-metionina como antioxidante.

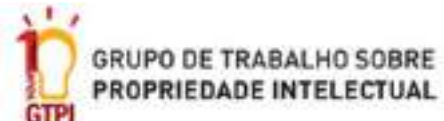
D3 é um artigo científico que avaliou o uso de antioxidantes, dentre os quais destaca-se a L-metionina livre, para estabilizar anticorpos monoclonais que sofreram oxidação de resíduos de metionina quando expostos à luz.

D4 é um artigo científico que relata a investigação do processo de oxidação de resíduos de metionina, triptofano e histidina em anticorpos monoclonais e a procura por estabilizadores adequados, dentre os quais destaca-se a L-metionina como antioxidante.

D5 é um artigo científico que avalia a estabilidade de proteínas submetidas a estresse oxidativo e avalia o uso de antioxidantes para controlar o processo, dentre os quais destaca-se a L-metionina livre.

6.2 Da falta de atividade inventiva (reivindicações 1 a 16)

As reivindicações 1 a 16 referem-se a uma formulação contendo um anticorpo anti-PD-1 humano, um tampão de histidina, um estabilizador, um tensoativo não iônico e um antioxidante.



Para a avaliação da atividade inventiva, emprega-se as etapas indicadas nas Diretrizes de Exame de Pedidos de Patente – Bloco II – Patenteabilidade, instituídas pela Resolução n° 169/2016:

> 5.9 Três etapas são empregadas para determinar se uma invenção reivindicada é óbvia quando em comparação com o estado da técnica:
> (i) determinar o estado da técnica mais próximo;
> (ii) determinar as características distintivas da invenção e/ou o problema técnico de fato solucionado pela invenção; e
> (iii) determinar se, diante do problema técnico considerado, e partindo-se do estado da técnica mais próximo, a invenção é ou não óbvia para um técnico no assunto.

O estado da técnica mais próximo do pedido BR2972 é **D1**, um documento que descreve formulações estáveis de anticorpos que inibem o receptor de morte programada PD-1 (página 2, parágrafo [0015]). A composição de **D1** contém cerca de 25 mg/mL a 100 mg/mL de um anticorpo anti-PD-1, um tampão de histidina, polissorbato 80 e sacarose (página 2, parágrafo [0016]).

De acordo com **D1**, a inclusão de sacarose, manitol, glicina ou lactose em composições de anticorpos favorece a estabilidade, por exemplo, relacionada à crioproteção e tonicidade (página 7, parágrafo [0074]). Outros agentes que promovem estabilidade, por meio de crioproteção são dextranos, polietilenoglicol, glicose, trealose, polissorbatos e aminoácidos como glicina, arginina e serina (página 7, parágrafo [0076]).

Em **D1** são previstas soluções de anticorpo ou fragmentos de ligação a antígenos em concentrações de 10, 15, 20, 25, 30, 40, 50, 60, 75, 80, 90 ou 100 mg/mL ou concentrações mais altas de 150 mg/mL, 200 mg/mL, 250 mg/mL, até 300 mg/mL. Tais soluções têm um pH entre 5 e 6, preferencialmente 5,5 (página 10, parágrafo [0111]).

Dessa forma, <u>D1 é o estado da técnica mais próximo do pedido BR2972</u>, sendo um documento que ensina a obtenção de formulações:

- Do mesmo campo técnico e com o mesmo uso pretendido: **D1** descreve composições de anticorpo anti-PD-1 ou fragmentos de ligação para administração endovenosa ou subcutânea em um ser humano.
- Em que o problema técnico solucionado é a obtenção de formulação de alta concentração que seja estável.
- E com diversas características técnicas da invenção pleiteada. O Quadro 4 detalha a comparação das características técnicas de **D1** e do pedido BR2972.

Quadro 4: Comparação entre o conteúdo das formulações e seus usos de **D1** e do pedido de patente **BR112019022972-8**.

BR2972	D1
Formulação de anticorpo anti-PD-1 humano, caracterizada pelo fato de que compreende:	Formulação de anticorpo anti-PD-1 humano, caracterizada pelo fato de que compreende:
a) 100 mg/mL a 200 mg/mL de um anticorpo anti-PD-1 humano ou fragmento de ligação ao antígeno do mesmo (reiv 1, 4, 5, 6, 13, 15)	10 mg/mL a 300 mg/mL de um anticorpo anti-PD-1 humano ou fragmento de ligação ao antígeno do mesmo; (página 10, parágrafo [0111] e reiv 1) Revela especificamente formulação com anticorpo anti-PD-1 humano ou fragmento na concentração de 25 a 100 mg/mL (reiv 3)
b) 5 mM a 20 mM de tampão de histidina (reiv 1, 4, 5, 6, 13, 15) em que o pH é entre 5 e 6 (reiv 2)	Tampão de histidina, preferencialmente a 10 mM como tampão para pH entre 5 e 6 (página 2, parágrafos [0017] e [0021] e reiv 6).
c) um estabilizador selecionado a partir do grupo que consiste em: i) 6% a 8% peso/volume (p/v) de sacarose, trealose ou (2-hidroxipropil)-β-ciclodextrina; ii) 3% a 5% p/v de manitol, sorbitol, L-arginina, um sal farmacêuticamente aceitável de L-arginina, L-prolina ou um sal farmaceuticamente aceitável de L-prolina; e iii) 1,8% a 2,2% p/v de glicina ou um sal farmaceuticamente aceitável da mesma; (reiv 1, 3, 4, 5, 6, 13, 15) mais especificamente 1,25% a 2,5% p/v de L-arginina ou um sal farmaceuticamente aceitável (reiv 8)	Estabilizadores como sacarose, manitol, glicina, lactose, dextranos, polietilenoglicol, glicose, trealose, polissorbatos e aminoácidos como glicina, arginina e serina em concentrações diversas. Revela especificamente a sacarose em cerca de 7% (p/v) (reiv 5).
d) 0,01% a 0,10% de tensoativo não iônico (reiv 1) em que o tensoativo não iônico é polissorbato 80 (reiv 4, 5, 6, 13, 15)	O tensoativo não iônico polissorbato 80, preferencialmente a 0,02% (página 2, parágrafos [0016] e [0019] e reiv 4).
e) 1 mM a 20 mM de antioxidante (reiv 1) em que o antioxidante é L-metionina ou um sal farmaceuticamente aceitável (reiv 4, 5, 6, 13, 15), mais especificamente HCl de L-metionina (reiv 7)	-
solução reconstituída a partir de uma formulação liofilizada (reiv 9)	A formulação “estável” pode ser liofilizada, um líquido reconstituído ou uma formulação líquida (não previamente liofilizada) (página 7, parágrafo [0084]).
Uso da formulação para tratar câncer (reiv 18)	A formulação pode ser para tratar câncer (página 1, parágrafo [0015]).
Uso da formulação para tratar infecção crônica (reiv 17)	A formulação pode ser para tratar infecção crônica (página 1, parágrafo [0015]).
Uso da formulação para administração subcutânea (reiv 19)	A formulação pode ser para administração subcutânea (página 2, parágrafo [0016]).

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Depreende-se, portanto, que a característica distintiva da matéria pleiteada no BR2972 frente ao estado da técnica mais próximo **D1** é a presença de um 1 mM a 20 mM de antioxidante, em que o antioxidante pode ser L-metionina entre 5 mM e 10 mM, preferencialmente 10 mM.

O problema técnico está indicado no relatório descritivo como a degradação do pembrolizumabe exposto à luz, peróxido e alto pH, sendo principalmente relacionada à oxidação de metionina 105 tanto no CDR quanto na porção Fc (Relatório descritivo, parágrafo [029] - Petição 870220077996, de 29/08/2022, pág. 21/259).

Segundo o relatório descritivo do pedido BR2972-8:

> As formulações da invenção se destinam a problemas de alta viscosidade e agregação aumentada associada a formulações de anticorpo que compreendem uma alta concentração de anticorpos anti-PD1. A invenção fornece adicionalmente formulações que compreendem pembrolizumabe ou um fragmento de ligação ao antígeno das mesmas com oxidação de metionina reduzida, (...) (RD, parágrafo [027], página 7)

A este respeito, é importante retomar a última etapa da avaliação da atividade inventiva, na qual deve-se determinar se, diante de um problema técnico, o técnico no assunto é capaz de encontrar uma solução no estado da técnica. Nesse sentido, esta subsidiante discorda do argumento da depositante de que **o problema** da oxidação não estaria revelado no estado da técnica para defender a atividade inventiva do pedido BR2972. Pelo contrário, a avaliação da atividade inventiva é feita para determinar se **a solução** proposta, e não o problema, é ou não óbvia e poderá ser considerada **uma invenção.**

Portanto, para avaliação da atividade inventiva, é preciso determinar se, **diante da oxidação** de resíduos de metionina no CDR3 da cadeia pesada e na porção Fc do pembrolizumabe e partindo do estado da técnica mais próximo, **o uso de L-metionina como antioxidante** é ou não óbvio para um técnico no assunto[19].

A oxidação de resíduos de aminoácidos era conhecida, à época da data de prioridade mais antiga do pedido BR2972, como uma das principais causas de baixa estabilidade das formulações farmacêuticas que contêm proteínas e anticorpos monoclonais, sendo a mais comum a oxidação de resíduos de metionina[20].

A importância de monitorar oxidação em resíduos de metionina também já era conhecimento geral de um técnico no assunto. Também já era parte do conhecimento

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[19] Resolução n° 169/2016, item 5.9 (iii) determinar se, diante do problema técnico considerado, e partindo-se do estado da técnica mais próximo, a invenção é ou não óbvia para um técnico no assunto.
[20] Sokolowska I, Mo J, Dong J, Lewis MJ, Hu P. Subunit mass analysis for monitoring antibody oxidation. Mabs, 9(3):498-505, 2017.

geral do técnico no assunto que os malefícios da oxidação de resíduos de metionina na estabilidade da anticorpos sintéticos podem ser reduzidos por meio de rigoroso controle da oxidação de metionina com estratégias de formulação e por condições adequadas de armazenamento do produto[21].

Com base nos ensinamentos de **D2**, o técnico no assunto saberia que a oxidação de metionina ocorre independentemente do pH e por meio de uma ampla gama de espécies reativas de oxigênio, inclusive o oxigênio molecular. Além disso, essas reações são propagadas por radicais livres e há muitas fontes potenciais de radicais livres, incluindo material de embalagem e excipientes. O grau de acessibilidade do resíduo é determinante para que as espécies oxidantes ataquem a cadeia lateral. Portanto, um resíduo de metionina que é totalmente exposto exibirá uma taxa maior de oxidação do que um resíduo que esteja protegido na estrutura tridimensional. Conforme **D2**, a oxidação de resíduos de metionina foi amplamente relatada para anticorpos monoclonais.

Conforme os ensinamentos de **D2**, uma das estratégias amplamente utilizadas para prevenir a oxidação de resíduos de metionina em anticorpos monoclonais é o uso de aditivos de sacrifício que serão oxidados no lugar do ingrediente ativo e o uso de L-metionina livre na solução se mostrou como uma estratégia eficaz (**D2**, página 552).

Outros documentos do estado da técnica reforçam que a estratégia pode ser implementada com sucesso em diferentes proteínas, inclusive em anticorpos monoclonais.

D3 ensina que, em formulações do anticorpo monoclonal rhuMAb HER2, o uso de antioxidantes como a L-metionina, tiossulfato de sódio, catalase ou platina impediram a oxidação de resíduos de metionina por atuarem como radicais livres ou eliminadores de oxigênio. O nível mínimo eficaz necessário para que a L-metionina livre possa inibir a oxidação do anticorpo induzida foi 1:5 (razão molar de proteína para antioxidante), o que foi bastante superado nas proporções pleiteadas no presente pedido (entre 1:7 e 1:15, considerando a massa molecular do pembrolizumabe próxima a 149 kDA e as concentrações de 100 mg/mL a 200 mg/mL).

D4 realizou diversos testes de oxidação com uma proteína experimental chamada PTH e concluiu que a presença de L-metionina livre protegeu os resíduos de metonina da proteína de sofrerem oxidação.

Complementarmente, os subsídios ao exame técnico enviados anteriormente também mencionam exemplos de documentos do estado da técnica (**D5**) que antecipam o uso de L-metionina livre, inclusive na concentração de 10 mM, como o

[21] Liu D, Ren D, Huang H, Dankberg J, Rosenfeld R, Cocco MJ, Li L, Brems DN, Remmele RL Jr. Structure and stability changes of human IgG1 Fc as a consequence of methionine oxidation. Biochemistry, 47(18):5088-100, 2008.

antioxidante com melhor desempenho na formulação de anticorpos monoclonais. O trecho segue reproduzido na Figura 1 para pronta referência:

[16] Assim, D3 revela e sugere o **uso de L-metionina** como um **antioxidante** em formulações de proteína, uma vez que nos testes de estabilidade e caracterização da formulação, ficou comprovado que a **L-metionina** foi o composto mais eficiente em **suprimir a oxidação de proteínas**. Sendo até mesmo concluído que **L-metionina** é o composto **mais eficiente** em **suprimir a oxidação de aminoácidos** (Página 199, *"5. Conclusão"* de D3), em que também foi revelada uma concentração de **10 mM de L-metionina** (Página 197, Tabela 5 de D3).

in the oxidized protein molecules. These changes implicate methionine as the most probable site of oxidation in lysozyme. Among the three antioxidants used, methionine was the most efficient in suppressing oxidation, suggesting amino acids as a promising component in the protein formulation. These results provide a basis

Página 199, "5. Conclusão" de D3.

S. Hada et al. / International Journal of Biological Macromolecules 82 (2016) 192–200 197

Table 5
Percentage of secondary structures of lysozyme in presence of different concentration of methionine.

Samples	Control	Lysozyme + H_2O_2	10 mM methionine	50 mM methionine	100 mM methionine
α-Helix	24.83	18.14	19.26	22.75	22.84
Antiparallel β-sheet	15.11	19.20	20.16	17.54	17.25
Parallel β-sheet	9.72	10.88	10.45	9.95	9.95
β-Turn	17.90	17.61	18.07	18.20	18.10
Random Coil	32.34	34.16	32.06	31.66	31.85
Total	100.00	100.00	100.00	100.00	100.00

Página 197, Tabela 5 de D3.

Figura 1: Petição n° 870240062616, de 24/07/2024 (página 13/237)

Dessa forma, é bastante difundido no estado da técnica que a oxidação de resíduos de metionina em anticorpos monoclonais pode afetar a estabilidade e que, uma vez identificada, pode ser devidamente controlada pelo uso de antioxidantes, como a L-metionina. O histórico de uso da L-metionina como antioxidante em diferentes proteínas, inclusive em anticorpos monoclonais, e, em diversos casos, o relato de ser este o antioxidante mais eficiente levariam o técnico no assunto a utilizar esta estratégia com expectativa razoável de sucesso.

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Portanto, retomando as etapas de avaliação da atividade inventiva da Resolução n° 169/2016 (item 5.9), conclui-se que diante do problema técnico considerado, e partindo-se dos ensinamentos do estado da técnica mais próximo **D1** em combinação com **D2**, **D3**, **D4 ou D5**, **a invenção é óbvia para um técnico no assunto.**

Estes fatos evidenciam que a matéria reivindicada no pedido **BR112019022972-8** não pode ser concedida por **falta de atividade inventiva, estando em desacordo com os artigos 8° e 13 da LPI**.

7. DO PEDIDO

A subsidiante, assim, acredita haver demonstrado que a matéria para qual se requer proteção no pedido **BR112019022972-8** não é dotada de novidade e atividade inventiva, além de não estar suficientemente descrita, infringindo os artigos 8°, 13, 24 e 25 da LPI, razão pela qual aguarda que o pedido de patente seja prontamente **INDEFERIDO**.

Rio de Janeiro, 23 de maio de 2025

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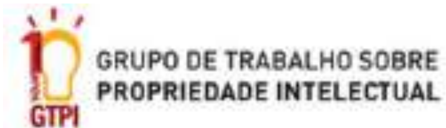

LISTA DE DOCUMENTOS ANEXOS:

ANEXO 1: US2014234296. Stable formulations of antibodies to human programmed death receptor PD-1 and related treatments**.** Data de publicação: 21/08/2014. (**D1**)
ANEXO 2: Manning MC, Chou DK, Murphy BM, Payne RW, Katayama DS. Stability of protein pharmaceuticals: an update. Pharm Res. 2010 Apr;27(4):544-75. doi: 10.1007/s11095-009-0045-6. Epub 2010 Feb 9. PMID: 20143256. Data de publicação: 09/12/2010 (**D2**)
ANEXO 3: Lam XM, Yang JY, Cleland JL. Antioxidants for prevention of methionine oxidation in recombinant monoclonal antibody HER2. J Pharm Sci. 1997 Nov;86(11):1250-5. doi: 10.1021/js970143s. PMID: 9383735. Data de publicação: 1997 (**D3**)
ANEXO 4: Ji JA, Zhang B, Cheng W, Wang YJ. Methionine, tryptophan, and histidine oxidation in a model protein, PTH: mechanisms and stabilization. J Pharm Sci. 2009 Dec;98(12):4485-500. doi: 10.1002/jps.21746. PMID: 19455640. Data de publicação 2009 (**D4**)
ANEXO 5: Hada S, Kim NA, Lim DG, Lim JY, Kim KH, Adhikary P, Jeong SH. Evaluation of antioxidants in protein formulation against oxidative stress using various biophysical methods. Int J Biol Macromol. 2016 Jan;82:192-200. doi: 10.1016/j.ijbiomac.2015.10.048. Epub 2015 Oct 21. PMID: 26499086. Data de publicação: 21/10/2015 (**D5**)

ANEXO 6: Estatuto Social da Abia
ANEXO 7: Ata de eleição de Diretoria da Abia
ANEXO 8: Procuração da Abia

ANEXO 9: Estatuto Social da Fenafar
ANEXO 10: Ata de eleição de Diretoria da Fenafar
ANEXO 11: Procuração da Fenafar

ANEXO 12: Estatuto Social do Foars
ANEXO 13: Ata de eleição de Diretoria do Foars
ANEXO 14: Procuração do Foars

ANEXO 15: Estatuto Social da Agani
ANEXO 16: Ata de eleição de Diretoria da Agani
ANEXO 17: Procuração da Agani

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(12) **Patent Application Publication** (10) **Pub. No.: US 2014/0234296 A1**

Sharma et al. (43) **Pub. Date: Aug. 21, 2014**

(54) **STABLE FORMULATIONS OF ANTIBODIES TO HUMAN PROGRAMMED DEATH RECEPTOR PD-1 AND RELATED TREATMENTS**

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(21) Appl. No.: **14/008,604**

(22) PCT Filed: **Mar. 29, 2012**

(86) PCT No.: **PCT/US12/31063**

§ 371 (c)(1),
(2), (4) Date: **Sep. 30, 2013**

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C07K 16/28 (2006.01)

(52) **U.S. Cl.**

CPC ***A61K 39/39591*** (2013.01); ***C07K 16/2818*** (2013.01); *A61K 2039/505* (2013.01)

USPC **424/133.1**

(57) **ABSTRACT**

The present invention relates to stable formulations of antibodies against human programmed death receptor PD-1, or antigen binding fragments thereof. The present invention further provides methods for treating various cancers and chronic infections with stable formulations of antibodies against human programmed death receptor PD-1, or antigen binding fragments thereof.

Stabililty Data for h409A11 Anti-PD-1 Powder for injection, 50 mg/Vial									
Storage Condition	5℃								
Batch Number	1								
		Stability Test Interval							
Test	**Clinical Acceptance Criteria**	**Initial**	**1-month**	**3-month**	**6-month**	**9-month**	**12-month**	**18-month**	**24-month**
Description Lyophilized Powder	White to off-white powder	White cake	White cake	White cake	White cake	White cake	White cake	White cake	White cake
Reconstitution Time (seconds)	Report Results	39	36	35	42	43	34	28	45
Description Reconstituted Solution									
Clarity	Clear to opalescent solution: May contain particulates	Opalescent solution	Opalescent solution	Opalescent solution	Opalescent solution	Opalescent solution	Opalescent solution	Opalescent solution	Opalescent solution
Color	Report results by "Y" ref solution	Colorless #	Colorless#	Colorless#	Colorless#	Colorless#	Colorless#	Colorless#	Colorless#
pH	5.0 - 6.0	5.6	5.5	5.5	5.6	5.6	5.6	5.6	5.6
Assay UV A_{280} nm	21.3 - 28.8 mg/mL	24.9	24.8	24.1	24.8	24.4	25.5	24.6	23.6
Biological Potency Anti-PD-1 Competitive ELISA (% Relative to control)	50-150% of Reference	95	80	86	116	93	87	76	83
Purity									
HPSEC									
High Molecular Weight Species (%)	≤ 5.00	ND	ND	<QL	<QL	<QL	<QL	<DL	<QL
Late Eluting Peaks (%)	Report Results	ND	ND	ND	ND	ND	ND	ND	ND
Monomer (%)	≥ 90.0	100.0	100.0	99.8	99.8	99.8	99.8	100.0	99.8
CE-SDS Reducing									
% Impurity	≤ 10.00% species other than heavy and light chains	0.38	0.35	0.39	0.41	0.40	0.44	0.38	0.39

FIG.1A

Stabililty Data for h409A11 Anti-PD-1 Powder for injection, 50 mg/Vial									
Storage Condition	5℃								
Batch Number	1								
	Stability Test Interval								
Test	Clinical Acceptance Criteria	Initial	1-month	3-month	6-month	9-month	12-month	18-month	24-month
CE-SDS Non-Reducing									
% Impurity	≤ 10.00% species other than main band	0.59	0.57	0.53	0.56	1.12	0.94	0.70	1.22
HP-IEX									
Acidic variants (%)	Report Results	2.5	2.5	3.3	3.2	3.3	3.4	3.2	3.3
Acidic 1 (%)	Report Results	3.7	3.7	3.6	3.9	3.9	3.9	3.7	3.8
Acidic 2 (%)	Report Results	8.2	8.5	7.7	8.0	8.1	8.3	7.8	8.0
Main (%)	Report Results	50.8	54.0	49.6	47.7	47.1	48.1	45.6	47.3
Basic 1 (%)	Report Results	11.0	10.6	10.7	11.3	11.7	10.4	11.5	11.0
Basic 2 (%)	Report Results	8.9	8.4	8.9	8.9	9.3	9.0	9.7	8.6
Basic Variants (%)	Report Results	14.9	12.4	16.1	17.0	16.5	16.8	18.5	17.9
Moisture (%)	≤ 5.0%	0.3	0.4	0.3	0.5	0.4	0.4	0.7	0.6
Particulate Matter (HIAC*)	Complies USP<788>								
≥ 10 μm per container	NMT 6000	51	50	51	50	51	57	43	44
≥ 25 μm per container	NMT 600	2	3	2	0	4	1	1	0
Bacterial Endotoxin	≤ 0.25 EU/mg	NT	NT	NT	NT	NT	NT	NT	NT
Sterility	Meets Sterility Test Requirement	NT	NT	NT	NT	NT	NT	NT	NT
Container Closure Integrity	No leakage detected	NT	NT	NT	NT	NT	NT	NT	NT

Quantization limit (QL) = 0.25%, Detection Limit (DL) = 0.10%, NT = not tested, ND = not detected
not tested according to "Y" ref solution
* A modified version of USP<788> was used for pre-clinical batch 1

FIG.1B

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Stability Data for h409A11 Anti-PD-1 Powder for injection, 50 mg/Vial						
Storage Condition	**25H (25°C, 60% RH)**					
Batch Number	**1**					
		Stability Test Interval				
Test	**Clinical Acceptance Criteria**	**Initial**	**1-month**	**3-month**	**6-month**	**12-month**
Description Lyophilized Powder	White to off-white powder	White cake	White cake	White cake	White cake	White cake
Reconstitution Time (seconds)	Report Results	39	39	37	36	32
Description Reconstituted Solution						
Clarity	Clear to opalescent solution: May contain particulates	Opalescent solution	Opalescent solution	Opalescent solution	Opalescent solution	Opalescent solution
Color	Report results by "Y" ref solution	Colorless#	Colorless#	Colorless#	Colorless#	Colorless#
pH	5.0 − 6.0	5.6	5.6	5.5	5.6	5.6
Assay UV A_{280} nm	21.3 − 28.8 mg/mL	24.9	25.1	23.5	24.2	24.8
Biological Potency Anti-PD-1 Competitive ELISA (% Relative to control)	50-150% of Reference	95	80	81	105	96
Purity						
HPSEC						
High Mol. Wt. Species (%)	≤ 5.00	ND	ND	<QL	<QL	<QL
Late Eluting Peaks (%)	Report Results	ND	ND	ND	ND	ND
Monomer (%)	≥ 90.0	100.0	100.0	99.8	99.8	99.8
CE-SDS Reducing						
% Impurity	≤ 10.00% species other than heavy and light chains	0.38	0.38	0.39	0.41	0.33
CE-SDS Non-Reducing						
% Impurity	≤ 10.00% species other than main band	0.59	0.57	0.71	0.58	0.98

FIG.2A

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Stabililty Data for h409A11 Anti-PD-1 Powder for injection, 50 mg/Vial						
Storage Condition	25H (25°C, 60% RH)					
Batch Number						
	Stability Test Interval					
Test	**Clinical Acceptance Criteria**	**Initial**	**1-month**	**3-month**	**6-month**	**12-month**
HP-IEX						
Acidic variants (%)	Report Results	2.5	2.8	3.1	3.2	3.4
Acidic 1 (%)	Report Results	3.7	3.5	3.7	4.0	4.0
Acidic 2 (%)	Report Results	8.2	8.6	8.3	8.4	9.1
Main (%)	Report Results	50.8	53.4	48.6	46.7	46.6
Basic 1 (%)	Report Results	11.0	10.8	10.8	11.3	10.4
Basic 2 (%)	Report Results	8.9	8.4	9.0	8.9	9.1
Basic Variants (%)	Report Results	14.9	12.6	16.5	17.5	17.4
Moisture (%)	≤ 5.0%	0.3	0.5	0.6	0.8	0.9
Particulate Matter (HIAC*)	Complies USP<788>					
≥ 10 μm per container	NMT 6000	51	31	37	31	59
≥ 25 μm per container	NMT 600	2	1	1	1	0
Bacterial Endotoxin	≤ 0.25 EU/mg	NT	NT	NT	NT	NT
Sterility	Meets Sterility Test Requirement	NT	NT	NT	NT	NT
Container Closure Integrity	No leakage detected	NT	NT	NT	NT	NT

Quantization limit (QL) = 0.25%, Detection Limit (DL) = 0.10%, NT = not tested, ND = not detected
not tested according to "Y" ref solution
* A modified version of USP<788> was used for pre-clinical batch 1

FIG.2B

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Stability Data for h409A11 Anti-PD-1 Powder for injection, 50 mg/Vial								
Storage Condition	**RH4 (40℃, 75% RH)**							
Batch Number	1							
		Stability Test Interval						
Test	**Clinical Acceptance Criteria**	**Initial**	**0.5-month**	**1-month**	**2-month**	**3-month**	**6-month**
Description Lyophilized Powder	White to off-white powder	White cake	White cake	White cake	White cake	White cake	White cake
Reconstitution Time (seconds)	Report Results	39	31	33	37	33	39
Description Reconstituted Solution							
Clarity	Clear to opalescent solution: May contain particulates	Opalescent solution	Opalescent solution	Opalescent solution	Opalescent solution	Opalescent solution	Opalescent solution
Color	Report results by "Y" ref solution	Colorless#	Colorless#	Colorless#	Colorless#	Colorless#	Colorless#
pH	5.0 – 6.0	5.6	5.5	5.6	5.6	5.5	5.6
Assay UV A_{280} **nm**	21.3 – 28.8 mg/mL	24.9	25.5	24.2	24.1	23.9	24.3
Biological Potency Anti-PD-1 Competitive ELISA (% Relative to control)	50-150% of Reference	95	85	83	77	86	97
Purity							
HPSEC							
High Mol. Wt. Species (%)	≤ 5.00	ND	ND	<DL	<QL	0.27	0.30
Late Eluting Peaks (%)	Report Results	ND	ND	ND	ND	ND	ND
Monomer (%)	≥ 90.0	100.0	100.0	99.9	99.8	99.7	99.7
CE-SDS Reducing							
% Impurity	≤ 10.00% species other than heavy and light chains	0.38	0.38	0.33	0.39	0.42	0.40
CE-SDS Non-Reducing							
% Impurity	≤ 10.00% species other than main band	0.59	0.58	0.56	0.50	0.60	0.72

FIG.3A

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Stabililty Data for h409A11 Anti-PD-1 Powder for injection, 50 mg/Vial							
Storage Condition	**RH4 (40°C, 75% RH)**						
Batch Number	**1**						
		Stability Test Interval					
Test	**Clinical Acceptance Criteria**	**Initial**	**0.5-month**	**1-month**	**2-month**	**3-month**	**6-month**
HP-IEX							
Acidic variants (%)	Report Results	2.5	2.7	2.4	2.5	3.0	3.3
Acidic 1 (%)	Report Results	3.7	3.6	4.0	3.9	3.8	4.0
Acidic 2 (%)	Report Results	8.2	8.7	9.2	9.4	9.5	10.0
Main (%)	Report Results	50.8	50.2	52.3	50.4	46.0	43.4
Basic 1 (%)	Report Results	11.0	11.0	10.8	10.8	10.7	11.8
Basic 2 (%)	Report Results	8.9	8.8	8.4	8.7	8.7	8.8
Basic Variants (%)	Report Results	14.9	15.0	12.9	14.4	18.2	18.7
Moisture (%)	≤ 5.0%	0.3	0.6	0.6	0.7	0.9	1.1
Particulate Matter (HIAC*)	Complies USP<788>						
≥ 10 μm per container	NMT 6000	51	40	37	65	28	43
≥ 25 μm per container	NMT 600	2	3	2	2	1	1
Bacterial Endotoxin	≤ 0.25 EU/mg	NT	NT	NT	NT	NT	NT
Sterility	Meets Sterility Test Requirement	NT	NT	NT	NT	NT	NT
Container Closure Integrity	No leakage detected	NT	NT	NT	NT	NT	NT

Quantization limit (QL) = 0.25%, Detection Limit (DL) = 0.10%, NT = not tested, ND = not detected
not tested according to "Y" ref solution
* A modified version of USP<788> was used for pre-clinical batch 1

FIG.3B

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Stabililty Data for h409A11 Anti-PD-1 Powder for injection, 50 mg/Vial									
Storage Condition	5℃								
Batch Number	2								
		Stability Test Interval							
Test	**Clinical Acceptance Criteria**	**Initial**	**1-month**	**3-month**	**6-month**	**9-month**	**12-month**	**18-month**	**24-month**
Description Lyophilized Powder	White to off-white powder	White cake	White cake	White cake	White cake	White cake	White cake	White cake	White cake
Reconstitution Time (seconds)	Report Results	49	30	36	27	22	23	27	36
Description Reconstituted Solution									
Clarity	Clear to opalescent solution: May contain particulates	Opalescent solution	Opalescent solution	Opalescent solution	Opalescent solution	Opalescent solution	Opalescent solution	Opalescent solution	Opalescent solution
Color	Report results by "Y" ref solution	Colorless #	Colorless#	Colorless#	Colorless#	Colorless#	Colorless#	Colorless#	Colorless#
pH	5.0 – 6.0	5.6	5.6	5.6	5.6	5.6	5.6	5.6	5.5
Assay UV A_{280} nm	21.3 – 28.8 mg/mL	25.6	26.4	25.2	26.1	23.2	24.6	24.7	25.5
Biological Potency Anti-PD-1 Competitive ELISA (% Relative to control)	50-150% of Reference	108	88	86	94	86	82	90	103
Purity									
HPSEC									
High Molecular Weight Species (%)	≤ 5.00	0.52	030	0.31	0.31	0.32	<DL	<QL	0.40
Late Eluting Peaks (%)	Report Results	ND	ND	ND	ND	ND	ND	ND	ND
Monomer (%)	≥ 90.0	99.5	99.7	99.7	99.7	99.7	99.9	99.8	99.6
CE-SDS Reducing									
% Impurity	≤ 10.00% species other than heavy and light chains	0.37	0.36	0.38	0.37	0.38	0.39	0.45	0.64

FIG.4A

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Stabililty Data for h409A11 Anti-PD-1 Powder for injection, 50 mg/Vial									
Storage Condition	5°C								
Batch Number	2								
	Stability Test Interval								
Test	Clinical Acceptance Criteria	Initial	1-month	3-month	6-month	9-month	12-month	18-month	24-month
CE-SDS Non-Reducing									
% Impurity	≤ 10.00% species other than main band	0.42	0.38	0.77	0.90	0.94	0.73	1.25	1.39
HP-IEX									
Acidic variants (%)	Report Results	3.1	3.4	3.3	3.4	3.4	3.2	3.3	4.2
Acidic 1 (%)	Report Results	4.0	3.9	4.0	4.0	4.0	3.8	3.8	3.9
Acidic 2 (%)	Report Results	8.0	8.1	8.0	8.4	8.3	7.8	8.0	8.1
Main (%)	Report Results	47.4	47.8	47.4	47.9	47.3	45.8	47.6	46.1
Basic 1 (%)	Report Results	11.4	11.4	11.6	10.5	12.3	11.6	10.9	11.2
Basic 2 (%)	Report Results	8.8	8.9	9.2	8.9	9.4	9.6	8.5	8.7
Basic Variants (%)	Report Results	17.3	16.5	16.4	16.8	15.4	18.2	17.8	17.9
Moisture (%)	≤ 5.00%	0.8	0.8	0.8	1.1	1.3	1.1	1.1	1.3
Particulate Matter (HIAC*)	Complies USP<788>								
≥ 10 μm per container	NMT 6000	55	6	35	17	23	1	46	20
≥ 25 μm per container	NMT 600	0	0	1	1	1	0	0	2
Bacterial Endotoxin	≤ 0.25 EU/mg	NT	NT	NT	NT	NT	NT	NT	NT
Sterility	Meets Sterility Test Requirement	NT	NT	NT	NT	NT	NT	NT	NT
Container Closure Integrity	No leakage detected	NT	NT	NT	NT	NT	NT	NT	NT

NT = not tested, ND = not detected,
# not tested according to "Y" ref solution
* A modified version of USP<788> was used for pre-clinical batch 2

FIG.4B

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Stabililty Data for h409A11 Anti-PD-1 Powder for injection, 50 mg/Vial					
Storage Condition	**25H (25°C, 60% RH)**				
Batch Number	B				
		Stability Test Interval			
Test	**Clinical Acceptance Criteria**	**Initial**	**1-month**	**3-month**	**6-month**
Description Lyophilized Powder	White to off-white powder	White powder	White powder	White powder	White powder
Reconstitution Time (seconds)	Report Results	49	33	31	23
Description Reconstituted Solution					
Clarity	Clear to opalescent solution: May contain particulates	Opalescent solution	Opalescent solution	Opalescent solution	Opalescent solution
Color	Report results by "Y" ref solution	Colorless#	Colorless#	Colorless#	Colorless#
pH	5.0 – 6.0	5.6	5.6	5.6	5.6
Assay UV A_{280} nm	21.3 – 28.8 mg/mL	25.6	25.8	24.8	26.4
Biological Potency Anti-PD-1 Competitive ELISA (%Relative to control)	50–150% of Reference	108	76	78	89
Purity					
HPSEC					
High Mol. Wt. Species (%)	≤ 5.00	0.52	0.30	0.34	0.33
Late Eluting Peaks (%)	Report Results	ND	ND	ND	ND
Monomer (%)	≥ 90.0	99.5	99.7	99.7	99.7
CE-SDS Reducing					
% Impurity	≤ 10.00% species other than heavy and light chains	0.37	0.31	0.39	0.27
CE-SDS Non-Reducing					
% Impurity	≤ 10.00% species other than main band	0.42	0.44	1.10	0.96

FIG.5A

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Stabililty Data for h409A11 Anti-PD-1 Powder for injection, 50 mg/Vial					
Storage Condition	**25H (25°C, 60% RH)**				
Batch Number	B				
		Stability Test Interval			
Test	**Clinical Acceptance Criteria**	**Initial**	**1-month**	**3-month**	**6-month**
HP-IEX					
Acidic variants (%)	Report Results	3.1	3.3	3.3	3.4
Acidic 1 (%)	Report Results	4.0	4.0	4.0	4.0
Acidic 2 (%)	Report Results	8.0	8.2	8.4	8.9
Main (%)	Report Results	47.4	47.5	47.1	47.2
Basic 1 (%)	Report Results	11.4	11.4	11.5	10.5
Basic 2 (%)	Report Results	8.8	9.0	9.1	8.8
Basic Variants (%)	Report Results	17.3	16.6	16.5	17.2
Moisture (%)	≤ 5.0%	0.8	0.9	1.1	1.2
Particulate Matter (HIAC*)	Complies USP<788>				
≥ 10 μm per container	NMT 6000	55	11	34	6
≥ 25 μm per container	NMT 600	0	0	1	0
Bacterial Endotoxin	≤ 0.25 EU/mg	NT	NT	NT	NT
Sterility	Meets Sterility Test Requirement	NT	NT	NT	NT
Container Closure Integrity	No leakage detected	NT	NT	NT	NT

NT = Not tested, ND = Not detected
# not tested according to "Y" ref solution
* A modified version of USP<788> was used for pre-clinical batch 2

FIG.5B

Stabililty Data for h409A11 Anti-PD-1 Powder for injection, 50 mg/Vial					
Storage Condition	**RH4 (40°C, 75% RH)**				
Batch Number	2				
		Stability Test Interval			
Test	**Clinical Acceptance Criteria**	**Initial**	**1-month**	**3-month**	**6-month**
Description Lyophilized Powder	White to off-white powder	White powder	White powder	White powder	White powder
Reconstitution Time (seconds)	Report Results	49	32	35	20
Description Reconstituted Solution					
Clarity	Clear to opalescent solution: May contain particulates	Opalescent solution	Opalescent solution	Opalescent solution	Opalescent solution
Color	Report results by "Y" ref solution	Colorless#	Colorless#	Colorless#	Colorless#
pH	5.0 – 6.0	5.6	5.6	5.6	5.6
Assay UV A_{280} nm	21.3 – 28.8 mg/mL	25.6	24.9	24.9	25.9
Biological Potency Anti-PD-1 Competitive ELISA (% Relative to control)	50-150% of Reference	108	76	60	66
Purity					
HPSEC					
High Mol. Wt. Species (%)	≤ 5.00	0.52	0.34	0.37	0.38
Late Eluting Peaks (%)	Report Results	ND	ND	ND	ND
Monomer (%)	≥ 90.0	99.5	99.7	99.6	99.6
CE-SDS Reducing					
% Impurity	≤ 10.00% species other than heavy and light chains	0.37	0.39	0.45	0.34
CE-SDS Non-Reducing					
% Impurity	≤ 10.00% species other than main band	0.42	0.43	1.16	0.97

FIG.6A

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Stabililty Data for h409A11 Anti-PD-1 Powder for injection, 50 mg/Vial					
Storage Condition	**RH4 (40°C, 75% RH)**				
Batch Number	3				
		Stability Test Interval			
Test	**Clinical Acceptance Criteria**	**Initial**	**1-month**	**3-month**	**6-month**
HP-IEX					
Acidic variants (%)	Report Results	3.1	3.4	3.3	3.5
Acidic 1 (%)	Report Results	4.0	4.0	4.3	4.1
Acidic 2 (%)	Report Results	8.0	8.8	9.4	10.2
Main (%)	Report Results	47.4	46.8	45.9	44.8
Basic 1 (%)	Report Results	11.4	11.7	11.4	10.4
Basic 2 (%)	Report Results	8.8	9.1	8.9	8.9
Basic Variants (%)	Report Results	17.3	16.4	16.8	18.0
Moisture (%)	$\leq$ 5.0%	0.8	1.1	1.3	1.7
Particulate Matter (HIAC*)	Complies USP<788>				
$\geq$ 10 μm per container	NMT 6000	55	11	14	35
$\geq$ 25 μm per container	NMT 600	0	0	0	1
Bacterial Endotoxin	$\leq$ 0.25 EU/mg	NT	NT	NT	NT
Sterility	Meets Sterility Test Requirement	NT	NT	NT	NT
Container Closure Integrity	No leakage detected	NT	NT	NT	NT

NT = not tested, ND = not detected
# not tested according to "Y" ref solution
* A modified version of USP<788> was used for pre-clinical batch 2

FIG.6B

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Stabililty Data for h409A11 Anti-PD-1 Powder for injection, 50 mg/Vial									
Storage Condition	5°C								
Batch Number	3								
		Stability Test Interval							
Test	**Clinical Acceptance Criteria**	**Initial**	**1-month**	**3-month**	**6-month**	**9-month**	**12-month**	**18-month**	**24-month**
Description Lyophilized Powder	White to off-white powder	White powder	White powder	White powder	White powder	White powder	White powder	Off-white powder	White powder
Reconstitution Time (seconds)	Report Results	41	35	34	32	32	38	42	32
Description Reconstituted Solution									
Clarity	Clear to opalescent solution: May contain particulates	Opalescent solution does not contain particulates	Clear solution does not contain particulates	Clear solution does not contain particulates	Opalescent solution does not contain particulates	Opalescent solution does not contain particulates	Opalescent solution does not contain particulates	Opalescent solution does not contain particulates	Opalescent solution does not contain particulates
Color	Report results by "Y" ref solution	Equivalent to ref solution Y7	Equivalent to ref solution Y7	Equivalent to ref solution Y7	Equivalent to ref solution Y7	Equivalent to ref solution Y7	Equivalent to ref solution Y7	Equivalent to ref solution Y7	Equivalent to ref solution Y7
pH	5.0 - 6.0	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5
Assay UV A_{280} nm	21.3 - 28.8 mg/mL	25.6	25.3	25.5	25.5	25.5	26.3	26.6	25.7
Biological Potency	50-150% of Reference	90	102	96	102	106	105	71	98
Purity									
HPSEC									
High Molecular Weight Species (%)	≤ 5.00	0.32	0.27	0.28	0.25	0.31	0.36	0.32	0.36
Late Eluting Peaks (%)	Report Results	<0.13	<0.13	<0.13	<0.13	<0.13	<0.13	<0.13	<0.13
Monomer (%)	≥ 90.0	99.7	99.7	99.7	99.8	99.7	99.6	99.7	99.6
CE-SDS Reducing									
% Impurity	≤ 10.00% species other than heavy and light chains	0.42	0.42	0.46	0.44	0.41	0.56	0.44	0.44

FIG. 7A

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Stabililty Data for h409A11 Anti-PD-1 Powder for injection, 50 mg/Vial									
Storage Condition	**5°C**								
Batch Number	**3**								
	Stability Test Interval								
Test	**Clinical Acceptance Criteria**	**Initial**	**1-month**	**3-month**	**6-month**	**9-month**	**12-month**	**18-month**	**24-month**
	light chains								
CE-SDS Non-Reducing									
% Impurity	≤ 10.00% species other than main band	0.45	0.47	0.42	0.55	0.78	0.31	0.60	0.55
HP-IEX									
Acidic variants (%)	Report Results	3.97	8.45	8.06	6.79	8.52	9.29	8.71	9.70
Acidic 1 (%)	Report Results	5.45	4.99	5.05	4.97	4.75	5.07	4.90	4.91
Acidic 2 (%)	Report Results	7.76	8.26	8.26	8.02	7.91	8.48	8.48	8.59
Main (%)	Report Results	54.4	48.3	47.3	48.9	46.2	47.0	46.6	44.9
Basic 1 (%)	Report Results	7.23	7.63	7.82	7.98	7.69	7.77	8.04	8.43
Basic 2 (%)	Report Results	7.92	8.65	9.23	9.33	9.07	8.87	9.08	9.08
Basic Variants (%)	Report Results	13.22	13.75	14.29	14.03	15.82	13.51	14.20	14.38
Moisture (%)	≤ 5.0%	0.8	0.8	0.9	0.8	0.8	1.0	1.0	1.0
Particulate Matter (HIAC*)	Complies USP<788>	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies
≥ 10 μm per container	NMT 6000	39	24	16	18	18	16	16	47
≥ 25 μm per container	NMT 600	0	1	0	1	0	0	0	2
Bacterial Endotoxin	≤ 0.25 EU/mg	<0.05	NT	NT	NT	NT	<0.05	NT	<0.05
Sterility	Meets Sterility Test Requirement	Meets Requirements	NT	NT	NT	NT	NT	NT	NT
Container Closure Integrity	No leakage detected	No leakage detected	NT	NT	NT	NT	No leakage detected	NT	No leakage detected
NT: Not tested									

FIG. 7B

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Stabililty Data for h409A11 Anti-PD-1 Powder for injection, 50 mg/Vial					
Storage Condition	**25H (25°C, 60% RH)**				
Batch Number	**3**				
		Stability Test Interval			
Test	**Clinical Acceptance Criteria**	**Initial**	**1-month**	**3-month**	**6-month**
Description Lyophilized Powder	White to off-white powder	White powder	White powder	White powder	White powder
Reconstitution Time (seconds)	Report Results	41	35	33	35
Description Reconstituted Solution					
Clarity	Clear to opalescent solution: May contain particulates	Opalescent solution does not contain particulates	Clear solution does not contain particulates	Clear solution does not contain particulates	Opalescent solution does not contain particulates
Color	Report results by "Y" ref solution	Equivalent to ref solution Y7	Equivalent to ref solution Y7	Equivalent to ref solution Y7	Equivalent to ref solution Y7
pH	5.0 – 6.0	5.5	5.5	5.5	5.5
Assay UV A_{280} nm	21.3 – 28.8 mg/mL	25.6	25.4	25.8	25.9
Biological Potency	50–150% of Reference	90	107	89	99
Purity					
HPSEC					
High Mol. Wt. Species (%)	≤ 5.00	0.32	0.28	0.25	0.32
Late Eluting Peaks (%)	Report Results	<0.13	<0.13	<0.13	<0.13
Monomer (%)	≥ 90.0	99.7	99.7	99.7	99.7
CE-SDS Reducing					
% Impurity	≤ 10.00% species other than heavy and light chains	0.42	0.42	0.43	0.45
CE-SDS Non-Reducing					
% Impurity	≤ 10.00% species other than main band	0.45	0.71	0.43	0.53

FIG.8A

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Stabililty Data for h 409 All Anti-PD-1 Powder for injection, 50 mg/Vial					
Storage Condition	**25H (25°C, 60% RH)**				
Batch Number	**3**				
		Stability Test Interval			
Test	**Clinical Acceptance Criteria**	**Initial**	**1-month**	**3-month**	**6-month**
HP-IEX					
Acidic variants (%)	Report Results	3.97	8.52	8.67	9.79
Acidic 1 (%)	Report Results	5.45	4.97	5.09	5.04
Acidic 2 (%)	Report Results	7.76	8.40	8.60	8.49
Main (%)	Report Results	54.4	48.0	45.9	44.9
Basic 1 (%)	Report Results	7.23	7.65	7.89	8.09
Basic 2 (%)	Report Results	7.92	8.60	9.22	9.37
Basic Variants (%)	Report Results	13.22	13.88	14.67	14.35
Moisture (%)	≤ 5.0%	0.8	0.9	1.0	1.2
Particulate Matter (HIAC)	Complies USP<788>	Complies	Complies	Complies	Complies
≥ 10 μm per container	NMT 6000	39	10	18	21
≥ 25 μm per container	NMT 600	0	0	0	0
Bacterial Endotoxin	≤ 0.25 EU/mg	<0.05	NT	NT	NT
Sterility	Meets Sterility Test Requirement	Meets requirements	NT	NT	NT
Container Closure Integrity	No leakage detected	No leakage detected	NT	NT	NT
NT: Not tested					

FIG.8B

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Stability Data for h409A11 Anti-PD-1 Powder for injection, 50 mg/Vial						
Storage Condition	**RH4 (40°C, 75% RH)**					
Batch Number	3					
		Stability Test Interval				
Test	**Clinical Acceptance Criteria**	**Initial**	**1-month**	**3-month**	**6-month**
Description Lyophilized Powder	White to off-white powder	White powder	White powder	White powder	White powder
Reconstitution Time (seconds)	Report Results	41	35	34	33
Description Reconstituted Solution					
Clarity	Clear to opalescent solution: May contain particulates	Opalescent solution does not contain particulates	Clear solution does not contain particulates	Clear solution does not contain particulates	Opalescent solution does not contain particulates
Color	Report results by "Y" ref solution	Equivalent to ref solution Y7	Equivalent to ref solution Y7	Equivalent to ref solution Y7	Equivalent to ref solution Y7
pH	5.0 – 6.0	5.5	5.5	5.5	5.5
Assay UV A_{280} nm	21.3 – 28.8 mg/mL	25.6	25.4	25.5	25.9
Biological Potency	50–150% of Reference	90	101	105	104
Purity					
HPSEC					
High Mol. Wt. Species (%)	≤ 5.00	0.32	0.30	0.33	0.40
Late Eluting Peaks (%)	Report Results	<0.13	<0.13	<0.13	<0.13
Monomer (%)	≥ 90.0	99.7	99.7	99.7	99.6
CE-SDS Reducing					
% Impurity	≤ 10.00% species other than heavy and light chains	0.42	0.50	0.48	0.45
CE-SDS Non-Reducing					
% Impurity	≤ 10.00% species other than main band	0.45	0.52	0.67	0.35

FIG.9A

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Stabililty Data for h409A11 Anti-PD-1 Powder for injection, 50 mg/Vial					
Storage Condition	**RH4 (40℃,75% RH)**				
Batch Number	3				
		Stability Test Interval			
Test	**Clinical Acceptance Criteria**	Initial	**1-month**	**3-month**	**6-month**
HP-IEX					
Acidic variants (%)	Report Results	3.97	8.16	9.32	9.47
Acidic 1 (%)	Report Results	5.45	5.11	5.12	5.08
Acidic 2 (%)	Report Results	7.76	8.88	9.47	10.11
Main (%)	Report Results	54.4	47.1	43.4	42.2
Basic 1 (%)	Report Results	7.23	7.75	8.22	8.41
Basic 2 (%)	Report Results	7.92	8.51	9.11	9.12
Basic Variants (%)	Report Results	13.22	14.48	15.32	15.66
Moisture (%)	≤ 5.0%	0.8	1.1	1.3	1.5.
Particulate Matter (HIAC)	Complies USP<788>	Complies	Complies	Complies	Complies
≥ 10 μm per container	NMT 6000	39	14	19	26
≥ 25 μm per container	NMT 600	0	0	0	1
Bacterial Endotoxin	≤ 0.25 EU/mg	<0.05	NT	NT	NT
Sterility	Meets Sterility Test Requirement	Meets requirements	NT	NT	NT
Container Closure Integrity	No leakage detected	No leakage detected	NT	NT	NT
NT: Not tested					

FIG.9B

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STABLE FORMULATIONS OF ANTIBODIES TO HUMAN PROGRAMMED DEATH RECEPTOR PD-1 AND RELATED TREATMENTS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. provisional patent application No. 61/470,121, filed Mar. 31, 2011, which is herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

[0002] The present invention relates to stable formulations of antibodies against human programmed death receptor PD-1, or antigen binding fragments thereof. The present invention further provides methods for treating various cancers and chronic infections with stable formulations of antibodies against human PD-1, or antigen binding fragments thereof.

BACKGROUND OF THE INVENTION

[0003] Programmed Death 1 (PD-1), a member of the CD28 costimulatory gene family, is moderately expressed on naive T, B and NKT cells and up-regulated by TB cell receptor signaling on lymphocytes, monocytes and myeloid cells (1). PD-1 has two known ligands with distinct expression profiles, PD-L1 (B7-H1) and PD-L2 (B7-DC). PD-L2 expression is relatively restricted and is found on activated dendritic cells, macrophages and monocytes and on vascular endothelial cells (1-3). In contrast, PD-L1 is expressed more broadly including on naive lymphocytes and its expression is induced on activated B and T cells, monocytes and dendritic cells. Furthermore, by mRNA, it is expressed by non-lymphoid tissues including vascular endothelial cells, epithelial cells and muscle cells.

[0004] PD-1 is recognized as an important player in immune regulation and the maintenance of peripheral tolerance. In the mouse, this was shown to require PD-L1 expression on peripheral tissues and ligation of PD-1 on potentially autoreactive T cells to negatively modulate T cell activation involving an ITIM sequence in the PD-1 cytoplasmic domain (1, 4).

[0005] Depending on the specific genetic background, pdcd1$^{-/-}$ mice spontaneously develop lupus-like phenomena or dilated cadiomyopathy (5, 6). Furthermore, antibody-induced blockade of the PD-1/PD-L 1 pathway was demonstrated to accelerate the onset of autoimmune insulitis and diabetes in NOD mice (7).

[0006] Human cancers arising in various tissues were found to over-express PD-L1 or PD-L2. In large sample sets of e.g. ovarian, renal, colorectal, pancreatic, liver cancers and melanoma it was shown that PD-L1 expression correlated with poor prognosis and reduced overall survival irrespective of subsequent treatment (15-26). Similarly, PD-1 expression on tumor infiltrating lymphocytes was found to mark dysfunctional T cells in breast cancer and melanoma (27-28) and to correlate with poor prognosis in renal cancer (29). Using primary patient samples, it was shown that blockade of PD-1 or PD-L1 in vitro results in enhancement of human tumor-specific T cell activation and cytokine production (30). Consequently, in several murine syngeneic tumor models, blockade of either PD-1 or PD-L1 significantly inhibited tumor growth or induced complete regression.

[0007] A PD-1 blocking mAb (h409A11) was discovered and developed for use to treat human cancer patients and chronic virus-infected patients (described in co-pending application WO2008/156712).

[0008] Antigen-specific T cell dysfunction or tolerance is exemplified by the accumulated loss of the potential to produce Interleukin 2 (IL-2), Tumor Necrosis factor (TNF) α, perforin, interferon (IFN) γ (8) and inability to mount a proliferative response to T cell receptor triggering (1). The PD-1 pathway controls antigen-specific T cell tolerance and was found to be exploited in viral infection and tumor development to control and evade effective T cell immunity.

[0009] In chronic infection with LCMV (mouse), HIV, HBV or HCV (human), antigen-specific T cells were found to express aberrantly high levels of PD-1 correlating with their state of anergy or dysfunction (9). Blocking the PD-1-PD-L1 interaction in vivo (LCMV) or in vitro (HIV, HCV, HBV) was shown to revive anti-viral T cell activity (10-12). PD-1 blockade in recently Simian Immunodeficiency Virus-infected macaques resulted in strong reduction of viral load and increased survival (13). Similarly, reduction in viral load was confirmed in second study using long-term SIV-infected rhesus macaques (14).

[0010] Overall, the PD-1/PD-L1 pathway is a well-validated target for the development of antibody therapeutics for cancer treatment. Anti-PD-1 antibodies are also useful for treating chronic viral infection. Memory CD8^{+} T cells generated after an acute viral infection are highly functional and constitute an important component of protective immunity. In contrast, chronic infections are often characterized by varying degrees of functional impairment (exhaustion) of virus-specific T-cell responses, and this defect is a principal reason for the inability of the host to eliminate the persisting pathogen. Although functional effector T cells are initially generated during the early stages of infection, they gradually lose function during the course of a chronic infection. Barber et al. (Barber et al., Nature 439: 682-687 (2006)) showed that mice infected with a laboratory strain of LCMV developed chronic infection resulting in high levels of virus in the blood and other tissues. These mice initially developed a robust T cell response, but eventually succumbed to the infection upon T cell exhaustion. The authors found that the decline in number and function of the effector T cells in chronically infected mice could be reversed by injecting an antibody that blocked the interaction between PD-1 and PD-L1.

[0011] PD-1 has also been shown to be highly expressed on T cells from HIV infected individuals and that receptor expression correlates with impaired T cell function and disease progression (Day et al., Nature 443:350-4 (2006); Trautmann L. et al., Nat. Med. 12: 1198-202 (2006)). In both studies, blockade of the PD-1 pathway using antibodies against the ligand PD-L1 significantly increased the expansion of HIV-specific, IFN-gamma producing cells in vitro.

[0012] Other studies also implicate the importance of the PD-1 pathway in controlling viral infection. PD-1 knockout mice exhibit better control of adenovirus infection than wild-type mice (Iwai et al., Exp. Med. 198:39-50 (2003)). Also, adoptive transfer of HBV-specific T cells into HBV transgenic animals initiated hepatitis (Isogawa M. et al., Immunity 23:53-63 (2005)). The disease state of these animals oscillates as a consequence of antigen recognition in the liver and PD-1 upregulation by liver cells.

[0013] Therapeutic antibodies may be used to block cytokine activity. A significant limitation in using antibodies as a

therapeutic agent in vivo is the immunogenicity of the antibodies. As most monoclonal antibodies are derived from non-human species, repeated use in humans results in the generation of an immune response against the therapeutic antibody. Such an immune response results in a loss of therapeutic efficacy at a minimum, and potentially a fatal anaphylactic response. Accordingly, antibodies of reduced immunogenicity in humans, such as humanized or fully human antibodies, are preferred for treatment of human subjects. Exemplary therapeutic antibodies specific for human PD-1 are disclosed in commonly-assigned U.S. Patent Application Publication No. US2010/0266617, and in International Patent Publication No. WO2008/156712, the disclosures of which are hereby incorporated by reference in their entireties.

[0014] Antibodies for use in human subjects must be stored prior to use and transported to the point of administration. Reproducibly attaining a desired level of antibody drug in a subject requires that the drug be stored in a formulation that maintains the bioactivity of the drug. The need exists for stable formulations of anti-human PD-1 antibodies for pharmaceutical use, e.g., for treating various cancers and infectious diseases. Preferably, such formulations will exhibit a long shelf-life, be stable when stored and transported, and will be amenable to administration at high concentrations, e.g. for use in subcutaneous administration, as well as low concentrations, e.g. for intravenous administration.

SUMMARY OF THE INVENTION

[0015] The present invention relates to stable formulations of antibodies against human programmed death receptor PD-1, or antigen binding fragments thereof. The present invention further provides methods for treating various cancers and chronic infections with stable formulations of antibodies against human programmed death receptor PD-1, or antigen binding fragments thereof.

[0016] In certain embodiments, the invention relates to a lyophilized formulation of an anti-human PD-1 antibody, or antigen binding fragment thereof, comprising: a) said anti-human PD-1 antibody, or antigen binding fragment thereof; b) histidine buffer; c) polysorbate 80; and d) sucrose.

[0017] In certain embodiments, the formulation has a pH between 5.0 and 6.0 when reconstituted.

[0018] In certain embodiments, the lyophilized formulation enables reconstitution of the antibody, or antigen binding fragment thereof, at a concentration of between about 25 mg/mL and 100 mg/mL.

[0019] In certain embodiments, polysorbate 80 is present at a weight ratio of approximately 0.02% (w/v).

[0020] In certain embodiments, sucrose is present at a weight ratio of approximately 7% (w/v).

[0021] In yet additional embodiments, the invention relates to a lyophilized pharmaceutical formulation of an anti-human PD-1 antibody, or antigen binding fragment thereof, made by lyophilizing an aqueous solution comprising: a) 25-100 mg/mL anti-antibody, or antigen binding fragment thereof; b) about 70 mg/mL sucrose; c) about 0.2 mg/mL polysorbate 80; and d) about 10 mM histidine buffer at pH 5.0-6.0.

[0022] In certain embodiments, the anti-human PD-1 antibody, or antigen binding fragment thereof, is present at about 25 mg/mL in the aqueous solution. In certain embodiments, the aqueous solution has a pH of about 5.5.

[0023] In yet additional embodiments, the invention relates to a lyophilized pharmaceutical formulation of an anti-human PD-1 antibody, or antigen binding fragment thereof; that when reconstituted comprises: a) 25-100 mg/mL anti-human PD-1 antibody, or antigen binding fragment thereof; b) about 70 mg/mL sucrose; c) about 0.2 mg/mL polysorbate 80; and d) about 10 mM Histidine buffer at about pH 5.0-pH 6.0.

[0024] In certain embodiments, the anti-human PD-1 antibody, or antigen binding fragment thereof; is present at about 25 mg/mL in the reconstituted solution. In certain embodiments, the reconstituted solution has a pH of about 5.5.

[0025] In yet additional embodiments, the invention relates to a liquid pharmaceutical formulation of an anti-human PD-1 antibody, or antigen binding fragment thereof comprising: a) 25-100 mg/mL anti-antibody, or antigen binding fragment thereof; b) about 70 mg/mL sucrose; c) about 0.2 mg/mL polysorbate 80; and d) about 10 mM histidine buffer at pH 5.0-6.0.

[0026] In yet additional embodiments, the invention relates to a pharmaceutical formulation of an anti-human PD-1 antibody, or antigen binding fragment thereof comprising: a) said anti-human PD-1 antibody, or antigen binding fragment thereof; b) histidine buffer; c) polysorbate 80; and d) sucrose. In certain embodiments, the formulation has a pH between 5.0 and 6.0 when reconstituted. In certain embodiments, the polysorbate 80 is present at a weight ratio of approximately 0.02% (w/v). In certain embodiments, the sucrose is present at a weight ratio of approximately 7% (w/v).

[0027] In yet additional embodiments, the invention relates to any of the formulations described herein, wherein the antibody, or antigen binding fragment thereof; comprises a light chain comprising three CDR sequences selected from the group consisting of SEQ ID NOs: 9, 10, 11, 15, 16, and 17.

[0028] In yet additional embodiments, the invention relates to any of the formulations described herein, wherein the antibody, or antigen binding fragment thereof; comprises a heavy chain comprising three CDR sequences selected from the group consisting of SEQ ID NOs: 12, 13, 14, 18, 19, and 20.

[0029] In yet additional embodiments, the invention relates to any of the formulations described herein, wherein the antibody, or antigen binding fragment thereof, comprises: i) a light chain comprising three CDR sequences SEQ ID NOs: 15, 16, and 17; and ii) a heavy chain comprising three CDR sequences SEQ ID NOs: 8, 19, and 20.

[0030] In yet additional embodiments, the invention relates to any of the formulations described herein, wherein the antibody, or antigen binding fragment thereof, comprises a light chain variable domain comprising amino acid residues 20 to 130 of SEQ ID NO:32.

[0031] In yet additional embodiments, the invention relates to any of the formulations described herein, wherein the antibody, or antigen binding fragment thereof, comprises a heavy chain variable domain comprising SEQ ID NO:31.

[0032] In yet additional embodiments, the invention relates to any of the formulations described herein, wherein the antibody, or antigen binding fragment thereof, comprises: i) a light chain comprising amino acid residues 20 to 237 of SEQ ID NO: 36 and ii) a heavy chain comprising amino acid residues 20 to 466 of SEQ ID NO: 31.

[0033] In yet additional embodiments, the invention relates to any of the formulations described herein, wherein the antibody is selected from the group consisting of h409A11, h409A16, and h409A17.

[0034] In yet additional embodiments, the invention relates to a method of treating chronic infection in a mammalian subject in need thereof comprising: administering an effective amount of any of the formulations described herein.

[0035] In yet additional embodiments, the invention relates to a method of treating cancer in a mammalian subject in need thereof, the method comprising administering an effective amount of any of the formulations described herein. In certain embodiments, the effective amount comprises a dose of anti-human PD-1 antibody selected from the group consisting of the 1.0, 3.0, and 10 mg/kg.

BRIEF DESCRIPTION OF THE DRAWINGS

[0036] FIGS. **1**A-B show stability data for lyophilized formulations of h409A11 at pH 5.5 stored at 5° C. (24 months).

[0037] FIGS. **2**A-B show stability data for lyophilized formulations of h409A11 at pH 5.5 stored at 25H conditions (25° C., 60% RH, 12 months).

[0038] FIGS. **3**A-B show stability data for lyophilized formulations of h409A11 at pH 5.5 stored at RH4 conditions (40° C., 75% RH, 6 months).

[0039] FIGS. **4**A-B show stability data for lyophilized formulations of h409A11 stored at 5° C. (24 months).

[0040] FIGS. **5**A-B show stability data for lyophilized formulations of h409A11 at pH 5.5 stored at 25H conditions (25° C., 60% RH, 6 months).

[0041] FIGS. **6**A-B show stability data for lyophilized formulations of h409A11 at pH 5.5 stored at RH4 conditions (40° C., 75% RH, 6 months).

[0042] FIGS. **7**A-B show stability data for lyophilized formulations of h409A11 stored at 5° C. (24 months).

[0043] FIGS. **8**A-B show stability data for lyophilized formulations of h409A11 25H conditions (25° C., 60% RH, 6 months).

[0044] FIGS. **9**A-B show stability data for lyophilized formulations of h409A11 at RH4 conditions (40° C., 75% RH, 6 months).

DETAILED DESCRIPTION

[0045] The present invention provides formulations of anti-PD-1 antibodies and uses thereof for treating various cancers and infectious diseases.

[0046] Anti-PD-1 antibody h409A11 is an exemplary antibody in the stable formulations described herein. Three humanized anti-PD-1 monoclonal antibodies (i.e., h409A11, h409A16, and h509A17) suitable for the present formulations are described in co-pending patent publication WO2008/156712. Additionally, formulations described herein are useful for treating certain cancers as well as chronic infections. Table 2 provides a list of the corresponding CDR sequences for h409A11. Table 6 provides a list of sequences of exemplary anti-PD-1 antibodies.

[0047] In accordance with the present invention there may be employed conventional molecular biology, microbiology, protein expression and purification, antibody, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook et al. (2001) *Molecular Cloning: A Laboratory Manual.* 3rd ed. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, N.Y.; Ausubel et al. eds. (2005) *Current Protocols in Molecular Biology*. John Wiley and Sons, Inc.: Hoboken, N.J.; Bonifacino et al. eds. (2005) *Current Protocols in Cell Biology*. John Wiley and Sons, Inc.: Hoboken, N.J.; Coligan et al. eds. (2005) *Current Protocols in Immunology*, John Wiley and Sons, Inc.: Hoboken, N.J.; Coico et al. eds. (2005) *Current Protocols in Microbiology*, John Wiley and Sons, Inc.: Hoboken, N.J.; Coligan et al. eds. (2005) *Current Protocols in Protein Science*, John Wiley and Sons, Inc.: Hoboken, N.J.; and Enna et al. eds. (2005) *Current Protocols in Pharmacology*, John Wiley and Sons, Inc.: Hoboken, N.J.; *Nucleic Acid Hybridization*, Hames & Higgins eds. (1985); *Transcription And Translation*, Hames & Higgins, eds. (1984); *Animal Cell Culture Freshney*, ed. (1986); *Immobilized Cells And Enzymes*, IRL Press (1986); Perbal, *A Practical Guide To Molecular Cloning* (1984); and Harlow and Lane. *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory Press: 1988).

I. DEFINITIONS

[0048] As used herein, the term "antibody" refers to any form of antibody that exhibits the desired biological activity. Thus, it is used in the broadest sense and specifically covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), chimeric antibodies, humanized antibodies, fully human antibodies, etc. so long as they exhibit the desired biological activity.

Adjuvant

[0049] As used herein, the term "adjuvant" refers to a compound or mixture that enhances the immune response to an antigen. An adjuvant can serve as a tissue depot that slowly releases the antigen and also as a lymphoid system activator that non-specifically enhances the immune response (Hood et al., *Immunology, Second Ed.,* 1984, Benjamin/Cummings: Menlo Park, Calif., p. 384). Often, a primary challenge with an antigen alone, in the absence of an adjuvant, will fail to elicit a humoral or cellular immune response. Adjuvants include, but are not limited to, complete Freund's adjuvant, incomplete Freund's adjuvant, saponin, mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil or hydrocarbon emulsions, keyhole limpet hemocyanins, and potentially useful human adjuvants such as N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine, N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine, BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*. Preferably, the adjuvant is pharmaceutically acceptable.

Cytokine

[0050] The term "cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, chemokines, and traditional polypeptide hormones. Examplary cytokines include: human IL-2, IFN-γ, IL-6, TNFα, IL-17, and IL-5.

Cytotoxic Agent

[0051] The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g., I^{131}, I^{125}, Y^{90} and Re^{186}) chemotherapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

Therapeutic Uses and Methods

[0052] The PD-1 blocking agents include those which specifically bind to human PD-1, can be used to increase, enhance, stimulate or up-regulate an immune response. Desirable subjects include human patients in need of enhancement of an immune response including patients with cancer and/or a chronic viral infection.

Cancer

[0053] The terms “cancer”, “cancerous”, or “malignant” refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to, carcinoma, lymphoma, leukemia, blastoma, and sarcoma. More particular examples of such cancers include squamous cell carcinoma, myeloma, small-cell lung cancer, non-small cell lung cancer, glioma, hodgkin’s lymphoma, non-hodgkin’s lymphoma, gastrointestinal (tract) cancer, renal cancer, ovarian cancer, liver cancer, lymphoblastic leukemia, lymphocytic leukemia, colorectal cancer, endometrial cancer, kidney cancer, prostate cancer, thyroid cancer, melanoma, chondrosarcoma, neuroblastoma, pancreatic cancer, glioblastoma multiforme, cervical cancer, brain cancer, stomach cancer, bladder cancer, hepatoma, breast cancer, colon carcinoma, and head and neck cancer.

[0054] PD-1 blocking agents include those used to treat cancer (i.e., to inhibit the growth or survival of tumor cells). Preferred cancers whose growth may be inhibited using anti-PD-1 antibodies such as humanized anti-PD-1 antibody h409A11 and include cancers typically responsive to immunotherapy, but also cancers that have not hitherto been associated with immunotherapy. Non-limiting examples of preferred cancers for treatment include melanoma (e.g., metastatic malignant melanoma), renal cancer (e.g. clear cell carcinoma), prostate cancer (e.g. hormone refractory prostate adenocarcinoma), pancreatic adenocarcinoma, breast cancer, colon cancer, lung cancer (e.g. non-small cell lung cancer), esophageal cancer, squamous cell carcinoma of the head and neck, liver cancer, ovarian cancer, cervical cancer, thyroid cancer, glioblastoma, glioma, leukemia, lymphoma, and other neoplastic malignancies. Malignancies that demonstrate improved disease-free and overall survival in relation to the presence of tumor-infiltrating lymphocytes in biopsy or surgical material, e.g. melanoma, colorectal, liver, kidney, stomach/esophageal, breast, pancreas, and ovarian cancer are encompassed in the methods and treatments described herein. Such cancer subtypes are known to be susceptible to immune control by T lymphocytes. Additionally, included are refractory or recurrent malignancies whose growth may be inhibited using the antibodies described herein. Particularly preferred cancers include those characterized by elevated expression of PD-1 and/or its ligands PD-L1 and/or PD-L2 in tested tissue samples, including: ovarian, renal, colorectal, pancreatic, breast, liver, gastric, esophageal cancers and melanoma. Additional cancers that can benefit from treatment with anti-PD-1 antibodies such as humanized anti-PD-1 antibody h409A11 include those associated with persistent infection with viruses such as human immunodeficiency viruses, hepatitis viruses class A, B and C, Epstein Barr virus, human papilloma viruses that are known to be causally related to for instance Kaposi’s sarcoma, liver cancer, nasopharyngeal cancer, lymphoma, cervical, vulval, anal, penile and oral cancers.

Chemotherapeutic Agent

[0055] A “chemotherapeutic agent” is a chemical compound useful in the treatment of cancer. Anti-PD-1 antibodies can be used with any one or more suitable chemotherapeutic agent. Examples of such chemotherapeutic agents include alkylating agents such as thiotepa and cyclosphosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphoramide and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); a camptothecin (including the synthetic analogue topotecan); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CBI-TMI); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibiotics such as the enediyne antibiotics (e.g. calicheamicin, especially calicheamicin gamma1I and calicheamicin phiI1, see, e.g., Agnew, Chem. Intl. Ed. Engl., 33:183-186 (1994); dynemicin, including dynemicin A; bisphosphonates, such as clodronate; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antibiotic chromomophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabicin, caminomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; losoxantrone; podophyllinic acid; 2-ethylhydrazide; procarbazine; razoxane; rhizoxin; sizofuran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacy-

tosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, e.g. paclitaxel and doxetaxel; chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine; vinorelbine; novantrone; teniposide; edatrexate; daunomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoids such as retinoic acid; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included are anti-hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens and selective estrogen receptor modulators (SERMs), including, for example, tamoxifen, raloxifene, droloxifene, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and toremifene (Fareston); aromatase inhibitors that inhibit the enzyme aromatase, which regulates estrogen production in the adrenal glands, such as, for example, 4(5)-imidazoles, aminoglutethimide, megestrol acetate, exemestane, formestane, fadrozole, vorozole, letrozole, and anastrozole; and anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

Growth Inhibitory Agent

[0056] A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell, especially cancer cell over expressing any of the genes identified herein, either in vitro or in vivo. Thus, the growth inhibitory agent is one which significantly reduces the percentage of cells over expressing such genes in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine) taxanes, and topo II inhibitors such as doxorubicin, epirubicin, daunorubicin, and etoposide. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as dacarbazine, mechlorethamine, and cisplatin. Further information can be found in *The Molecular Basis of Cancer*, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogens, and antineoplastic drugs" by Murakami et al. (WB Saunders: Philadelphia, 1995).

Antibody or Antibody Fragments in Combination with Additional Agents

[0057] Anti-PD-1 antibody or antibody fragments can be used alone or in combination with: other anti-neoplastic agents or immunogenic agents (for example, attenuated cancerous cells, tumor antigens (including recombinant proteins, peptides, and carbohydrate molecules), antigen presenting cells such as dendritic cells pulsed with tumor derived antigen or nucleic acids, immune stimulating cytokines (for example, IL-2, IFNα2, GM-CSF), and cells transfected with genes encoding immune stimulating cytokines such as but not limited to GM-CSF); standard cancer treatments (for example, chemotherapy, radiotherapy or surgery); or other antibodies (including but not limited to antibodies to VEGF, EGFR, Her2/neu, VEGF receptors, other growth factor receptors, CD20, CD40, CD-40L, CTLA-4, OX-40, 4-1BB, and ICOS).

Infectious Diseases

[0058] Antagonist anti-PD-1 antibodies or antibody fragments can also be used to prevent or treat infections and infectious disease. These agents can be used alone, or in combination with vaccines, to stimulate the immune response to pathogens, toxins, and self-antigens. The antibodies or antigen-binding fragment thereof can be used to stimulate immune response to viruses infectious to humans, including but not limited to: human immunodeficiency viruses, hepatitis viruses class A, B and C, Epstein Barr virus, human cytomegalovirus, human papilloma viruses, and herpes viruses. Antagonist anti-PD-1 antibodies or antibody fragments can be used to stimulate immune response to infection with bacterial or fungal parasites, and other pathogens. Viral infections with hepatitis B and C and HIV are among those considered to be chronic viral infections.

[0059] As used herein, the terms "PD-1 binding fragment," "antigen binding fragment thereof," "binding fragment thereof" or "fragment thereof" encompass a fragment or a derivative of an antibody that still substantially retains its biological activity of binding to antigen (human PD-1) and inhibiting its activity (e.g., blocking the binding of PD-1 to PDL1 and PDL2). Therefore, the term "antibody fragment" or PD-1 binding fragment refers to a portion of a full length antibody, generally the antigen binding or variable region thereof. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules, e.g., sc-Fv; and multispecific antibodies formed from antibody fragments. Typically, a binding fragment or derivative retains at least 10% of its PD-1 inhibitory activity. Preferably, a binding fragment or derivative retains at least 25%, 50%, 60%, 70%, 80%, 90%, 95%, 99% or 100% (or more) of its PD-1 inhibitory activity, although any binding fragment with sufficient affinity to exert the desired biological effect will be useful. It is also intended that a PD-1 binding fragment can include variants having conservative amino acid substitutions that do not substantially alter its biologic activity.

[0060] A "domain antibody" is an immunologically functional immunoglobulin fragment containing only the variable region of a heavy chain or the variable region of a light chain. In some instances, two or more V_H regions are covalently joined with a peptide linker to create a bivalent domain antibody. The two V_H regions of a bivalent domain antibody may target the same or different antigens.

[0061] A "bivalent antibody" comprises two antigen binding sites. In some instances, the two binding sites have the same antigen specificities. However, bivalent antibodies may be bispecific. As used herein, the term "bispecific antibody" refers to an antibody, typically a monoclonal antibody, having binding specificities for at least two different antigenic epitopes. In one embodiment, the epitopes are from the same antigen. In another embodiment, the epitopes are from two different antigens. Methods for making bispecific antibodies are known in the art. For example, bispecific antibodies can be produced recombinantly using the co-expression of two immunoglobulin heavy chain/light chain pairs. See, e.g., Milstein et al. (1983) *Nature* 305: 537-39. Alternatively, bispecific antibodies can be prepared using chemical linkage. See, e.g., Brennan et al. (1985) *Science* 229:81. Bispecific antibodies include bispecific antibody fragments. See, e.g., Holliger et al. (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:6444-48, Gruber et al. (1994) *J. Immunol.* 152:5368.

[0062] As used herein, the term "single-chain Fv" or "scFv" antibody refers to antibody fragments comprising the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Generally, the Fv

polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv, see Pluckthun (1994) THE PHARMACOLOGY OF MONOCLONAL ANTIBODIES, vol. 113, Rosenburg and Moore eds. Springer-Verlag, New York, pp. 269-315.

[0063] The monoclonal antibodies herein also include camelized single domain antibodies. See, e.g., Muyldermans et al. (2001) *Trends Biochem. Sci.* 26:230; Reichmann et al. (1999) *J. Immunol. Methods* 231:25; WO 94/04678; WO 94/25591; U.S. Pat. No. 6,005,079). Single domain antibodies comprising two V_H domains with modifications such that single domain antibodies are formed are also included.

[0064] As used herein, the term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy chain variable domain (V_H) connected to a light chain variable domain (V_L) in the same polypeptide chain (V_H-V_L or V_L-V_H). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, e.g., EP 404,097; WO 93/11161; and Holliger et al. (1993) *Proc. Natl. Acad. Sci. USA* 90: 6444-6448. For a review of engineered antibody variants generally see Holliger and Hudson (2005) *Nat. Biotechnol.* 23:1126-1136.

[0065] As used herein, the term "humanized antibody" refers to forms of antibodies that contain sequences from non-human (e.g., murine) antibodies as well as human antibodies. Such antibodies contain minimal sequence derived from non-human immunoglobulin. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. The humanized forms of rodent antibodies will generally comprise the same CDR sequences of the parental rodent antibodies, although certain amino acid substitutions may be included to increase affinity, increase stability of the humanized antibody, or for other reasons.

[0066] The antibodies of the present invention also include antibodies with modified (or blocked) Fc regions to provide altered effector functions. See, e.g., U.S. Pat. No. 5,624,821; WO2003/086310; WO2005/120571; WO2006/0057702; Presta (2006) *Adv. Drug Delivery Rev.* 58:640-656. Such modification can be used to enhance or suppress various reactions of the immune system, with possible beneficial effects in diagnosis and therapy. Alterations of the Fc region include amino acid changes (substitutions, deletions and insertions), glycosylation or deglycosylation, and adding multiple Fc. Changes to the Fc can also alter the half-life of antibodies in therapeutic antibodies, and a longer half-life would result in less frequent dosing, with the concomitant increased convenience and decreased use of material. See Presta (2005) *J. Allergy Clin. Immunol.* 116:731 at 734-35.

[0067] The term "fully human antibody" refers to an antibody that comprises human immunoglobulin protein sequences only. A fully human antibody may contain murine carbohydrate chains if produced in a mouse, in a mouse cell, or in a hybridoma derived from a mouse cell. Similarly, "mouse antibody" refers to an antibody which comprises mouse immunoglobulin sequences only. A fully human antibody may be generated in a human being, in a transgenic animal having human immunoglobulin germline sequences, by phage display or other molecular biological methods.

[0068] As used herein, the term "hypervariable region" refers to the amino acid residues of an antibody that are responsible for antigen-binding. The hypervariable region comprises amino acid residues from a "complementarity determining region" or "CDR" (e.g. residues 24-34 (CDRL1), 50-56 (CDRL2) and 89-97 (CDRL3) in the light chain variable domain and residues 31-35 (CDRH1), 50-65 (CDRH2) and 95-102 (CDRH3) in the heavy chain variable domain (Kabat et al. (1991) Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md.) and/or those residues from a "hypervariable loop" (i.e. residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain (Chothia and Lesk (1987) *J. Mol. Biol.* 196: 901-917). As used herein, the term "framework" or "FR" residues refers to those variable domain residues other than the hypervariable region residues defined herein as CDR residues. The residue numbering above relates to the Kabat numbering system and does not necessarily correspond in detail to the sequence numbering in the accompanying Sequence Listing.

[0069] "Conservatively modified variants" or "conservative substitution" refers to substitutions of amino acids are known to those of skill in this art and may be made generally without altering the biological activity of the resulting molecule, even in essential regions of the polypeptide. Such exemplary substitutions are preferably made in accordance with those set forth in Table 1 as follows:

TABLE 1

Exemplary Conservative Amino Acid Substitutions

Original residue	Conservative substitution
Ala (A)	Gly; Ser
Arg (R)	Lys, His
Asn (N)	Gln; His
Asp (D)	Glu; Asn
Cys (C)	Ser; Ala
Gln (Q)	Asn
Glu (E)	Asp; Gln
Gly (G)	Ala
His (H)	Asn; Gln
Ile (I)	Leu; Val
Leu (L)	Ile; Val
Lys (K)	Arg; His
Met (M)	Leu; Ile; Tyr
Phe (F)	Tyr; Met; Leu
Pro (P)	Ala
Ser (S)	Thr
Thr (T)	Ser
Trp (W)	Tyr; Phe
Tyr (Y)	Trp; Phe
Val (V)	Ile; Leu

[0070] In addition, those of skill in this art recognize that, in general, single amino acid substitutions in non-essential regions of a polypeptide do not substantially alter biological activity. See, e.g., Watson et al. (1987) *Molecular Biology of the Gene*, The Benjamin/Cummings Pub. Co., p. 224 (4th Edition).

[0071] The phrase "consists essentially of," or variations such as "consist essentially of" or "consisting essentially of," as used throughout the specification and claims, indicate the inclusion of any recited elements or group of elements, and the optional inclusion of other elements, of similar or different nature than the recited elements, that do not materially change the basic or novel properties of the specified dosage regimen, method, or composition. As a non-limiting example, a binding compound that consists essentially of a recited amino acid sequence may also include one or more amino acids, including substitutions of one or more amino acid residues, that do not materially affect the properties of the binding compound.

[0072] "Immune condition" or "immune disorder" encompasses, e.g., pathological inflammation, an inflammatory disorder, and an autoimmune disorder or disease. "Immune condition" also refers to infections, persistent infections, and proliferative conditions, such as cancer, tumors, and angiogenesis, including infections, tumors, and cancers that resist eradication by the immune system. "Cancerous condition" includes, e.g., cancer, cancer cells, tumors, angiogenesis, and precancerous conditions such as dysplasia.

[0073] The antibody, or binding composition derived from the antigen-binding site of an antibody, of the contemplated formulation or method binds to its antigen with an affinity that is at least two fold greater, preferably at least ten times greater, more preferably at least 20-times greater, and most preferably at least 100-times greater than the affinity with unrelated antigens. In a preferred embodiment the antibody will have an affinity that is greater than about 10^9 liters/mol, as determined, e.g., by Scatchard analysis. Munsen et al. (1980) *Analyt. Biochem.* 107:220-239.

Pharmaceutical Composition Definitions

[0074] The term "bulking agents" comprise agents that provide the structure of the freeze-dried product. Common examples used for bulking agents include mannitol, glycine, lactose and sucrose. In addition to providing a pharmaceutically elegant cake, bulking agents may also impart useful qualities in regard to modifying the collapse temperature, providing freeze-thaw protection, and enhancing the protein stability over long-term storage. These agents can also serve as tonicity modifiers.

[0075] The term "buffer" encompasses those agents which maintain the solution pH in an acceptable range prior to lyophilization and may include succinate (sodium or potassium), histidine, phosphate (sodium or potassium), Tris (tris (hydroxymethyl)aminomethane), diethanolamine, citrate (sodium) and the like. The buffer of this invention has a pH in the range from about 5.0 to about 6.0; and preferably has a pH of about 5.5. Examples of buffers that will control the pH in this range include succinate (such as sodium succinate), gluconate, histidine, citrate and other organic acid buffers. In arriving at the exemplary formulation, histidine, acetate and citrate buffers in the pH range of 5.0-6.0 were explored for suitability. Histine and acetate buffer systems performed better than the citrate system. Histidine buffer is a preferred buffer system, because acetate buffer systems are not compatible with the lyophilization process.

[0076] The term "cryoprotectants" generally includes agents which provide stability to the protein against freezing-induced stresses, presumably by being preferentially excluded from the protein surface. They may also offer protection during primary and secondary drying, and long-term product storage. Examples are polymers such as dextran and polyethylene glycol; sugars such as sucrose, glucose, trehalose, and lactose; surfactants such as polysorbates; and amino acids such as glycine, arginine, and serine.

[0077] The terms "lyophilization," "lyophilized," and "freeze-dried" refer to a process by which the material to be dried is first frozen and then the ice or frozen solvent is removed by sublimation in a vacuum environment. An excipient may be included in pre-lyophilized formulations to enhance stability of the lyophilized product upon storage.

[0078] The term "lyoprotectant" includes agents that provide stability to the protein during the drying or 'dehydration' process (primary and secondary drying cycles), presumably by providing an amorphous glassy matrix and by binding with the protein through hydrogen bonding, replacing the water molecules that are removed during the drying process. This helps to maintain the protein conformation, minimize protein degradation during the lyophilization cycle and improve the long-term product stability. Examples include polyols or sugars such as sucrose and trehalose.

[0079] The term "pharmaceutical formulation" refers to preparations which are in such form as to permit the active ingredients to be effective, and which contains no additional components which are toxic to the subjects to which the formulation would be administered.

[0080] "Pharmaceutically acceptable" excipients (vehicles, additives) are those which can reasonably be administered to a subject mammal to provide an effective dose of the active ingredient employed.

[0081] "Reconstitution time" is the time that is required to rehydrate a lyophilized formulation with a solution to a particle-free clarified solution.

[0082] A "stable" formulation is one in which the protein therein essentially retains its physical stability and/or chemical stability and/or biological activity upon storage. Various analytical techniques for measuring protein stability are available in the art and are reviewed in Peptide and Protein Drug Delivery, 247-301, Vincent Lee Ed., Marcel Dekker, Inc., New York, N.Y., Pubs. (1991) and Jones, A. Adv. Drug Delivery Rev. 10:29-90 (1993). Stability can be measured at a selected temperature for a selected time period.

[0083] A "stable" lyophilized antibody formulation is a lyophilized antibody formulation with no significant changes observed at a refrigerated temperature (2-8° C.) for at least 12 months, preferably 2 years, and more preferably 3 years; or at room temperature (23-27° C.) for at least 3 months, preferably 6 months, and more preferably 1 year. Typical acceptable criteria for stability are as follows. No more than 10%, preferably 5%, of antibody monomer is degraded as measured by SEC-HPLC. The rehydrated solution is typically colorless, or clear to slightly opalescent by visual analysis. The concentration, pH and osmolality of the formulation have no more than $^{+/-}$10% change. Potency is typically within a range of 50-150% of the reference. No more than 10%, preferably 5% of clipping is observed. No more than 10%, preferably 5% of aggregation is formed.

[0084] A "stable" pharmaceutical antibody formulation (including a lyophilized formulation, a reconstituted liquid, as well as a liquid formulation that is a "final" formulation (i.e., has not been previously lyophilized)) is a pharmaceutical antibody formulation with no significant changes observed at a refrigerated temperature (2-8° C.) for at least 3 months, preferably 6 months, and more preferably 1 year, and even more preferably up through 2 years. Additionally, a

"stable" liquid formulation includes one that exhibits desired features at temperatures including at 25° C. and 40° C. for periods including 1 month, 3 months, 6 months, 12 months, and/or 24 months. Typical acceptable criteria for stability stability are as follows. Typically, no more than about 10%, preferably about 5%, of antibody monomer is degraded as measured by SEC-HPLC. The pharmaceutical antibody formulation is colorless, or clear to slightly opalescent by visual analysis. The concentration, pH and osmolality of the formulation have no more than $^{+/-}$10% change. Potency is typically within 50-150 of the reference. Typically, no more than about 10%, preferably about 5% of clipping is observed. Typically, no more than about 10%, preferably about 5% of aggregation is formed.

[0085] An antibody "retains its physical stability" in a pharmaceutical formulation if it shows no significant increase of aggregation, precipitation and/or denaturation upon visual examination of color and/or clarity, or as measured by UV light scattering, size exclusion chromatography (SEC) and dynamic light scattering. The changes of protein conformation can be evaluated by fluorescence spectroscopy, which determines the protein tertiary structure, and by FTIR spectroscopy, which determines the protein secondary structure.

[0086] An antibody "retains its chemical stability" in a pharmaceutical formulation, if it shows no significant chemical alteration. Chemical stability can be assessed by detecting and quantifying chemically altered forms of the protein. Degradation processes that often alter the protein chemical structure include hydrolysis or clipping (evaluated by methods such as size exclusion chromatography and SDS-PAGE), oxidation (evaluated by methods such as by peptide mapping in conjunction with mass spectroscopy or MALDI/TOF/MS), deamidation (evaluated by methods such as ion-exchange chromatography, capillary isoelectric focusing, peptide mapping, isoaspartic acid measurement), and isomerization (evaluated by measuring the isoaspartic acid content, peptide mapping, etc.).

[0087] An antibody "retains its biological activity" in a pharmaceutical formulation, if the biological activity of the antibody at a given time is within a predetermined range of the biological activity exhibited at the time the pharmaceutical formulation was prepared. The biological activity of an antibody can be determined, for example, by an antigen binding assay.

[0088] The term "isotonic" means that the formulation of interest has essentially the same osmotic pressure as human blood. Isotonic formulations will generally have an osmotic pressure from about 270-328 mOsm. Slightly hypotonic pressure is 250-269 and slightly hypertonic pressure is 328-350 mOsm. Osmotic pressure can be measured, for example, using a vapor pressure or ice-freezing type osmometer.

[0089] Tonicity Modifiers: Salts (NaCl, KCl, $MgCl_2$, $CaCl_2$, etc.) are used as tonicity modifiers to control osmotic pressure. In addition, cryprotecants/lyoprotectants and/or bulking agents such as sucrose, mannitol, glycine etc. can serve as tonicity modifiers.

Analytical Methods

[0090] Analytical methods suitable for evaluating the product stability include size exclusion chromatography (SEC), dynamic light scattering test (DLS), differential scanning calorimetery (DSC), iso-asp quantification, potency, UV at 340 nm, UV spectroscopy, and FTIR. SEC (J. Pharm. Scien., 83:1645-1650, (1994); Pharm. Res., 11:485 (1994); J. Pharm. Bio. Anal., 15:1928 (1997); J. Pharm. Bio. Anal., 14:1133-1140 (1986)) measures percent monomer in the product and gives information of the amount of soluble aggregates. DSC (Pharm. Res., 15:200 (1998); Pharm. Res., 9:109 (1982)) gives information of protein denaturation temperature and glass transition temperature. DLS (American Lab., November (1991)) measures mean diffusion coefficient, and gives information of the amount of soluble and insoluble aggregates. UV at 340 nm measures scattered light intensity at 340 nm and gives information about the amounts of soluble and insoluble aggregates. UV spectroscopy measures absorbance at 278 nm and gives information of protein concentration. FTIR (Eur. J. Pharm. Biopharm., 45:231 (1998); Pharm. Res., 12:1250 (1995); J. Pharm. Scien., 85:1290 (1996); J. Pharm. Scien., 87:1069 (1998)) measures IR spectrum in the amide one region, and gives information of protein secondary structure.

[0091] The iso-asp content in the samples is measured using the Isoquant Isoaspartate Detection System (Promega). The kit uses the enzyme Protein Isoaspartyl Methyltransferase (PIMT) to specifically detect the presence of isoaspartic acid residues in a target protein. PIMT catalyzes the transfer of a methyl group from S-adenosyl-L-methionine to isoaspartic acid at the .alpha.-carboxyl position, generating S-adenosyl-L-homocysteine (SAH) in the process. This is a relatively small molecule, and can usually be isolated and quantitated by reverse phase HPLC using the SAH HPLC standards provided in the kit.

[0092] The potency or bioidentity of an antibody can be measured by its ability to bind to its antigen. The specific binding of an antibody to its antigen can be quantitated by any method known to those skilled in the art, for example, an immunoassay, such as ELISA (enzyme-linked immunosorbant assay).

[0093] A "reconstituted" formulation is one that has been prepared by dissolving a lyophilized protein formulation in a diluent such that the protein is dispersed in the reconstituted formulation. The reconstituted formulation is suitable for administration, e.g. parenteral administration), and may optionally be suitable for subcutaneous administration.

Humanized Anti-PD-1 Antibodies

[0094] DNA constructs encoding the variable regions of the heavy and light chains of the humanized antibodies h409A11, h409A16 and h409A17 are described in WO2008/156712.

[0095] The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the culture deposited, since the deposited embodiment is intended as a single illustration of one aspect of the invention and any culture that is functionally equivalent is within the scope of this invention. The deposit of material herein does not constitute an admission that the written description herein contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific illustration that it represents. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

[0096] Sequences are provided for exemplary anti-human PD-1 antibodies; a summary table of the sequences is pro-

vided in Table 6. CDRs are provided under separate sequence identifiers, as indicated in Table 2 for h409A11.

[0097] Ordinarily, amino acid sequence variants of the humanized anti-PD-1 antibody will have an amino acid sequence having at least 75% amino acid sequence identity with the original humanized antibody amino acid sequences of either the heavy or the light chain more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, and most preferably at least 95, 98, or 99%. Identity or homology with respect to this sequence is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the humanized anti-PD-1 residues, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. None of N-terminal, C-terminal, or internal extensions, deletions, or insertions into the antibody sequence shall be construed as affecting sequence identity or homology.

[0098] The humanized antibody can be selected from any class of immunoglobulins, including IgM, IgG, IgD, IgA, and IgE. Preferably, the antibody is an IgG antibody. Any isotype of IgG can be used, including IgG_1, IgG_2, IgG_3, and IgG_4. Different constant domains may be appended to the humanized V_L and V_H regions provided herein. For example, if a particular intended use of an antibody (or fragment) of the present invention were to call for altered effector functions, a heavy chain constant domain other than IgG1 may be used. Although IgG1 antibodies provide for long half-life and for effector functions, such as complement activation and antibody-dependent cellular cytotoxicity, such activities may not be desirable for all uses of the antibody. In such instances an IgG4 constant domain, for example, may be used.

[0099] Likewise, either class of light chain can be used in the compositions and methods herein. Specifically, kappa, lambda, or variants thereof are useful in the present compositions and methods.

[0100] CDR and FR residues are determined according to the standard sequence definition of Kabat. Kabat et al. (1987) Sequences of Proteins of Immunological Interest, National Institutes of Health, Bethesda Md.

[0101] The signal sequences, or nucleic acid sequences encoding the signal sequences, may be appended to the N-terminus of the respective antibody chains to create a precursor protein for secretion from a host cell. Alternative signal sequences may also be used, and several can be found at "SPdb: a Signal Peptide Database." Choo et al. (2005) *BMC Bioinformatics* 6:249.

TABLE 2

H409A11 CDR Sequences

Antibody	CDR Sequence	SEQ ID NO:
H409A11	Light chain CDR1 (equivalent to hPD-1.09A light chain CDR1) RASKGVSTSGYSYLH	15
H409A11	Light chain CDR2 (equivalent to hPD-1.09A light chain CDR2) LASYLES	16
H409A11	Light chain CDR3 (equivalent to hPD-1.09A light chain CDR3) QHSRDLPLT	17
H409A11	Heavy chain CDR1 (equivalent to hPD-1.09A heavy chain CDR1) NYYMY	18
H409A11	Heavy chain CDR2 (equivalent to hPD-1.09A heavy chain CDR2) GINPSNGGTNFNEKFKN	19
H409A11	Heavy chain CDR3 (equivalent to hPD-1.09A heavy chain CDR3) RDYRFDMGFDY	20

Biological Activity of Humanized Anti-PD-1

[0102] Formulations of the present invention include antibodies and fragments thereof that are biologically active when reconstituted or in liquid form. As used herein, the term "biologically active" refers to an antibody or antibody fragment that is capable of binding the desired the antigenic epitope and directly or indirectly exerting a biologic effect. Typically, these effects result from the failure of PD-1 to bind its ligands. As used herein, the term "specific" refers to the selective binding of the antibody to the target antigen epitope. Antibodies can be tested for specificity of binding by comparing binding to PD-1 to binding to irrelevant antigen or antigen mixture under a given set of conditions.

Lyophilized Pharmaceutical Compositions

[0103] Lyophilized formulations of therapeutic proteins provide several advantages. Lyophilized formulations in general offer better chemical stability than solution formulations, and thus increased half-life. A lyophilized formulation may also be reconstituted at different concentrations depending on clinical factors, such as route of administration or dosing. For example, a lyophilized formulation may be reconstituted at a high concentration (i.e. in a small volume) if necessary for subcutaneous administration, or at a lower concentration if administered intravenously. High concentrations may also be necessary if high dosing is required for a particular subject, particularly if administered subcutaneously where injection volume must be minimized. One such lyophilized antibody formulation is disclosed at U.S. Pat. No. 6,267,958, which is hereby incorporated by reference in its entirety. Lyophilized formulations of another therapeutic protein are disclosed at U.S. Pat. No. 7,247,707, which is hereby incorporated by reference in its entirety.

[0104] Typically, the lyophilized formulation is prepared in anticipation of reconstitution at high concentration of drug product (DP, in an exemplary embodiment humanized anti-PD-1 antibody h409A11, or antigen binding fragment thereof), i.e. in anticipation of reconstitution in a low volume of water. Subsequent dilution with water or isotonic buffer can then readily be used to dilute the DP to a lower concentration. Typically, excipients are included in a lyophilized formulation of the present invention at levels that will result in a roughly isotonic formulation when reconstituted at high DP concentration, e.g. for subcutaneous administration. Reconstitution in a larger volume of water to give a lower DP concentration will necessarily reduce the tonicity of the reconstituted solution, but such reduction may be of little

significance in non-subcutaneous, e.g. intravenous, administration. If isotonicity is desired at lower DP concentration, the lyophilized powder may be reconstituted in the standard low volume of water and then further diluted with isotonic diluent, such as 0.9% sodium chloride.

[0105] In an embodiment of the present invention, humanized anti-PD-1 antibody (or antigen binding fragment thereof) is formulated as a lyophilized powder for reconstituting and utilizing for intravenous administration. Exemplary formulations are described in Tables 3-4, and in FIGS. **1-9**. In certain embodiments, the antibody (or antigen binding fragment thereof) is provided at about 50 mg/vial, and is reconstituted with sterile water for injection prior to use. If desired, the reconstituted antibody may be aseptically diluted with 0.9% Sodium Chloride Injection USP in a sterile IV container. The target pH of the reconstituted formulation is 5.5±0.5. In various embodiments, the lyophilized formulation of the present invention enables reconstitution of the anti-PD-1 antibody to high concentrations, such as about 20, 25, 30, 40, 50, 60, 75, 100 or more mg/mL.

[0106] The present invention provides in certain embodiments, a lyophilized formulation comprising humanized anti-PD-1 antibody, a histidine buffer at about pH 5.5, or at about pH 5.0, for example at about 5.1, 5.2, 5.3, 5.4, 5.6, 5.7, 5.8, 5.9, or 6.0.

[0107] When a range of pH values is recited, such as "a pH between pH 5.5 and 6.0," the range is intended to be inclusive of the recited values. Unless otherwise indicated, the pH refers to the pH after reconstitution of the lyophilized formulations of the present invention. The pH is typically measured at 25° C. using standard glass bulb pH meter. As used herein, a solution comprising "histidine buffer at pH X" refers to a solution at pH X and comprising the histidine buffer, i.e. the pH is intended to refer to the pH of the solution.

[0108] The formulation in Table 3 reflects the weight of the components in a batch formulation, as lyophilized in vials, and as reconstituted. Lyophilized formulations are by definition essentially dry, and thus the concept of concentration is not useful in describing them. Describing a lyophilized formulation in the terms of the weight of the components in a unit dose vial is more useful, but is problematic because it varies for different doses or vial sizes. In describing the lyophilized formulations of the present invention, it is useful to express the amount of a component as the ratio of the weight of the component compared to the weight of the drug substance (DS) in the same sample (e.g. a vial). This ratio may be expressed as a percentage. Such ratios reflect an intrinsic property of the lyophilized formulations of the present invention, independent of vial size, dosing, and reconstitution protocol.

[0109] In other embodiments, the lyophilized formulation of anti-human PD-1 antibody, or antigen binding fragment, is defined in terms of the pre-lyophilization solution used to make the lyophilized formulation, such as the pre-lyophilization solution. In one embodiment the pre-lyophilization solution comprises antibody, or antigen-binding fragment thereof, at a concentration of about 25 mg/mL. Such pre-lyophilization solutions may be at pH 4.4-5.2 (including about 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, 5.1. and 5.2), e.g. preferably about pH 4.8, or about pH 5.5.

[0110] In yet other embodiments, the lyophilized formulation of anti-human PD-1 antibody, or antigen binding fragment, is defined in terms of the reconstituted solution generated from the lyophilized formulation, such as the reconstituted solution disclosed at Table 4.

[0111] Reconstituted solutions may comprise antibody, or antigen-binding fragment thereof, at concentrations of about 10, 15, 20, 25, 30, 40, 50, 60, 75, 80, 90 or 100 mg/mL or higher concentrations such as 150 mg/mL, 200 mg/mL, 250 mg/mL, or up to about 300 mg/mL. Such reconstituted solutions may be at about pH 5.5, or range from about pH 5.0 to about 6.0

[0112] The lyophilized formulations of the present invention are formed by lyophilization (freeze-drying) of a pre-lyophilization solution. Freeze-drying is accomplished by freezing the formulation and subsequently subliming water at a temperature suitable for primary drying. Under this condition, the product temperature is below the eutectic point or the collapse temperature of the formulation. Typically, the shelf temperature for the primary drying will range from about −30 to 25° C. (provided the product remains frozen during primary drying) at a suitable pressure, ranging typically from about 50 to 250 mTorr. The formulation, size and type of the container holding the sample (e.g., glass vial) and the volume of liquid will dictate the time required for drying, which can range from a few hours to several days (e.g. 40-60 hrs). A secondary drying stage may be carried out at about 0-40° C., depending primarily on the type and size of container and the type of protein employed. The secondary drying time is dictated by the desired residual moisture level in the product and typically takes at least about 5 hours. Typically, the moisture content of a lyophilized formulation is less than about 5%, and preferably less than about 3%. The pressure may be the same as that employed during the primary drying step. Freeze-drying conditions can be varied depending on the formulation and vial size.

[0113] In some instances, it may be desirable to lyophilize the protein formulation in the container in which reconstitution of the protein is to be carried out in order to avoid a transfer step. The container in this instance may, for example, be a 3, 5, 10, 20, 50 or 100 cc vial.

[0114] The lyophilized formulations of the present invention are reconstituted prior to administration. The protein may be reconstituted at a concentration of about 10, 15, 20, 25, 30, 40, 50, 60, 75, 80, 90 or 100 mg/mL or higher concentrations such as 150 mg/mL, 200 mg/mL, 250 mg/mL, or 300 mg/mL up to about 500 mg/mL. High protein concentrations are particularly useful where subcutaneous delivery of the reconstituted formulation is intended. However, for other routes of administration, such as intravenous administration, lower concentrations of the protein may be desired (e.g. from about 5-50 mg/mL).

[0115] Reconstitution generally takes place at a temperature of about 25° C. to ensure complete hydration, although other temperatures may be employed as desired. The time required for reconstitution will depend, e.g., on the type of diluent, amount of excipient(s) and protein. Exemplary diluents include sterile water, bacteriostatic water for injection (BWFI), a pH buffered solution (e.g. phosphate-buffered saline), sterile saline solution, Ringer's solution or dextrose solution.

[0116] The lyophilized formulations of the present invention are expected to be stable for at least about 36 months (based on the stability data from FIGS. **1-9**). In addition, the liquid formulation is expected to exhibit stability for at least

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24 months, based on 24 months of stability data from reconstituted h409A11 formulation in polypropylene tubes at 2-8° C.

[0117] In line with the results shown in FIGS. **1-9**, stability has been observed through 2 years for a refrigerated reconstituted formulation of h409A11. 2 mL samples in polypropylene tubes were stored at 5° C., and 25H and RH4 conditions and tested at initial, 1, 3, 6, 9, 12, 18, and 24 month periods. This reconstituted h409A11 formulation has the same substituents in the same concentration as a liquid h409A11 formulation (i.e., a formulation that was not lyophilized) and the stability is expected to be the same.

Liquid Pharmaceutical Compositions

[0118] A liquid antibody formulation can be made by taking the drug substance (e.g., anti-humanized PD-1) which is in liquid form (e.g., h409A11 in an aqueous pharmaceutical formulation) and buffer exchanging it into the desired buffer as the last step of the purification process. There is no lyophilization step in this embodiment. The drug substance in the final buffer is concentrated to a desired concentration. Excipients such as sucrose and polysorbate 80 are added to the drug substance and it is diluted using the appropriate buffer to final protein concentration. The final formulated drug substance is filtered using 0.22 μm filters and filled into a final container (e.g. glass vials). Such a liquid formulation is exemplified by a final liquid formulation comprising 10 mM histidine pH 5.5, 7% sucrose, 0.02% polysorbate 80, and 25 mg/mL h409A11.

[0119] Various literature references are available to facilitate selection of pharmaceutically acceptable carriers or excipients. See, e.g., *Remington's Pharmaceutical Sciences and U.S. Pharmacopeia: National Formulary*, Mack Publishing Company, Easton, Pa. (1984); Hardman et al. (2001) *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, McGraw-Hill, New York, N.Y.; Gennaro (2000) *Remington: The Science and Practice of Pharmacy*, Lippincott, Williams, and Wilkins, New York, N.Y.; Avis et al. (eds.) (1993) *Pharmaceutical Dosage Forms: Parenteral Medications*, Marcel Dekker, NY; Lieberman, et al. (eds.) (1990) *Pharmaceutical Dosage Forms: Tablets*, Marcel Dekker, NY; Lieberman et al. (eds.) (1990) *Pharmaceutical Dosage Forms: Disperse Systems*, Marcel Dekker, NY; Weiner and Kotkoskie (2000) *Excipient Toxicity and Safety*, Marcel Dekker, Inc., New York, N.Y.

[0120] Toxicity is a consideration in selecting the proper dosing of a therapeutic agent, such as a humanized anti-PD-1 antibody (or antigen binding fragment thereof). Toxicity and therapeutic efficacy of the antibody compositions can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio of LD_{50} to ED_{50}. Antibodies exhibiting high therapeutic indices are preferred. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized.

[0121] Suitable routes of administration may, for example, include parenteral delivery, including intramuscular, intradermal, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal. Drugs can be administered in a variety of conventional ways, such as intraperitoneal, parenteral, intraarterial or intravenous injection. Modes of administration in which the volume of solution must be limited (e.g. subcutaneous administration) require a lyophilized formulation to enable reconstitution at high concentration.

[0122] Alternately, one may administer the antibody in a local rather than systemic manner, for example, via injection of the antibody directly into a pathogen-induced lesion characterized by immunopathology, often in a depot or sustained release formulation. Furthermore, one may administer the antibody in a targeted drug delivery system, for example, in a liposome coated with a tissue-specific antibody, targeting, for example, pathogen-induced lesion characterized by immunopathology. The liposomes will be targeted to and taken up selectively by the afflicted tissue.

[0123] Selecting an administration regimen for a therapeutic depends on several factors, including the serum or tissue turnover rate of the entity, the level of symptoms, the immunogenicity of the entity, and the accessibility of the target cells in the biological matrix. Preferably, an administration regimen maximizes the amount of therapeutic delivered to the patient consistent with an acceptable level of side effects. Accordingly, the amount of biologic delivered depends in part on the particular entity and the severity of the condition being treated. Guidance in selecting appropriate doses of antibodies, cytokines, and small molecules are available. See, e.g., Wawrzynczak (1996) *Antibody Therapy*, Bios Scientific Pub. Ltd, Oxfordshire, UK; Kresina (ed.) (1991) *Monoclonal Antibodies, Cytokines and Arthritis*, Marcel Dekker, New York, N.Y.; Bach (ed.) (1993) *Monoclonal Antibodies and Peptide Therapy in Autoimmune Diseases*, Marcel Dekker, New York, N.Y.; Baert et al. (2003) *New Engl. J. Med.* 348: 601-608; Milgrom et al. (1999) *New Engl. J. Med.* 341:1966-1973; Slamon et al. (2001) *New Engl. J. Med.* 344:783-792; Beniaminovitz et al. (2000) *New Engl. J. Med.* 342:613-619; Ghosh et al. (2003) *New Engl. J. Med.* 348:24-32; Lipsky et al. (2000) *New Engl. J. Med.* 343:1594-1602; Physicians' Desk Reference 2003 (Physicians' Desk Reference, 57th Ed); Medical Economics Company; ISBN: 1563634457; 57th edition (November 2002).

[0124] Determination of the appropriate dose is made by the clinician, e.g., using parameters or factors known or suspected in the art to affect treatment or predicted to affect treatment. The appropriate dosage ("therapeutically effective amount") of the protein will depend, for example, on the condition to be treated, the severity and course of the condition, whether the protein is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the protein, the type of protein used, and the discretion of the attending physician. Generally, the dose begins with an amount somewhat less than the optimum dose and it is increased by small increments thereafter until the desired or optimum effect is achieved relative to any negative side effects. Important diagnostic measures include those of symptoms of, e.g., the inflammation or level of inflammatory cytokines produced. The antibody is suitably administered to the patient at one time or repeatedly. The antibody may be administered alone or in conjunction with other drugs or therapies.

[0125] A pharmaceutical antibody formulation can be administered by continuous infusion, or by doses at intervals

of, e.g., one day, 1-7 times per week, one week, two weeks, three weeks, monthly, bimonthly, etc. A preferred dose protocol is one involving the maximal dose or dose frequency that avoids significant undesirable side effects. A total weekly dose is generally at least 0.05 μg/kg, 0.2 μg/kg, 0.5 μg/kg, 1 μg/kg, 10 μg/kg, 100 μg/kg, 0.2 mg/kg, 1.0 mg/kg, 2.0 mg/kg, 10 mg/kg, 25 mg/kg, 50 mg/kg body weight or more. See, e.g., Yang et al. (2003) *New Engl. J. Med.* 349:427-434; Herold et al. (2002) *New Engl. J. Med.* 346:1692-1698; Liu et al. (1999) *J. Neurol. Neurosurg. Psych.* 67:451-456; Portielji et al. (20003) *Cancer Immunol. Immunother.* 52:133-144. The desired dose of a small molecule therapeutic, e.g., a peptide mimetic, natural product, or organic chemical, is about the same as for an antibody or polypeptide, on a moles/kg basis.

[0126] In certain embodiments, dosing will comprise administering to a subject escalating doses of 1.0, 3.0, and 10 mg/kg of the pharmaceutical formulation, i.e, a formulation comprising h409A11, over the course of treatment. The formulation comprising h409A11 can be a reconstituted liquid formulation, or it can be a liquid formulation not previously lyophilized. Time courses can vary, and can continue as long as desired effects are obtained. In certain embodiments, dose escalation will continue up to a dose of about 10 mg/kg. In certain embodiments, the subject will have a histological or cytological diagnosis of melanoma, or other form of solid tumor, and in certain instances, a subject may have non-measurable disease. In certain embodiments, the subject will have been treated with other chemotherapeutics, while in other embodiments, the subject will be treatment naïve.

[0127] In yet additional embodiments, the dosing regimen will comprise administering a dose of 1, 3, or 10 mg/kg of any of the pharmaceutical formulations described herein (i.e, a formulation comprising h409A 11), throughout the course of treatment. For such a constant dosing regimen, the interval between doses will be about 14 days (±2 days). In certain embodiments, the interval between doses will be about 21 days (±2 days).

[0128] In certain embodiments, the dosing regimen will comprise administering a dose of from about 0.005 mg/kg to about 10 mg/kg, with intra-patient dose escalation. In certain embodiments, a dose of 5 mg/kg or 10 mg/kg will be administered at intervals of every 3 weeks, or every 2 weeks. In yet additional embodiments, a dose of 3 mg/kg will be administered at three week intervals for melanoma patients or patients with other solid tumors. In these embodiments, patients should have non-resectable disease; however, patients may have had previous surgery.

[0129] In certain embodiments, a subject will be administered a 30 minute IV infusion of any of the pharmaceutical formulations described herein. In certain embodiments for the escalating dose, the dosing interval will be about 28 days ((±1 day) between the first and second dose. In certain embodiments, the interval between the second and third doses will be about 14 days (±2 days). In certain embodiments, the dosing interval will be about 14 days (±2 days), for doses subsequent to the second dose.

[0130] In certain embodiments, the use of cell surface markers and/ore cytokine markers, as described in co-pending patent publications WO2012/018538 or WO2008/156712 will be used in bioassays for monitoring, diagnostic, patient selection, and/or treatment regimens involving blockade of the PD-1 pathway.

[0131] Subcutaneous administration may performed by injected using a syringe, or using other injection devices (e.g. the Inject-ease® device); injector pens; or needleless devices (e.g. MediJector and BioJector®).

[0132] The broad scope of this invention is best understood with reference to the following examples, which are not intended to limit the inventions to the specific embodiments. The specific embodiments described herein are offered by way of example only, and the invention is to be limited by the terms of the appended claims, along with the full scope of equivalents to which such claims are entitled.

EXAMPLES

Example 1

Antibody Production

[0133] h409A11 is a humanized monoclonal antibody that binds to human PD-1 and blocks the interaction between PD-1 and its ligands PDL1 and PDL2. The antibody is an IgG4/kappa isotype with a stabilizing S228P sequence alteration in the Fc region. Table 2 provides a list of the CDR sequences. The theoretical molecular weights of the heavy and light chains derived from the amino acid sequences, excluding glycosylation, are 49.3 kDa and 23.7 kDa, respectively. The parental antibody (hPD-1.09A) was produced by immunizing mice with hPD-1 DNA. The h409A11 antibody was generated by humanization of the parental murine anti-human PD-1 antibody by the Medical Research Council (Cambridge, UK) using CDR grafting technology, (e.g., U.S. Pat. No. 5,225,539), as described in co-pending WO2008/156712.

[0134] An expression plasmid was constructed for expression of heavy and light chains of h409A11. The nucleotide sequences encoding the heavy and light chains, along with their respective promoters and poly A signal sequence, were confirmed by DNA sequence analysis. The expression vector was subsequently used to transfect a CHO cell line. An antibody-expressing clone was selected for the generation of a Master Seed Bank (MSB), based on growth, productivity, and production stability. This MSB was then used to prepare the antibody and to generate the Master Cell Bank (MCB).

[0135] Cells from the MCB were expanded in shake flasks, culture bags, and a seed bioreactor to generate the inoculum for a production bioreactor to produce the antibody product. Further processing included three chromatography steps (protein A affinity, cation exchange and anion exchange chromatography), two orthogonal viral clearance steps (low pH viral inactivation and viral reduction filtration), ultrafiltration/diafiltration, and a final 0.2 μm filtration step.

h409A11 Structure and Features

[0136] h409A11 is a highly selective humanized monoclonal antibody that blocks the interaction between human PD-1 and its ligands PD-L1 and PD-L2. h409A11 is heterogeneously glycosylated at asparagine 297 within the Fc domain of each heavy chain, yielding molecular weights typically ranging between 148.9 and 149.5 kDa, depending on the attached glycan chains. The amino acid sequences of the heavy and light chains of h409A11 are found in SEQ ID NO:31 and SEQ ID NO:36. The light chain without the leader sequences comprises amino acid residues 20 to 237 of SEQ ID NO: 36 and the heavy chain without the leader sequences comprises amino acid residues 20 to 466 of SEQ ID NO: 31.

Stable Humanized PD-1 Formulations

[0137] In certain embodiments, stable humanized PD-1 e.g., h409A11 is an aqueous solution stored under refrigerated conditions (temp. range: typically about 2-8° C., but under certain circumstances, the aqueous formulation may exhibit stability at other temperatures including at about 25° C. and about 40° C. for periods of up to about 12 months) at a concentration of ≥25 mg/mL in 10 mM Histidine buffer, pH 5.0-6.0. In certain embodiments, stable humanized PD-1 e.g., h409A11 is an aqueous solution at a concentration of about 25 mg/mL in 10 mM Histidine buffer, pH 5.0-6.0. The stable formulation (i.e., drug substance) is typically a clear to opalescent solution and may contain particulates.

[0138] In certain embodiments, a liquid or frozen solution of h409A11 is formulated in histidine buffer (pH 5.5) containing sucrose and polysorbate 80.

[0139] An additional exemplary formulation includes: h409A11 formulated in histidine buffer (pH 5.5) containing sucrose and polysorbate 80 in lyophilized form.

[0140] In certain embodiments, stable humanized PD-1 formulation is provided as lyophilized powder in vials intended for single-use.

[0141] In certain embodiments, stable humanized PD-1 formulation is reconstituted with water for injection (WFI) and aseptically diluted with appropriate volumes of 0.9% sodium chloride for injection in a sterile IV container to form an admixture solution.

Biological Activity

[0142] Biological activity of the humanized anti-PD-1 antibody is measured by its ability to compete with PD-L1 (natural ligand of PD-1) in binding to human PD-1, quantified in competitive ELISA relative to a reference material. The stable formulations described herein exhibit biological activity for long periods of time, including up to at least about eighteen months. The stability of several batches of h409A11, under various storage conditions are illustrated in FIGS. **1-9**.

Stable Formulations of Humanized Anti-PD-1 Antibodies

[0143] Lyophilized formulations of anti-PD-1 antibody are prepared as follows. An exemplary batch formula for h409A11 antibody is provided in Table 3. The final concentration of antibody is 25 mg/mL. This batch formulation may be used to prepare the lyophilized 50 mg/vial units, as discussed with reference to Table 4, infra. Polysorbate 80 from a vegetable source is used. Additional hydrochloric acid or sodium hydroxide may be added to adjust the pH to the desired value of approximately 5.5 (±0.2). The components are brought to a final volume of 14 L with sterile water for injection (WFI). Correspondingly smaller lots may be prepared by proportional reduction of the amounts listed in Table 3.

[0144] An exemplary liquid antibody formulation is prepared by taking the drug substance (e.g., anti-humanized PD-1 from a batch formula described herein) which is in liquid form (e.g., h409A11 in an aqueous formulation) and buffer exchanging it into the desired buffer as the last step of the purification process. In this instance, there is no previous lyophilization step. The drug substance in the final histidine buffer is concentrated to a desired concentration. Excipients such as sucrose and polysorbate 80 are added to the drug substance and it is diluted using the appropriate buffer to final protein concentration. The final formulated drug substance is filtered using 0.22 μm filters and filled into a final container (e.g. glass vials). Such a liquid formulation includes final liquid formulation comprising 10 mM histidine pH 5.5, 7% sucrose, 0.02% polysorbate 80, and 25 mg/mL h409A11.

TABLE 3

Batch Formula of Representative 14.0 L Pre-lyophilization Solution for h409A11 Powder for Injection, 50 mg/vial

Component	Compendial Grade	Concentration (mg/mL)	Amount per Batch (g)
h409A11 antibody	N/A	25.0	350.0
L-Histidine	USP	1.55	21.7
Polysorbate 80	NF	0.2	2.8
Sucrose	NF	70	980
Hydrochloric acid [a]	NF	—	—
Sodium Hydroxide [a]	NF	—	pH adjustment
Water for injection [b]	USP	—	14.0 L @ q.s

[a] Hydrochloric acid and sodium hydroxide added if needed to adjust pH to 5.5
[b] Water removed by sublimation and desorption during lyophilization

[0145] The unit composition of an exemplary final lyophilized formulation of humanized anti-PD-1 is provided at Table 4.

TABLE 4

Unit Composition of Lyophilized Powder Formulation for Injection, 50 mg/vial

Component	Grade	Amount (mg/vial)	Concentration after Reconstitution (mg/mL)[b]	Function
h409A11	N/A	50	25	Drug Substance/Active Pharmaceutical ingredient
L-Histidine	USP	3.1	1.55	Buffer
Polysorbate 80	NF	0.4	0.2	Surfactant
Sucrose	NF	140	70	Stabilizer/ Tonicity Modifier
Hydrochloric acid [C]	NF	—	—	pH adjustment
Sodium Hydroxide [C]	NF	—	—	pH adjustment
Sterile Water for Injection (sWFI or WFI)[d]	USP	2.0 mL @ q.s.	—	Solvent

[a] An excess fill of 0.4 mL is provided to ensure the recovery of 50 mg h409A11 per vial.
[b] Following reconstitution with 2.3 mL sterile water for injection.
[C] Hydrochloric acid and sodium hydroxide added if needed to adjust pH to 5.5
[d] Water removed by sublimation and desorption during lyophilization

[0146] The unit formulation of Table 4 comprises $1/20{,}000^{th}$ of the batch formulation of Table 3 after lyophilization to remove the water. The 50 mg of DS is added as 2.0 mL of the 25 mg/mL batch formulation of Table 3. Each vial is filled with 2.4 mL and reconstituted with 2.3 mL sWFI, resulting in approximately 2.4 mL of reconstituted solution due to expansion volume of the lyophilized cake.

[0147] The drug is packaged in sterile 20 mm neck, 6R DIN, Type 1 glass tubing vials, closed with 20-mm gray butyl rubber stoppers and sealed with aluminum crimp seals. Vials are stored at 2-8° C., and refrigerated when shipped.

[0148] Compounding involves the following steps. Charge the required amount of water for injection (WFI) into a tared compounding vessel. Charge and dissolve with mixing, sucrose, histidine, and polysorbate 80 from a vegetable source. Measure the pH and adjust if needed to bring the pH

to about 5.4-5.6. Use hydrochloric acid and/or sodium hydroxide to adjust the pH. Equilibrate the drug substance to ambient temperature and charge the drug substance slowly into the compounding vessel. Continue to mix gently to avoid foaming. Measure the pH again and adjust if needed to bring the pH to approximately 5.5. Charge WFI to the final weight of the bulk solution with continued gentle mixing.

[0149] Filtration involves the following steps. Connect clarifying filter (0.22 μm) and sterilizing filter (0.22 μm) to the compounding vessel. Collect an aliquot of the bulk solution for bioburden testing after clarifying filtration step. Perform aseptic filtration using a 0.22 μm filter into a sterile container. Remove aliquot of sample after aseptic filtration for bulk sterility testing. Perform filter integrity testing after product filtration.

[0150] Filling involves the following steps. Using suitable filling equipment, aseptically fill the product solution into sterilized Type I tubing glass vials to achieve a target fill volume of 2.4 mL. Perform fill weight checks during filling. Partially seat sterilized lyo-shape stoppers into filled vials. Load the filled vials into a suitable freeze-dryer.

[0151] Lyophilization, stoppering and capping involve the following steps. Lyophilize the filled vials using an appropriate lyophilization cycle. After lyophilization is complete, backfill the vials with 0.22 μm filtered nitrogen and fully stopper. Unload the stoppered vials from the lyophilizer and seal them.

[0152] The resulting vials are inspected for visual defects and stored at 2-8° C. Finished unit dosage vials are shipped under refrigerated conditions.

Example 2

Stability Testing of Lyophilized Formulations of Humanized Anti-PD-1 Antibodies

[0153] FIGS. **1-9** provide data of stability testing of lyophilized formulations of a humanized anti-human PD-1 antibody under various storage conditions. Vials were stored in upright configurations. As discussed in more detail below, formulations of the present invention show stability through at least 24 months for antibodies lyophilized at pH 5.5 (histidine buffer), as well as similar liquid formulations.

[0154] Stability was assessed as follows. Samples were lyophilized in 6R DIN Type I glass vials, and sealed with 20 mm bromobutyl lyo stoppers (Helvoet Rubber & Plastic Technologies BV, Hellevoetsluis, The Netherlands) and flip-off aluminum seals. Vials were placed on stability stations under the following storage conditions: 5° C. (5±3° C.), 25H (25, 60% relative humidity), or RH4 (40° C., 70% relative humidity). Samples were obtained at an initial time point, and for certain samples at a variety of time points including 1, 2, 3, 6, 9, 12, 18, and 24 months.

[0155] The stability of the samples is illustrated by the various characteristics presented in the tables in FIGS. **1-9**. The lyophilized samples were visually inspected, reconstituted, and the reconstituted formulation was visually inspected. The pH of the samples after reconstitution was measured, and the protein concentration determined by U.V. absorbance. The samples were analyzed by CE-SDS technique in which protein was denatured with sodium dodecyl sulfate (SDS) under reducing and non-reducing conditions and separated using capillary electrophoresis (CE). The proteins separate based on their apparent molecular weight. Under non-reducing conditions, all species other than the main IgG peak are classified as impurities. Under reducing conditions, the IgG is resolved into the heavy and light chains. All other species are classified as impurities.

[0156] Purity of the sample was further assessed by high performance size exclusion chromatography (HPSEC) in which the percentage of monomer was determined, as well as the percentages of high molecular weight species (possibly aggregates) and late eluting peaks (possibly degradation products).

[0157] Additional sample characterization data are provided in FIGS. **1-9**. High performance ion-exchange chromatography (HP-IEX) was used to assess purity by revealing the presence of acidic or basic variants. Results are presented as a percentage of total observed material. The samples were further characterized for biological function using an enzyme-linked immunosorbent assay (ELISA) for binding to human PD-1. The antibody concentration necessary to achieve half-maximal binding is called EC_{50}. Potency of the test sample was assessed by comparing binding curves of the test samples to a reference material (or control) by the ration of EC_{50}'s. Potency was expressed as percent relative potency of reference material (or control). Moisture content of the lyophilized powder was also determined by coulometric titration. Particulate matter count measurements were performed to count particles ≥10 μm and ≥25 μm. The method used for these measurements was based on USP<788>.

[0158] These results demonstrate high stability formulations of the present invention over at least 24 months at about pH 5.5. The data reveal no trending over time that would reflect instability for samples the tested storage conditions.

Example 3

Initial Clinical Results

[0159] Phase 1 Study of h409A11 (Anti-PD-1 Monoclonal Antibody) in Patients with Advanced Solid Tumors

[0160] A phase 1 trial examined safety, PK, PD, and anti-tumor activity of h409A11. An open-label, dose escalation study was conducted in patients with advanced malignancy refractory to standard chemotherapy. In the initial patient set, patients with advanced solid tumors were treated with a stable h409A11 formulation as described herein. There was no limitation/restriction regarding surgery; however, patients were not currently surgical candidates. Cohorts of 3-6 patients were enrolled (3+3 design) at IV doses of 1, 3, or 10 mg/kg. Following an initial dose and 28-day Cycle 1, patients were allowed to subsequently receive multiple doses given every 2 wks. For phase 1 part A, three patients were treated at 1 mg/kg, three patients were treated at 3 mg/kg, and nine patients were treated at 10 mg/kg and all were dosed every 2 weeks. There was no intrapatient dose escalation. Radiographic assessment was conducted every 8 wks using RECIST 1.1 guidelines.

[0161] Nine patients, 3 at each dose level, completed the dose-limiting toxicity (DLT) period (28 d). Patients had non-small cell lung cancer (NSCLC, n=3), rectal cancer (n=2), melanoma (MEL, n=2), sarcoma (n=1), or carcinoid (n=1). To date, a total of 63 doses were administered (median 7/patient; max 12) without DLT. Drug-related adverse events (AEs) across all doses included Grade 1 fatigue (n=3), nausea (n=2), diarrhea (n=1), dysgeusia (n=1), breast pain (n=1), and pruritus (N=1). One drug-related Grade 2 AE of pruritus was reported. No drug-related AEs ≥grade 3 were observed. PK data are shown in Table 5. Based on RECIST, 1 patient with

MEL on therapy >6 mths had a partial response, and preliminary evidence of tumor size reduction (stable disease) was observed in 3 additional patients with advanced cancer. These results show that h409A11 was well-tolerated without DLT across 3 tested dose levels. (i.e., 1, 3, and 5 mg/kg). Evidence of antitumor activity was observed.

TABLE 5

Mean (CV %) PK Parameter Values of MK-3475 Following Single IV Dose of 1, 3, or 10 mg/kg in Cycle 1

Dose (mg/kg)	N	C_{max} (μg/mL)	$AUC_{(0\text{-}28day)}$ (μg · day/mL)	$t_{1/2}{}^{a}$ (day)
1	4	16.8 (23)	163 (20) [b]	15.1 (41)[b]
3	3	109 (26)	990 (23)	21.7 (11)
10	2	337 (8)	2640 (30)	13.6 (28)

[a]PK sampling up to 28 days following first IV administration, therefore $t_{1/2}$ not fully characterized.
[b]N = 3 due to subject discontinuation.

TABLE 6

provides a brief description of the sequences in the sequence listing. Sequence Identifiers

SEQ ID NO:	Description
1	hPD-1.08A heavy chain variable region (DNA)
2	hPD-1.08A light chain variable region (DNA)
3	hPD-1.09A heavy chain variable region (DNA)
4	hPD-1.09A light chain variable region (DNA)
5	hPD-1.08A heavy chain variable region (AA)
6	hPD-1.08A light chain variable region (AA)
7	hPD-1.09A heavy chain variable region (AA)
8	hPD-1.09A light chain variable region (AA)
9	hPD-1.08A light chain CDR1 (AA)
10	hPD-1.08A light chain CDR2 (AA)
11	hPD-1.08A light chain CDR3 (AA)
12	hPD-1.08A heavy chain CDR1 (AA)
13	hPD-1.08A heavy chain CDR2 (AA)
14	hPD-1.08A heavy chain CDR3 (AA)
15	hPD-1.09A light chain CDR1 (AA)
16	hPD-1.09A light chain CDR2 (AA)
17	hPD-1.09A light chain CDR3 (AA)
18	hPD-1.09A heavy chain CDR1 (AA)
19	hPD-1.09A heavy chain CDR2 (AA)
20	hPD-1.09A heavy chain CDR3 (AA)
21	109A-H heavy chain variable region (DNA)
22	Codon optimized 109A-H heavy chain variable region (DNA)
23	Codon optimized 409A-H heavy chain full length (DNA)
24	K09A-L-11 light chain variable region (DNA)
25	K09A-L-16 light chain variable region (DNA)
26	K09A-L-17 light chain variable region (DNA)
27	Codon optimized K09A-L-11 light chain variable region (DNA)
28	Codon optimized K09A-L-16 light chain variable region (DNA)
29	Codon optimized K09A-L-17 light chain variable region (DNA)
30	109A-H heavy chain variable region (AA)
31	409A-H heavy chain full length (AA)
32	K09A-L-11 light chain variable region (AA)
33	K09A-L-16 light chain variable region (AA)
34	K09A-L-17 light chain variable region (AA)
35	109A-H heavy chain full length (AA)
36	K09A-L-11 light chain full length (AA)
37	K09A-L-16 light chain full length (AA)
38	K09A-L-17 light chain full length (AA)

[0162] As used herein, including the appended claims, the singular forms of words such as "a," "an," and "the," include their corresponding plural references unless the context clearly dictates otherwise. Unless otherwise indicated, the proteins and subjects referred to herein are human proteins and subject, rather than another species.

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prognostic factors of human ovarian cancer. *Proceeding of the National Academy of Sciences* (2007): 104: 3360-3365.

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[0189] All references cited herein are incorporated by reference to the same extent as if each individual publication, database entry (e.g. Genbank sequences or GeneID entries), patent application, or patent, was specifically and individually indicated to be incorporated by reference. This statement of incorporation by reference is intended by Applicants, pursuant to

[0190] 37 C.F.R. §1.57(b)(1), to relate to each and every individual publication, database entry (e.g. Genbank sequences or GeneID entries), patent application, or patent, each of which is clearly identified in compliance with 37 C.F.R. §1.57(b)(2), even if such citation is not immediately adjacent to a dedicated statement of incorporation by reference. The inclusion of dedicated statements of incorporation by reference, if any, within the specification does not in any way weaken this general statement of incorporation by reference. Citation of the references herein is not intended as an admission that the reference is pertinent prior art, nor does it constitute any admission as to the contents or date of these publications or documents.

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ggacaaggcc ttgagtggat tggggggggtt aatcctagta atggtggtac taacttcagt     240

gagaagttca agagcaaggc cacactgact gtagacaaat cctccagcac agcctacatg     300

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Gly Gly Val Asn Pro Ser Asn Gly Gly Thr Asn Phe Ser Glu Lys Phe
    50                  55                  60

Lys Ser Lys Ala Thr Leu Thr Val Asp Lys Ser Ser Ser Thr Ala Tyr
65                  70                  75                  80

Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys
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Gly Phe Ser Tyr Leu His Trp Tyr Gln Gln Lys Pro Gly Gln Pro Pro
        35                  40                  45

Lys Leu Leu Ile Phe Leu Ala Ser Asn Leu Glu Ser Gly Val Pro Ala
    50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Asn Ile His
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Tyr Met Tyr Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile
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Gly Gly Ile Asn Pro Ser Asn Gly Gly Thr Asn Phe Asn Glu Lys Phe
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Ser

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tgcaaggctt ctggctacac ctttaccaac tactatatgt actgggtgcg acaggcccct   180

ggacaagggc ttgagtggat gggagggatt aatcctagca atggtggtac taacttcaat   240

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Codon optimized 109A-H heavy chain variable region
<220> FEATURE:
<221> NAME/KEY: sig_peptide
<222> LOCATION: (1)..(57)

<400> SEQUENCE: 22

atggactgga cctggtctat cctgttcctg gtggccgctc ctaccggcgc tcactcccag 60

gtgcagctgg tgcagtccgg cgtggaggtg aagaagcctg gcgcctccgt caaggtgtcc 120

tgcaaggcct ccggctacac cttcaccaac tactacatgt actgggtgcg gcaggctccc 180

ggccagggac tggagtggat gggcggcatc aacccttcca acggcggcac caacttcaac 240

gagaagttca agaaccgggt gaccctgacc accgactcct ccaccaccac cgcctacatg 300

gagctgaagt ccctgcagtt cgacgacacc gccgtgtact actgcgccag gcgggactac 360

cggttcgaca tgggcttcga ctactggggc cagggcacca ccgtgaccgt gtcctcc 417

<210> SEQ ID NO 23
<211> LENGTH: 1398
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Codon optimized 409A-H heavy chain full length
<220> FEATURE:
<221> NAME/KEY: sig_peptide
<222> LOCATION: (1)..(57)

<400> SEQUENCE: 23

atggccgtgc tgggcctgct gttctgcctg gtgaccttcc cttcctgcgt gctgtcccag 60

gtgcagctgg tgcagtccgg cgtggaggtg aagaagcctg gcgcctccgt caaggtgtcc 120

tgtaaggcct ccggctacac cttcaccaac tactacatgt actgggtgcg gcaggcccca 180

ggccagggac tggagtggat gggcggcatc aacccttcca acggcggcac caacttcaac 240

gagaagttca agaaccgggt gaccctgacc accgactcct ccaccacaac cgcctacatg 300

gaactgaagt ccctgcagtt cgacgacacc gccgtgtact actgcgccag gcgggactac 360

cggttcgaca tgggcttcga ctactggggc cagggcacca ccgtgaccgt gtcctccgct 420

agcaccaagg gcccttccgt gttccctctg gccccttgct cccggtccac ctccgagtcc 480

accgccgctc tgggctgtct ggtgaaggac tacttccctg agcctgtgac cgtgagctgg 540

aactctggcg ccctgacctc cggcgtgcac accttccctg ccgtgctgca gtcctccggc 600

ctgtactccc tgtcctccgt ggtgaccgtg ccttcctcct ccctgggcac caagacctac 660

acctgcaacg tggaccacaa gccttccaac accaaggtgg acaagcgggt ggagtccaag 720

tacggccctc cttgccctcc ctgccctgcc cctgagttcc tgggcggacc ctccgtgttc 780

ctgttccctc ctaagcctaa ggacaccctg atgatctccc ggacccctga ggtgacctgc 840

gtggtggtgg acgtgtccca ggaagatcct gaggtccagt tcaattggta cgtggatggc 900

gtggaggtgc acaacgccaa gaccaagcct cgggaggaac agttcaactc cacctaccgg 960

gtggtgtctg tgctgaccgt gctgcaccag gactggctga acggcaagga atacaagtgc 1020

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```
aaggtcagca acaagggcct gccctcctcc atcgagaaaa ccatctccaa ggccaagggc   1080

cagcctcgcg agcctcaggt gtacaccctg cctcctagcc aggaagagat gaccaagaat   1140

caggtgtccc tgacatgcct ggtgaagggc ttctaccctt ccgatatcgc cgtggagtgg   1200

gagagcaacg gccagccaga gaacaactac aagaccaccc ctcctgtgct ggactccgac   1260

ggctccttct tcctgtactc caggctgacc gtggacaagt cccggtggca ggaaggcaac   1320

gtcttttcct gctccgtgat gcacgaggcc ctgcacaacc actacaccca gaagtccctg   1380

tccctgtctc tgggcaag                                                 1398


<210> SEQ ID NO 24
<211> LENGTH: 393
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: K09A-L-11 light chain variable region
<220> FEATURE:
<221> NAME/KEY: sig_peptide
<222> LOCATION: (1)..(60)

<400> SEQUENCE: 24

atggaagccc cagctcagct tctcttcctc ctgctactct ggctcccaga taccaccgga     60

gaaattgtgt tgacacagtc tccagccacc ctgtctttgt ctccagggga aagagccacc    120

ctctcctgca gggccagcaa aggtgtcagt acatctggct atagttattt gcactggtat    180

caacagaaac ctggccaggc tcccaggctc ctcatctatc ttgcatccta cctagaatct    240

ggcgtcccag ccaggttcag tggtagtggg tctgggacag acttcactct caccatcagc    300

agcctagagc ctgaagattt tgcagtttat tactgtcagc acagcaggga ccttccgctc    360

acgttcggcg gagggaccaa agtggagatc aaa                                 393


<210> SEQ ID NO 25
<211> LENGTH: 393
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: K09A-L-16 light chain variable region
<220> FEATURE:
<221> NAME/KEY: sig_peptide
<222> LOCATION: (1)..(60)

<400> SEQUENCE: 25

atggaaaccc cagcgcagct tctcttcctc ctgctactct ggctcccaga taccaccgga     60

gaaattgtgc tgactcagtc tccactctcc ctgcccgtca cccctggaga gccggcctcc    120

atctcctgca gggccagcaa aggtgtcagt acatctggct atagttattt gcattggtac    180

ctccagaagc cagggcagtc tccacagctc ctgatctatc ttgcatccta cctagaatct    240

ggggtccctg acaggttcag tggcagtgga tcaggcacag attttacact gaaaatcagc    300

agagtggagg ctgaggatgt tggggtttat tactgccagc atagtaggga ccttccgctc    360

acgtttggcc aggggaccaa gctggagatc aaa                                 393


<210> SEQ ID NO 26
<211> LENGTH: 393
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: K09A-L-17 light chain variable region
<220> FEATURE:
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<221> NAME/KEY: sig_peptide
<222> LOCATION: (1)..(60)

<400> SEQUENCE: 26

atgaggctcc ctgctcagct cctggggctg ctaatgctct gggtctctgg atccagtggg     60

gatattgtga tgacccagac tccactctcc ctgcccgtca cccctggaga gccggcctcc    120

atctcctgca gggccagcaa aggtgtcagt acatctggct atagttattt gcattggtat    180

ctgcagaagc cagggcagtc tccacagctc ctgatctatc ttgcatccta cctagaatct    240

ggagtcccag acaggttcag tggcagtggg tcaggcactg ctttcacact gaaaatcagc    300

agggtggagg ctgaggatgt tggactttat tactgccagc atagtaggga ccttccgctc    360

acgtttggcc aggggaccaa gctggagatc aaa                                 393


<210> SEQ ID NO 27
<211> LENGTH: 390
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Codon optimized K09A-L-11 light chain variable
      region
<220> FEATURE:
<221> NAME/KEY: sig_peptide
<222> LOCATION: (1)..(57)

<400> SEQUENCE: 27

atggcccctg tgcagctgct gggcctgctg gtgctgttcc tgcctgccat gcggtgcgag     60

atcgtgctga cccagtcccc tgccaccctg tccctgagcc ctggcgagcg ggctaccctg    120

agctgcagag cctccaaggg cgtgtccacc tccggctact cctacctgca ctggtatcag    180

cagaagccag gccaggcccc tcggctgctg atctacctgg cctcctacct ggagtccggc    240

gtgcctgccc ggttctccgg ctccggaagc ggcaccgact tcaccctgac catctcctcc    300

ctggagcctg aggacttcgc cgtgtactac tgccagcact cccgggacct gcctctgacc    360

tttggcggcg gaacaaaggt ggagatcaag                                     390


<210> SEQ ID NO 28
<211> LENGTH: 390
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Codon optimized K09A-L-16 light chain variable
      region
<220> FEATURE:
<221> NAME/KEY: sig_peptide
<222> LOCATION: (1)..(57)

<400> SEQUENCE: 28

atggcccctg tgcagctgct gggcctgctg gtgctgttcc tgcctgccat gcggtgcgag     60

atcgtgctga cccagtcccc tctgtccctg cctgtgaccc ctggcgagcc tgcctccatc    120

tcctgccggg cctccaaggg cgtgtccacc tccggctact cctacctgca ctggtatctg    180

cagaagcctg gccagtcccc ccagctgctg atctacctgg cctcctacct ggagtccggc    240

gtgcctgacc ggttctccgg ctccggcagc ggcaccgact tcaccctgaa gatctcccgg    300

gtggaggccg aggacgtggg cgtgtactac tgccagcact cccgggacct gcctctgacc    360

ttcggccagg gcaccaagct ggagatcaag                                     390
```

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<210> SEQ ID NO 29
<211> LENGTH: 390
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Codon optimized K09A-L-17 light chain variable
      region
<220> FEATURE:
<221> NAME/KEY: sig_peptide
<222> LOCATION: (1)..(57)

<400> SEQUENCE: 29

atggcccctg tgcagctgct gggcctgctg gtgctgttcc tgcctgccat gcggtgcgac     60

atcgtgatga cccagacccc tctgtccctg cctgtgaccc ctggcgagcc tgcctccatc    120

tcctgccggg cctccaaggg cgtgtccacc tccggctact cctacctgca ctggtatctg    180

cagaagcctg gccagtcccc tcagctgctg atctacctgg cctcctacct ggagtccggc    240

gtgcctgacc ggttctccgg ctccggaagc ggcaccgctt ttaccctgaa gatctccaga    300

gtggaggccg aggacgtggg cctgtactac tgccagcact cccgggacct gcctctgacc    360

ttcggccagg gcaccaagct ggagatcaag                                     390


<210> SEQ ID NO 30
<211> LENGTH: 139
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 109A-H heavy chain variable region
<220> FEATURE:
<221> NAME/KEY: sig_peptide
<222> LOCATION: (1)..(19)

<400> SEQUENCE: 30

Met Asp Trp Thr Trp Ser Ile Leu Phe Leu Val Ala Ala Pro Thr Gly
1               5                   10                  15

Ala His Ser Gln Val Gln Leu Val Gln Ser Gly Val Glu Val Lys Lys
            20                  25                  30

Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe
        35                  40                  45

Thr Asn Tyr Tyr Met Tyr Trp Val Arg Gln Ala Pro Gly Gln Gly Leu
    50                  55                  60

Glu Trp Met Gly Gly Ile Asn Pro Ser Asn Gly Gly Thr Asn Phe Asn
65                  70                  75                  80

Glu Lys Phe Lys Asn Arg Val Thr Leu Thr Thr Asp Ser Ser Thr Thr
                85                  90                  95

Thr Ala Tyr Met Glu Leu Lys Ser Leu Gln Phe Asp Asp Thr Ala Val
            100                 105                 110

Tyr Tyr Cys Ala Arg Arg Asp Tyr Arg Phe Asp Met Gly Phe Asp Tyr
        115                 120                 125

Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser
    130                 135


<210> SEQ ID NO 31
<211> LENGTH: 466
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: 409A-H heavy chain full length
<220> FEATURE:
<221> NAME/KEY: sig_peptide
```

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<222> LOCATION: (1)..(19)

<400> SEQUENCE: 31

```
Met Ala Val Leu Gly Leu Leu Phe Cys Leu Val Thr Phe Pro Ser Cys
1               5                   10                  15

Val Leu Ser Gln Val Gln Leu Val Gln Ser Gly Val Glu Val Lys Lys
            20                  25                  30

Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe
        35                  40                  45

Thr Asn Tyr Tyr Met Tyr Trp Val Arg Gln Ala Pro Gly Gln Gly Leu
    50                  55                  60

Glu Trp Met Gly Gly Ile Asn Pro Ser Asn Gly Gly Thr Asn Phe Asn
65                  70                  75                  80

Glu Lys Phe Lys Asn Arg Val Thr Leu Thr Thr Asp Ser Ser Thr Thr
                85                  90                  95

Thr Ala Tyr Met Glu Leu Lys Ser Leu Gln Phe Asp Asp Thr Ala Val
            100                 105                 110

Tyr Tyr Cys Ala Arg Arg Asp Tyr Arg Phe Asp Met Gly Phe Asp Tyr
        115                 120                 125

Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Ala Ser Thr Lys Gly
    130                 135                 140

Pro Ser Val Phe Pro Leu Ala Pro Cys Ser Arg Ser Thr Ser Glu Ser
145                 150                 155                 160

Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val
                165                 170                 175

Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe
            180                 185                 190

Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
        195                 200                 205

Thr Val Pro Ser Ser Ser Leu Gly Thr Lys Thr Tyr Thr Cys Asn Val
    210                 215                 220

Asp His Lys Pro Ser Asn Thr Lys Val Asp Lys Arg Val Glu Ser Lys
225                 230                 235                 240

Tyr Gly Pro Pro Cys Pro Pro Cys Pro Ala Pro Glu Phe Leu Gly Gly
                245                 250                 255

Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile
            260                 265                 270

Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser Gln Glu
        275                 280                 285

Asp Pro Glu Val Gln Phe Asn Trp Tyr Val Asp Gly Val Glu Val His
    290                 295                 300

Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Phe Asn Ser Thr Tyr Arg
305                 310                 315                 320

Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys
                325                 330                 335

Glu Tyr Lys Cys Lys Val Ser Asn Lys Gly Leu Pro Ser Ser Ile Glu
            340                 345                 350

Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr
        355                 360                 365

Thr Leu Pro Pro Ser Gln Glu Glu Met Thr Lys Asn Gln Val Ser Leu
    370                 375                 380
```

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```
Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp
385                 390                 395                 400

Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val
                405                 410                 415

Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Arg Leu Thr Val Asp
            420                 425                 430

Lys Ser Arg Trp Gln Glu Gly Asn Val Phe Ser Cys Ser Val Met His
        435                 440                 445

Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Leu
    450                 455                 460

Gly Lys
465
```

<210> SEQ ID NO 32
<211> LENGTH: 130
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: K09A-L-11 light chain variable region
<220> FEATURE:
<221> NAME/KEY: sig_peptide
<222> LOCATION: (1)..(19)

<400> SEQUENCE: 32

```
Met Ala Pro Val Gln Leu Leu Gly Leu Leu Val Leu Phe Leu Pro Ala
1               5                   10                  15

Met Arg Cys Glu Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser Leu
            20                  25                  30

Ser Pro Gly Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Lys Gly Val
        35                  40                  45

Ser Thr Ser Gly Tyr Ser Tyr Leu His Trp Tyr Gln Gln Lys Pro Gly
    50                  55                  60

Gln Ala Pro Arg Leu Leu Ile Tyr Leu Ala Ser Tyr Leu Glu Ser Gly
65                  70                  75                  80

Val Pro Ala Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu
                85                  90                  95

Thr Ile Ser Ser Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln
            100                 105                 110

His Ser Arg Asp Leu Pro Leu Thr Phe Gly Gly Gly Thr Lys Val Glu
        115                 120                 125

Ile Lys
    130
```

<210> SEQ ID NO 33
<211> LENGTH: 130
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: K09A-L-16 light chain variable region
<220> FEATURE:
<221> NAME/KEY: sig_peptide
<222> LOCATION: (1)..(19)

<400> SEQUENCE: 33

```
Met Ala Pro Val Gln Leu Leu Gly Leu Leu Val Leu Phe Leu Pro Ala
1               5                   10                  15

Met Arg Cys Glu Ile Val Leu Thr Gln Ser Pro Leu Ser Leu Pro Val
            20                  25                  30
```

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```
Thr Pro Gly Glu Pro Ala Ser Ile Ser Cys Arg Ala Ser Lys Gly Val
        35                  40                  45

Ser Thr Ser Gly Tyr Ser Tyr Leu His Trp Tyr Leu Gln Lys Pro Gly
    50                  55                  60

Gln Ser Pro Gln Leu Leu Ile Tyr Leu Ala Ser Tyr Leu Glu Ser Gly
65                  70                  75                  80

Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu
                85                  90                  95

Lys Ile Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Gln
            100                 105                 110

His Ser Arg Asp Leu Pro Leu Thr Phe Gly Gln Gly Thr Lys Leu Glu
        115                 120                 125

Ile Lys
    130


&lt;210&gt; SEQ ID NO 34
&lt;211&gt; LENGTH: 130
&lt;212&gt; TYPE: PRT
&lt;213&gt; ORGANISM: Artificial Sequence
&lt;220&gt; FEATURE:
&lt;223&gt; OTHER INFORMATION: K09A-L-17 light chain variable region
&lt;220&gt; FEATURE:
&lt;221&gt; NAME/KEY: sig_peptide
&lt;222&gt; LOCATION: (1)..(19)

&lt;400&gt; SEQUENCE: 34

Met Ala Pro Val Gln Leu Leu Gly Leu Leu Val Leu Phe Leu Pro Ala
1               5                   10                  15

Met Arg Cys Asp Ile Val Met Thr Gln Thr Pro Leu Ser Leu Pro Val
            20                  25                  30

Thr Pro Gly Glu Pro Ala Ser Ile Ser Cys Arg Ala Ser Lys Gly Val
        35                  40                  45

Ser Thr Ser Gly Tyr Ser Tyr Leu His Trp Tyr Leu Gln Lys Pro Gly
    50                  55                  60

Gln Ser Pro Gln Leu Leu Ile Tyr Leu Ala Ser Tyr Leu Glu Ser Gly
65                  70                  75                  80

Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu
                85                  90                  95

Lys Ile Ser Arg Val Glu Ala Glu Asp Val Gly Leu Tyr Tyr Cys Gln
            100                 105                 110

His Ser Arg Asp Leu Pro Leu Thr Phe Gly Gln Gly Thr Lys Leu Glu
        115                 120                 125

Ile Lys
    130


&lt;210&gt; SEQ ID NO 35
&lt;211&gt; LENGTH: 469
&lt;212&gt; TYPE: PRT
&lt;213&gt; ORGANISM: Artificial Sequence
&lt;220&gt; FEATURE:
&lt;223&gt; OTHER INFORMATION: 109A-H heavy chain full length
&lt;220&gt; FEATURE:
&lt;221&gt; NAME/KEY: sig_peptide
&lt;222&gt; LOCATION: (1)..(19)

&lt;400&gt; SEQUENCE: 35

Met Ala Val Leu Gly Leu Leu Phe Cys Leu Val Thr Phe Pro Ser Cys
```

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```
1               5                   10                  15

Val Leu Ser Gln Val Gln Leu Val Gln Ser Gly Val Glu Val Lys Lys
            20                  25                  30

Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe
        35                  40                  45

Thr Asn Tyr Tyr Met Tyr Trp Val Arg Gln Ala Pro Gly Gln Gly Leu
    50                  55                  60

Glu Trp Met Gly Gly Ile Asn Pro Ser Asn Gly Gly Thr Asn Phe Asn
65                  70                  75                  80

Glu Lys Phe Lys Asn Arg Val Thr Leu Thr Thr Asp Ser Ser Thr Thr
                85                  90                  95

Thr Ala Tyr Met Glu Leu Lys Ser Leu Gln Phe Asp Asp Thr Ala Val
            100                 105                 110

Tyr Tyr Cys Ala Arg Arg Asp Tyr Arg Phe Asp Met Gly Phe Asp Tyr
        115                 120                 125

Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser Ala Ser Thr Lys Gly
    130                 135                 140

Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly
145                 150                 155                 160

Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val
                165                 170                 175

Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe
            180                 185                 190

Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
        195                 200                 205

Thr Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val
    210                 215                 220

Asn His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys
225                 230                 235                 240

Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu
                245                 250                 255

Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr
            260                 265                 270

Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val
        275                 280                 285

Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val
    290                 295                 300

Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser
305                 310                 315                 320

Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu
                325                 330                 335

Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala
            340                 345                 350

Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro
        355                 360                 365

Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln
    370                 375                 380

Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala
385                 390                 395                 400

Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr
                405                 410                 415
```

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```
Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu
            420                 425                 430

Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser
        435                 440                 445

Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser
    450                 455                 460

Leu Ser Pro Gly Lys
465


<210> SEQ ID NO 36
<211> LENGTH: 237
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: K09A-L-11 light chain full length
<220> FEATURE:
<221> NAME/KEY: sig_peptide
<222> LOCATION: (1)..(19)

<400> SEQUENCE: 36

Met Ala Pro Val Gln Leu Leu Gly Leu Leu Val Leu Phe Leu Pro Ala
1               5                   10                  15

Met Arg Cys Glu Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser Leu
            20                  25                  30

Ser Pro Gly Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Lys Gly Val
        35                  40                  45

Ser Thr Ser Gly Tyr Ser Tyr Leu His Trp Tyr Gln Gln Lys Pro Gly
    50                  55                  60

Gln Ala Pro Arg Leu Leu Ile Tyr Leu Ala Ser Tyr Leu Glu Ser Gly
65                  70                  75                  80

Val Pro Ala Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu
                85                  90                  95

Thr Ile Ser Ser Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln
            100                 105                 110

His Ser Arg Asp Leu Pro Leu Thr Phe Gly Gly Gly Thr Lys Val Glu
        115                 120                 125

Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser
    130                 135                 140

Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn
145                 150                 155                 160

Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala
                165                 170                 175

Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys
            180                 185                 190

Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp
        195                 200                 205

Tyr Glu Lys His Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu
    210                 215                 220

Ser Ser Pro Val Thr Lys Ser Phe Asn Arg Gly Glu Cys
225                 230                 235


<210> SEQ ID NO 37
<211> LENGTH: 237
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
```

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<220> FEATURE:
<223> OTHER INFORMATION: K09A-L-16 light chain full length
<220> FEATURE:
<221> NAME/KEY: sig_peptide
<222> LOCATION: (1)..(19)

<400> SEQUENCE: 37

Met Ala Pro Val Gln Leu Leu Gly Leu Leu Val Leu Phe Leu Pro Ala
1 5 10 15

Met Arg Cys Glu Ile Val Leu Thr Gln Ser Pro Leu Ser Leu Pro Val
20 25 30

Thr Pro Gly Glu Pro Ala Ser Ile Ser Cys Arg Ala Ser Lys Gly Val
35 40 45

Ser Thr Ser Gly Tyr Ser Tyr Leu His Trp Tyr Leu Gln Lys Pro Gly
50 55 60

Gln Ser Pro Gln Leu Leu Ile Tyr Leu Ala Ser Tyr Leu Glu Ser Gly
65 70 75 80

Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu
85 90 95

Lys Ile Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Gln
100 105 110

His Ser Arg Asp Leu Pro Leu Thr Phe Gly Gln Gly Thr Lys Leu Glu
115 120 125

Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser
130 135 140

Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn
145 150 155 160

Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala
165 170 175

Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys
180 185 190

Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp
195 200 205

Tyr Glu Lys His Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu
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Ser Ser Pro Val Thr Lys Ser Phe Asn Arg Gly Glu Cys
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20 25 30

Thr Pro Gly Glu Pro Ala Ser Ile Ser Cys Arg Ala Ser Lys Gly Val
35 40 45

Ser Thr Ser Gly Tyr Ser Tyr Leu His Trp Tyr Leu Gln Lys Pro Gly

-continued

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    50                  55                  60

Gln Ser Pro Gln Leu Leu Ile Tyr Leu Ala Ser Tyr Leu Glu Ser Gly
65                  70                  75                  80

Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu
                85                  90                  95

Lys Ile Ser Arg Val Glu Ala Glu Asp Val Gly Leu Tyr Tyr Cys Gln
            100                 105                 110

His Ser Arg Asp Leu Pro Leu Thr Phe Gly Gln Gly Thr Lys Leu Glu
        115                 120                 125

Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser
    130                 135                 140

Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn
145                 150                 155                 160

Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala
                165                 170                 175

Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys
            180                 185                 190

Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp
        195                 200                 205

Tyr Glu Lys His Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu
    210                 215                 220

Ser Ser Pro Val Thr Lys Ser Phe Asn Arg Gly Glu Cys
225                 230                 235
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1-30. (canceled)

31. A lyophilized formulation of an anti-human PD-1 antibody, or antigen binding fragment thereof, made by lyophilizing an aqueous solution comprising:

a) 25-100 mg/mL of the anti-human PD-1 antibody, or antigen binding fragment thereof;
b) about 70 mg/mL sucrose;
c) about 0.2 mg/mL polysorbate 80; and
d) about 10 mM Histidine buffer at about pH 5.0-pH 6.0, and

wherein the antibody, or antigen binding fragment thereof, comprises:

i) a light chain comprising three CDR sequences SEQ ID NOs: 15, 16, and 17; and
ii) a heavy chain comprising three CDR sequences SEQ ID NOs: 18, 19, and 20.

32. The lyophilized pharmaceutical formulation of claim **31**, wherein the anti-human PD-1 antibody, or antigen binding fragment thereof, is present at about 25 mg/mL in the aqueous solution.

33. The lyophilized pharmaceutical formulation of claim **31**, wherein the aqueous solution has a pH of about 5.5.

34. The lyophilized pharmaceutical formulation of claim **31**, wherein the antibody comprises a light chain comprising amino acid residues 20 to 237 of SEQ ID NO: 36 and a heavy chain comprising amino acid residues 20 to 466 of SEQ ID NO:31.

35. The lyophilized pharmaceutical formulation of claim **31**, wherein the antibody is h409A11.

36. A liquid pharmaceutical formulation of an anti-human PD-1 antibody, or antigen binding fragment thereof comprising:

a) 25-100 mg/mL of the anti-human PD-1 antibody, or antigen binding fragment thereof;
b) about 70 mg/mL sucrose;
c) about 0.2 mg/mL polysorbate 80; and
d) about 10 mM histidine buffer at pH 5.0-6.0,

wherein the antibody, or antigen binding fragment thereof, comprises:

i) a light chain comprising three CDR sequences SEQ ID NOs: 15, 16, and 17; and
ii) a heavy chain comprising three CDR sequences SEQ ID NOs: 18, 19, and 20.

37. The liquid pharmaceutical formulation of claim **36**, wherein the antibody comprises a light chain comprising amino acid residues 20 to 237 of SEQ ID NO: 36 and a heavy chain comprising amino acid residues 20 to 466 of SEQ ID NO:31.

38. The liquid pharmaceutical formulation of claim **36**, wherein the antibody is h409A11.

39. A method of treating cancer in a mammalian subject in need thereof, the method comprising administering an effective amount of a pharmaceutical formulation of an anti-human PD-1 antibody, or antigen binding fragment thereof comprising:

a) 25-100 mg/mL of the anti-human PD-1 antibody, or antigen binding fragment thereof;
b) about 70 mg/mL sucrose;
c) about 0.2 mg/mL polysorbate 80; and
d) about 10 mM histidine buffer at pH 5.0-6.0,

wherein the antibody, or antigen binding fragment thereof, comprises:

i) a light chain comprising three CDR sequences SEQ ID NOs: 15, 16, and 17; and

ii) a heavy chain comprising three CDR sequences SEQ ID NOs: 18, 19, and 20.

40. The method of claim **39**, wherein the antibody comprises a light chain comprising amino acid residues 20 to 237 of SEQ ID NO: 36 and a heavy chain comprising amino acid residues 20 to 466 of SEQ ID NO:31 and wherein the effective amount comprises a dose selected from the group consisting of about 1.0, 3.0, and 10 mg/kg administered at intervals of about 14 days or about 21 days throughout the course of treatment.

41. The method of claim **39**, wherein the antibody comprises a light chain comprising amino acid residues 20 to 237 of SEQ ID NO: 36 and a heavy chain comprising amino acid residues 20 to 466 of SEQ ID NO:31 and wherein the effective amount comprises a dose of 5.0 mg/kg or 10 mg/kg administered at intervals of every 2 weeks or every 3 weeks throughout the course of treatment.

42. The method of claim **39**, wherein the subject has melanoma, the antibody comprises a light chain comprising amino acid residues 20 to 237 of SEQ ID NO: 36 and a heavy chain comprising amino acid residues 20 to 466 of SEQ ID NO:31, and the effective amount comprises a dose of 3.0 mg/kg administered at intervals of every 3 weeks throughout the course of treatment.

43. The method of claim **39**, wherein the subject is treatment naïve.

44. The method of claim **39**, wherein the pharmaceutical formulation is administered in a 30 minute IV infusion.

45. The method of claim **39**, wherein the subject has been previously treated with additional chemotherapeutics.

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Expert Review

Stability of Protein Pharmaceuticals: An Update

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Abstract. In 1989, Manning, Patel, and Borchardt wrote a review of protein stability (Manning *et al.*, Pharm. Res. 6:903–918, 1989), which has been widely referenced ever since. At the time, recombinant protein therapy was still in its infancy. This review summarizes the advances that have been made since then regarding protein stabilization and formulation. In addition to a discussion of the current understanding of chemical and physical instability, sections are included on stabilization in aqueous solution and the dried state, the use of chemical modification and mutagenesis to improve stability, and the interrelationship between chemical and physical instability.

KEY WORDS: formulation; protein stability; protein stabilization.

INTRODUCTION

In 1989, Manning, Patel and Borchardt wrote a review summarizing what was known at the time about the stability and stabilization of protein pharmaceuticals (1), an article that has been referenced almost 500 times. In the late 1980s, there were only three recombinant protein products on the US market: Orthoclone (OKT-3), human insulin, and tissue plasminogen activator. If one included plasma-derived products, the number of approved proteins only numbered about a dozen. Clearly, recombinant DNA technology has drastically changed the pharmaceutical market. Now there are nearly twenty antibody products and almost 150 approved protein-based products that are commercially available in the US alone. In addition, our knowledge regarding protein stability and formulation has increased dramatically. The purpose of this review is to provide an update regarding what we have learned in the past 20 years. In addition to updating the sections of the original review article, some discussion is provided regarding topics that were not found in the literature at the time, such as the interrelationship of chemical and physical instability, instabilities that occur during bioprocessing, the impact of lyophilization cycle on protein stability, and the importance of packaging in maintaining protein stability.

One can separate protein instabilities into two general classes: chemical instability and physical instability. Chemical instabilities involve processes that make or break covalent bonds, generating new chemical entities. A list of the more commonly observed chemical degradation processes is listed in Table I. Conversely, there are physical instabilities for proteins in which the chemical composition is unaltered, but the physical state of the protein does change. This includes denaturation, aggregation, precipitation, and adsorption (Table I). The term precipitation is used here to denote insolubility rather than insoluble aggregate formation.

Our knowledge of all protein degradation pathways is markedly greater than it was 20 years ago. Therefore, the emphasis of this review is on the progress that has been made since 1989. In addition, there were degradation processes and topics that were barely discussed or observed at that time. Those are now included as separate sections below. For example, there have been many articles on increasing conformational stability of proteins with various excipients, both in aqueous solution and in the dried state. In addition, a brief overview is provided of protein stabilization methods, including various drying methods, chemical modification, and site-directed mutagenesis. Finally, a discussion of the interrelationship between chemical and physical instability is provided.

CHEMICAL INSTABILITY

Deamidation

Twenty years ago, it was already appreciated that deamidation, which involves the hydrolysis of Asn and Gln side chain amides, was a common degradation pathway for proteins and peptides. It is still regarded as the most common chemical degradation pathway for peptides and proteins. From a regulatory perspective, deamidation generates process-related impurities and degradation products. In addition, it may contribute to increased immunogenicity (2).

At the time of the original review article, there were a few examples of deamidation in pharmaceutically relevant proteins, including human growth hormone (hGH) (3,4), insulin (5), γ-globulin (6), and hemoglobin (7). Moreover, the effect of extrinsic factors, such as pH, temperature, and ionic strength, were known as well (8). Since that time, the amount of information now available on deamidation and related

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Table I. Chemical Instabilities Reported for Proteins of Therapeutic Interest

Deamidation
Asp–isoAsp interconversion/isomerization
Racemization
Proteolysis
Beta-elimination
Oxidation
Metal-Catalyzed Oxidation (MCO)
Photooxidation
Free radical cascade oxidation
Disulfide exchange
DKP formation
Condensation reactions
pGlu formation
Hinge region hydrolysis
Trp hydrolysis

reactions has increased significantly, as can be found in a number of excellent review articles (9–12) as well as entire books on the subject (13,14). There is even a web site devoted to this topic (www.deamidation.org).

Asn Deamidation

For those unfamiliar with this reaction, deamidation of Asn residues under acidic conditions takes place by direct hydrolysis of the Asn side chain amide to form only Asp. Under these conditions, deamidation is subject to acid catalysis. Similarly, Gln residues are converted to Glu (as is described in more detail below). However, this mechanism is rarely observed, as the pH must be less than 3. In neutral to basic solution (i.e., above pH 6), the mechanism changes to an intramolecular cyclization reaction. The first step involves nucleophilic attack of the n+1 nitrogen of the protein backbone on the carbonyl group of the Asn side chain (Fig. 1). This step is base catalyzed, since abstraction or partial abstraction of the backbone amide proton makes the nitrogen more nucleophilic, accelerating the reaction. A cyclic imide (also called succinimide or Asu) intermediate is formed (Fig. 1) with loss of ammonia. Since ammonia is a gas and is typically not retained in solution, this step is effectively irreversible. While the Asu intermediate often can be detected as a degradation product in its own right (see below), it is readily hydrolyzed in aqueous solution to form the Asp and isoAsp products (Fig. 1). Formation of the Asu five-membered ring intermediate is thought to be the reason that Asn deamidation is more prevalent than Gln deamidation, as five-membered heterocyclic rings are more stable than the six-membered rings associated with Gln deamidation.

Consequently, deamidation generates two degradation products (Asp and isoAsp) at the site of the original Asn residue. Coupled with the possibility for racemization (15), four possible products (L-Asp, D-Asp, L-isoAsp, and D-isoAsp) could be formed. It is now known that racemization does not occur to any appreciable extent from the Asu intermediate, as was previously thought. Instead, it appears to be a parallel degradation pathway (16). Dehart and Anderson have provided a detailed kinetic description of the intramolecular cyclization (17). The same observation of a lack of racemization via the cyclic imide intermediate has been made for larger proteins as well (18).

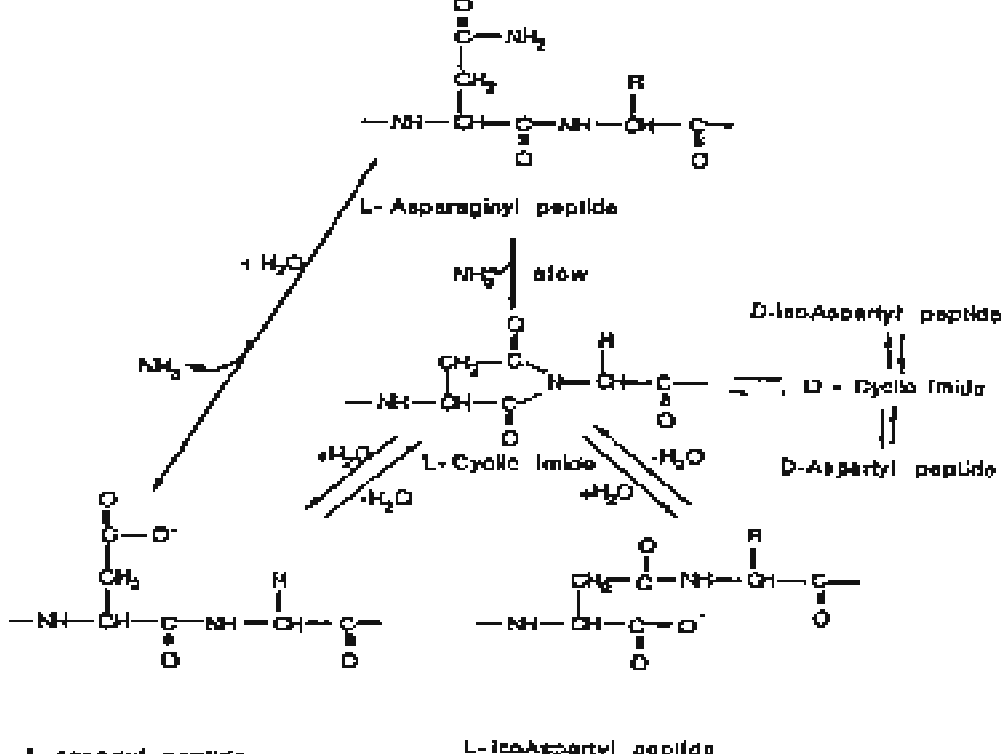

Fig. 1. General mechanism for deamidation of Asn residues and isomerization of Asp to isoAsp (taken from reference 1). Direct hydrolysis occurs below pH 4 while the cyclic imide pathway predominates at pH 6 and above.

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Effect of Sequence on Asn Deamidation

Some work had been done by 1989 on the effects of primary sequence on deamidation, especially recognizing that Asn–Gly were particularly prone to deamidation. Subsequently, the influence of sequence was examined by Robinson and coworkers in detail (19,20). Ultimately, their work on sequence effects resulted in effective predictive schemes (14,21–25). In general, two trends are apparent. First, having amino acids with smaller side chains after the Asn residues leads to faster deamidation, presumably due to lack of steric hindrance of the initial cyclization reaction. Second, succeeding amino acids that have side chains that can act as hydrogen bond donors tend to accelerate the reaction, likely due to intramolecular hydrogen binding to the carbonyl oxygen of Asn, making it more electrophilic and thereby more reactive to nucleophilic attack.

As a result, one does not need to be concerned about deamidation at every Asn residue. Only those Asn residues followed by a small or hydrogen bond-donating (e.g., Ser, Asn, or Asp) residues are found to exhibit deamidation on a time scale relevant to the pharmaceutical scientist. For example, Chelius *et al.* found that Asn deamidation in monoclonal antibodies (MAbs) occurred at Asn–Gly and Asn–Asn sequences (26), while Xiao and Bondarenko found deamidation at Asn–Asp sequences (27). Overall, Asn–Gly is the most reactive sequence in polypeptides, consistent with the schemes of Robinson and Robinson (Table II). For the most part, the preceding residue has little or no effect on deamidation rate, at least in solution. However, Li *et al.* have shown that Gln or Glu in that position appears to accelerate deamidation in the solid state, presumably by increasing hydration around the Asn residue (28).

For deamidation that occurs at acidic pH, the mechanism does not involve cyclic imide formation at all. Instead, the protonated amide side chain undergoes direct nucleophilic attack by water. Therefore, it is not surprising that sequence has been found to play a minimal role in controlling deamidation rates (29).

Table II. Relative Deamidation Rates for Asn–Xaa where Xaa is the Succeeding Amino Acid (Taken from Reference 21)

Residue	% deamidation after 100 d (Tris buffer)	% deamidation after 100 d (phosphate buffer)
Gly	38	87.3
Ser	9.8	34.6
His	7.7	33.2
Ala	5.6	17.9
Asp	4.8	19.0
Glu	3.0	13.1
Asn	1.5	13.5
Thr	2.0	12.3
Lys	1.8	10.4
Gln	1.7	10.1
Cys	1.1	7.3
Lys	1.8	10.4
Gln	1.7	10.1
Arg	1.6	9.4
Phe	1.1	7.3
Met	0.9	5.4
Tyr	0.9	5.4
Trp	0.4	5.0
Leu	0.3	5.4
Val		2.8
Ile		1.3

Effect of Higher Order Structure on Asn Deamidation

In 1989, the ability of higher order structure to influence deamidation rates was just starting to be appreciated. In 1988, Kossiakoff demonstrated that polypeptide chain flexibility impacted deamidation rates (30). Other studies have since arrived at the same conclusions, examining the relative deamidation rates for Asn residues dispersed across a given globular protein structure (31,32). In addition, a number of studies have shown that placement of the reactive Asn residue within an ordered secondary structure slows the reaction rate. This has been found for α-helices (33,34), β-sheets (35), and β-turns (36,37).

Combining information about primary sequence along with the location of an Asn residue within a three-dimensional structure leads to improved predictive accuracy for deamidation rates (22). Moreover, alterations in the three-dimensional structure can affect deamidation rates. For example, addition of ligands that induce α-helical structure in insulin slow deamidation at Asn^{B3} (38).

Deamidation in Monoclonal Antibodies (MAbs)

Our knowledge regarding the stability and structure of MAbs has increased exponentially over the past 20 years. This includes detailed studies of deamidation in these pharmaceutically important molecules. In general, deamidation is responsible for much of the heterogeneity observed in MAbs along with other kinds of chemical instability and glycosylation differences (39).

In 1992, Kroon *et al.* reported that OKT-3, the first marketed monoclonal antibody product, undergoes deamidation (40). Subsequently, there were sporadic reports of deamidation in MAbs over the next decade (41–43). In the last 5 years, the number of reports on deamidation in MAbs has increased significantly. Some focus on the effect of extrinsic factors (44), some on sequence effects (26), while others emphasize the analytical methods used to monitor and quantify deamidation, which is primarily done by some type of mass spectrometry (26,45–57). These studies provide a solid basis for monitoring and quantifying deamidation in any protein or peptide. Other groups have reported using charge separation methods to detect and quantify deamidation in peptides and proteins (58–61), while others have employed RP HPLC (62,63), peptide mapping (64), and even Raman spectroscopy, which was reported to detect deamidation (65). However, the latter is quite insensitive, requiring deamidation to exceed 10%.

Prolonged storage of a human MAb resulted in deamidation at both Asn and Gln residues, as well as other chemical instabilities, such as fragmentation and pGlu formation (66–68). Those other degradation pathways are discussed below. What appears to be true is that the factors controlling deamidation rate (primary sequence, temperature, pH, *etc.*) in peptides and smaller proteins are equally important in MAb degradation.

Deamidation of Other Protein Pharmaceuticals

In addition to the large amount of work on MAbs, a number of other studies have appeared describing deamida-

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Table III. Protein of Pharmaceutical Interst Where Deamidation has Been Observed

Compound	Reference
tPA	(69)
IL-11	(47,70)
rhGH	(71)
hGH	(72)
Tetanus vaccine	(73)
Anthrax vaccine	(74)
Anthrax protective antigen	(75)
Fc fusion protein	(76,77)
Glucagon	(78)
Lymphotoxin	(31)
Protein G	(79)
Hemoglobin	(80)
NGF	(81)
Hirudin	(82,83)
IL-1 receptor (type II)	(84)

tion in peptides and protein of pharmaceutical relevance, including vaccines and antigens (47,69–84). These include the entries in Table III. In general, any protein or peptide that contains one of these reactive Asn–Xaa sequences will be prone to deamidation over time.

Control of Deamidation Rates

A number of formulation approaches have been described to slow deamidation. The most effective approach is to control the pH. Deamidation for a single reactive Asn displays a V-shaped pH-rate profile, with the minimum being between pH 3 and 6. In addition, being a chemical reaction, it displays typical Arrhenius behavior, provided the protein or peptide does not change conformation appreciably across the temperature range of study.

Interestingly, it is possible to slow deamidation rates by altering the conformation of the protein. Even in 1989, it was known that a specific set of phi/psi angles is needed to allow the intramolecular nucleophilic attack to form the Asu intermediate (85). Phi and psi refer to the dihedral angles for the Cα–N bond and the C(O)-Cα bond, respectively. Therefore, limiting the flexibility of the peptide chain should and does slow deamidation. This is the basis for slower deamidation rates in well-defined and rigid higher-order structures (see above). It is possible to alter polypeptide chain flexibility using excluded solutes. Addition of sucrose to a flexible peptide caused it to adopt a β-turn conformation, thereby slowing deamidation (86). Sugars and polyols compact the structure of alcohol dehydrogenase and thereby slow deamidation in both the apo and holoenzymes (87). Similarly, removal of C-terminal amino acids in histidine-containing protein allows deamidation to proceed, presumably by removing steric constraints (88).

Finally, one can imagine that formulations that lower NH acidity would slow deamidation rates. This has been done using nonaqueous solvents (33,89), although these same solvents can also affect conformation, viscosity, and solvent dielectric, so the effect might not be entirely due to modulation of acid-base properties. The effect of viscosity has been described for model peptides (90,91). Similarly, dielectric and viscosity effects have been examined for Asp isomerization in MAbs (92,93). In that case, increased chemical stability was obtained at the expense of reduced conformational stability. Therefore, such approaches using nonaqueous solvents may not be viable for many globular proteins but could work for peptides, where solution conformation is less important to maintaining biological activity.

Prior to 1989, it was known that certain buffers exhibited buffer catalysis of Asn deamidation. Most buffers had been shown to exhibit some degree of buffer catalysis. Therefore, limiting the amount of buffer used should slow deamidation rates. In the last 20 years, relatively little has been done on this topic. Tyler-Cross and Schirch (29) demonstrated that deamidation of model peptides exhibited general base catalysis, but they did not observe specific base catalysis in their studies. So, apart from some observations on buffer effects, little has been done on mechanistic aspects of catalysis of deamidation. As for more recent observation on buffer effects, Girardet *et al.* reported that phosphate buffer increased deamidation rates in α-lactalbumin faster than tris buffer at pH 7.4 (94). Zheng and Janis conducted a detailed study on buffer effects on deamidation in a MAb, looking at tartrate, citrate, succinate, and phosphate (44). They found that citrate was the best choice, while the pH had to be less than 5.

Deamidation in the Solid State

The propensity of peptides and proteins to degrade chemically while in the solid state has been reviewed by Lai and Topp (95). Briefly, many of the reactions described here, including deamidation, have been observed for polypeptides in the solid state as well. For example, the deamidation rates of both cyclic and linear peptides were investigated in the solid state (37). A comparison of deamidation rates between solution and in the solid state can be found as well (96). Finally, Houchin and Topp (97) have recently reviewed the chemical degradation of peptides and proteins, including deamidation, encapsulated within PLGA microspheres.

Gln Deamidation

Our knowledge base regarding deamidation of Gln has increased tremendously over the past 20 years. It is still true that deamidation of Gln residues is less common than for Asn. Recall that cyclization of Asn residues leads to a five-membered ring. With Gln, that same intermediate is a six-membered ring, which is less favorable thermodynamically than the smaller ring. Certainly, Gln deamidation was known in 1989 (19). Yet, so little was reported that it was not discussed in our previous review. Since then, Joshi and Kirsch have reported some detailed mechanistic studies on Gln deamidation in peptides (78,98,99). A number of reports have found Gln deamidation in larger proteins, such as crystallins (100) and MAbs (101).

Theoetical Studies on Deamidation

In addition to the explosion of experimental studies on deamidation in peptides and proteins, a number of theoretical studies have emerged as well. These include molecular dynamics (MD) simulations (102) and *ab initio* calculations (103–105). Of note, Radkiewicz *et al.* 2001 showed that backbone conformation (i.e., phi–psi angles) affect acidity of the NH group (106). Gly, being able to sample more conformational space, shows

increased NH acidity, which would contribute to increased Asn deamidation rates. Therefore, the increased reactivity of Asn–Gly sequences might not be entirely due to lack of steric hindrance to intramolecular nucleophilic attack.

Succinimide Formation

In general, deamidated forms (Asp and isoAsp), as well as the corresponding cyclic imide (Asu) intermediates, have been isolated and identified, especially in peptides. The cyclic imide intermediate has been repeatedly isolated and characterized in monoclonal antibodies. Groups from Amgen used hydrophobic interaction chromatography (HIC), cation exchange chromatographjy (CEX), and liquid chromatography-mass spectrometry (LC–MS) to identify Asu formation in MAbs, especially IgG2s, that were stored at elevated temperatures (56,57,107,108). The primary degradation product appears to be the cyclic imide (Asu) intermediate at position 30 of the light chain (LC). Other studies have reported Asu formation at position LC32 (109) and residue 102 of the heavy chain (43).

Succinimide formation has been reported in other systems. For example, stressed samples of hGH form a succinimide product at an Asp–Gly site that was isolated and quantified using reversed-phase HPLC (110). Similar degradation has been reported for glial cell line-derived neurotrophic factor, which forms a succinimide product at position 96 (111). The degraded form was identical to the native protein in structure, pharmacokinetics and activity. Lysozyme has also been reported to form a succinimide product at a Asp–Gly site as well (112).

Asp Isomerization

Once the cyclic imide intermediate forms, it can open to form either Asp or isoAsp products (Fig. 1). Such a mechanism indicates that Asp itself could cyclize to form the same succinimide (Asu) species, thereby allowing conversion from Asp to isoAsp. This reaction has been called Asp–isoAsp interconversion, but is more commonly referred to as Asp isomerization. The rate-limiting step is the same for both deamidation and Asp isomerization, that is, the rate is controlled by formation of the cyclic imide intermediate. Consequently, the same approach can be taken to slow each reaction. In other words, pH provides the greatest degree of control by slowing deprotonation that leads to intramolecular cyclization. Early work on this reaction indicated that only the protonated form of Asp isomerizes, i.e., there is much lower reaction rate above pH 5 (113). In fact, above pH 8, the reaction is independent of pH and buffer concentration. Below pH 3, only hydrolysis is observed. The size of the C-terminal amino acid retards the formation of the cyclic imide intermediate (114), thereby slowing Asp isomerization. Steric constraints affect cyclization rates, as with deamidation (88).

Since the original review was published, Asp isomerization has been reported in many systems, especially monoclonal antibodies (27,43,55,92,93,107,108,115). Some of the same LC-MS methods used to identify deamidation in MAbs have been used to monitor Asp isomerization as well (27,55,116). Both degradation pathways have been observed in MAbs (43,55). Specifically, Asp isomerization has been reported at position 32 in the light chain (93,109) and position 102 in the heavy chain (43). For Asp–Asp sequences in MAbs, both Asp isomerization and Asp-assisted hydrolysis were observed (27).

Racemization (which is discussed in more detail below) has been observed concomitantly with Asp isomerization (63), similar to the observations with deamidation (13,15). This emphasizes once again how interconnected many of these chemical degradation pathways can be.

Other proteins of pharmaceutical interest besides MAbs have been reported to undergo Asp isomerization. For example, Asp^{93} isomerization has been shown to be the primary degradation pathway for NGF (81), while Asp isomerization (at Asp^{45} and Asp^{47}) has been found in IL-11 as well (70). Dette and Wätzig were able to resolve the isoAsp product of Asp isomerization in recombinant hirudin using capillary electrophoresis (117).

Outside of controlling pH and temperature (see above), little has been reported on formulation strategies to slow Asp isomerization. The use of excluded solutes to provide conformational stability in a MAb actually decreased chemical stability by accelerating Asp isomerization (92). Presumably, changing the succeeding amino acid (in the n+1 position) would also slow the reaction, but no detailed studies of that type have been reported.

Asp Hydrolysis

There is a third reaction that is associated with degradation at As/Asp residues and that is Asp-associated hydrolysis of the peptide backbone (also known as proteolysis). Unfortunately, there are few reviews available on the topic, with the most extensive dating back to 1983 (118). Since this reaction also involves intramolecular cyclization, it is not surprising that proteolysis shows the same pH-rate profile and sensitivity to buffer catalysis as deamidation (119). The mechanism was delineated in detail by Joshi and Kirsch (78), with nucleophilic attack occurring at the ionized side chain of Asp on the protonated carbonyl of the peptide backbone. This produces an anhydride species and release of the N-terminal portion of the peptide chain. There is some information available on the effect of primary structure on Asp hydrolysis. The presence of Ser or Tyr at position n+1 can accelerate reaction (98,99). Similarly, having Ser or Val at position n+1 accelerates hydrolysis relative to Asp isomerization (114).

Other similar hydrolysis reactions have been reported. For example, the Asn–Pro bond appears to be particularly labile in the presence of ammonia (120). A similar degradation process has been reported for the Asp^{60}–Pro^{61} bond in NGF (81). The peptide linkages in either side of Pro and Trp were found to hydrolyze in spantide II, a bioactive peptide (121).

Hinge Region Hydrolysis

Hydrolysis of the peptide backbone has been seen in antibodies even when Asp is not present. This reaction occurs most frequently within the hinge region of the antibody, so it is known as hinge region hydrolysis. However, it can occur at the C_H2–C_H3 interface as well (67). Typically, it occurs in IgG1s, so the reaction is likely influenced by the flexibility of the peptide chain. This reaction is distinct from the enzymatic hydrolysis that can occur in this region with antibodies (39).

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There have been a number of detailed studies on this process. The first study reported cleavage in the hinge region of mouse MAbs (122), showing that the reaction can occur under basic pH conditions (122). Fragmentation, along with other chemical instabilities, was reported in OKT3, which is a mouse IgG2a antibody (40,123). Using MALDI-TOF and capillary electrophoresis, Alexander and Hughes found hinge region hydrolysis to occur in chimeric mouse/human IgGs (124), as was also reported by Paborji *et al.* (125).

The general nature of this reaction was shown by Cordoba *et al.*, who showed that hinge region hydrolysis occurred in four different human IgG1s (126). The observed fragmentation pattern indicated that the hydrolysis reaction is not specific to a particular peptide bond, but occurs within a narrow range of residues. In this case, hydrolysis was limited to the heavy chain sequence Ser-Cys-Asp-Lys-Thr-His-Thr. Similarly, descriptions of hinge region hydrolysis, detected in the course of mass spectrometry studies on MAbs, were reported as well (127,128). While chain flexibility appears to be important, recently it was demonstrated that conformational instability of Fab region leads to increased rates of hinge region hydrolysis as well (129).

The pH-rate profile for hinge region hydrolysis is V-shaped (130), with a minimum near pH 6. The rate increases linearly with pH above pH 6. The study by Cordoba *et al.* indicated that EDTA and protease inhibitors have no effect on hydrolysis rates (126). In addition to the more general hinge region hydrolysis described above, there have been reports of metal-assisted hydrolysis of MAbs in the same region (131,132). In these cases, chelating agents have some ability to slow degradation.

Trp Hydrolysis

In addition to these better-known degradation processes, other functional groups are also sensitive to hydrolysis. For example, the side chain of Trp is known to undergo hydrolysis. The primary degradation product is called kynurenine (133–135), which fluoresces at much longer wavelength (450 nm) than Trp itself. Kynurenine and related substances can also form during oxidative degradation of Trp as well (see below).

Racemization and β-Elimination

These two degradation pathways are interrelated, as the initial step is the same: deprotonation of the hydrogen on the α-carbon (Fig. 2). Usually, C–H bonds have little acid-base reactivity, but the C–H bond of an amino acid does have some acidic character. As a result, racemization is usually a very slow process, so slow that it can be used to date artifacts. *In vivo*, a number of proteins have been reported to racemize, as in crystallins from the lens of the eye (136,137) and myelin in muscle (138).

Typically, the racemization occurs at Asp residues (138), although racemization at Asn[127] in murine lysozyme has been reported (139). Why this residue is more reactive is not yet known. A more extensive summary of amino acid racemization can be found in the review by McCudden and Kraus (140).

Once the Cα–H bond ionizes, recombination can lead to racemization (Fig. 2). On the other hand, the resulting carbanion can rearrange and eject a leaving group from the β-carbon, producing a double bond between the alpha- and beta-carbon. This is β-elimination. At high temperatures, it appears that β-elimination of Cys residues occurs readily in a number of proteins (141). Among proteins of pharmaceutical interest, β-elimination has been reported for IL-1ra (142) and insulin (143). It has also been shown that β-elimination occurs under conditions causing hinge region hydrolysis (144).

Fig. 2. General mechanism for racemization and β-elimination in proteins (taken from reference 1).

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DKP Formation

One other N-terminal cyclization process has been described in some detail. Note that the N-terminal amino group can be a potent nucleophile, especially above pH 8. If the amine attacks the second carbonyl group in the peptide backbone, a diketopiperazine (DKP) ring is formed. Degradation of the N-terminus of a peptide or protein by DKP formation has been commonly observed during long-term storage and during peptide synthesis (145–147).

This reaction was initially observed in peptides (148), where the DKP ring can rearrange, either with loss of the first two amino acids or reversal of their positions in the chain. The extent of DKP formation depends on percentage of terminal amino groups in the free base form (17,149,150). Under acidic conditions, the reaction is quite slow and pH-independent. Kinetic analyses of DKP formation in peptides have examined the effects of pH, buffer type and concentration and temperature (17,149–151). The first-order rate constant generally increases with increasing buffer concentration, except for carbonate, which shows no concentration dependence (150). Degradation caused by DKP formation was shown to be responsible for the N-terminal heterogeneity observed in hGH (145) and substance P (152). Further details of the reaction kinetics of DKP formation have been presented recently (17).

To the extent that DKP formation leads to reduction in the length of the polypeptide chain, it can be considered a proteolytic reaction. Rearrangement of a DKP from the first two amino acids, via cleavage of the peptide bond C-terminal to the second amino acid, produces a clipped protein reduced in molecular weight by the mass of the two amino acids. In solution, DKP formation is common for proteins with the N-terminal sequence NH_2-Gly-Pro (153).

pGlu Formation

This reaction was not covered in the original 1989 review, although there were some literature references prior to that date (e.g., references 154–156). This reaction involves nucleophilic attack of the N-terminal amine on the side chain of a Glu residue (and occasionally a Gln residue) to form a five-membered ring structure (Fig. 3). In other words, non-enzymatic formation of pyroGlu (pGlu) follows a mechanism similar to that for DKP formation in that it involves nucleophilic attack of the N-terminal amine on the polypeptide chain. In this case, site of attack is the carbonyl group of the N-terminal Glu side chain, resulting in elimination of water (Fig. 3). This cyclized, N-terminal structure is often observed in monoclonal antibodies due to the frequency of Glu in the first position of the light chain and occasionally in the heavy chain (45,67,157–162). Most often, the presence of pGlu is detected using mass spectrometric techniques. The pGlu degradation product has been seen to increase during prolonged storage of MAbs (67,160). In some cases, the conversion to pGlu at the N-terminus of the heavy chain has been quantitative (127). Formation of pGlu has also been reported in variants of BMP-15 (163).

As the reaction involves nucleophilic attack, rates of pGlu formation are typically pH-dependent. The pH dependence of the reaction has been reported (67,162), although the data are quite limited compared to the detailed pH profiles published for other hydrolytic reactions. It appears that the nature of the buffer has an effect on the rate of pGlu formation (162,164). Phosphate appears to cause more rapid cyclization, at least in model peptides (164). At lower pH, acetate appears to be the best buffer species for slowing pGlu formation (162). Finally, it has been reported that pGlu can be formed from N-terminal Gln residues as well as Glu, although the reaction appears to be slower with Gln than with Glu (154,165).

Glycation of Proteins

Glycation occurs when a protein is incubated in the presence of a reducing sugar. It involves the reaction with a base, typically the side chain of lysine and a carbonyl group of a reducing sugar. This leads to formation of a Schiff base, which can undergo rearrangement to more stable products. Altogether, these associated reactions are termed the Maillard reaction or non-enzymatic browning after the color that evolves.

The Maillard reaction can occur in the solid state as well as in aqueous solution (95). For example, recombinant DNAse I undergoes glycation in dried formulations (166). Glycation occurs *in vivo* as well as *in vitro*. In fact, the extent of glycation of hemoglobin *in vivo* is a distinctive marker for diabetes (167).

The mechanism of glycation has been outlined in some detail recently (168). When glycation occurs, it is known that it can affect function (169), although it has been shown not to affect binding affinity of certain antibodies (168). However, it can affect the overall stability of the molecule. For example, the Maillard reaction can lead to more labile peptide linkage, as in relaxin (170).

This degradation pathway is one of the primary reasons that formulation scientists tend to avoid using reducing sugars (glucose, lactose, fructose, maltose) in formulations. However, reducing sugars can be generated *in situ* by hydrolysis of sucrose. Smales *et al.* demonstrated this for sucrose-based formulations undergoing viral inactivation at elevated temperatures (171,172). Similarly, glycation in sucrose-based formulations has been observed during storage studies as

$-H_2O$

Fig. 3. Conversion of Glu to pGlu.

well (173–175). However, these usually require elevated temperatures and acidic pH. Note that the glycosidic bond of trehalose appears to be much stronger than for sucrose, as trehalose formulations rarely, if ever, display this type of decomposition. On the other hand, low pH has been shown to lead to sucrose instability and subsequent glycation (174). In fact, glycation happens about 2,000-fold faster with sucrose than trehalose at pH 2.5 (176) due to formation of glucose and fructose upon sucrose hydrolysis.

The sites of glycation usually involve lysine residues, although Arg residues and the N-terminus can be involved as well. It is known that certain lysines are more reactive in MAbs (177) than others with respect to glycation. The basis for the enhanced reactivity is unclear, although basicity and solvent accessibility are likely to be important. Glycation has been seen for IgG2s (178), as well as IgG1s (177). Some buffer catalysis has been observed for glycation (179), at least with γ-globulin, with phosphate accelerating the reaction. However, buffer catalysis was not seen with BSA or ovalbumin.

Oxidation

Chemical degradation of a protein due to oxidation is the other primary degradation process that occurs, along with the hydrolytic reactions described above. Any protein that contains His, Met, Cys, Tyr and Trp amino acids can be potentially damaged by reaction with any of a number of reactive oxygen species (ROS) (180–184). Oxidation of these reactive side chains in a protein can occur during any stage of protein production, purification, formulation and storage (40,185–187).

Our understanding of chemical degradation caused by oxidation of proteins has greatly expanded over the last 20 years. The rate of oxidation is affected by both intrinsic and extrinsic factors. Intrinsic actors include the flexibility of the peptide backbone (188) and the overall structure of the protein (188–190). In addition, extrinsic factors, such as pH (191–193) and buffer type (191,194), can affect oxidation rates of proteins as well.

Oxidation of proteins and peptides are usually broken down into two general categories: site-specific (i.e., metal catalyzed oxidation or MCO) and non-site specific reactions, which includes photooxidation and free radical cascades. The latter can be initiated from a variety of sources, leading primarily to Met and Trp oxidation.

Met Oxidation

The chemical stability of methionine (Met) residues has been shown to be important for conformational stability and protein function (191,195–197). Unlike hydrolysis reactions, it appears that Met oxidation is nearly pH-independent (191). Therefore, one cannot control oxidation by adjusting pH. Oxidation of Met can be accomplished with a wide range of ROS. Even molecular oxygen is potent enough to convert the side chain of Met to its corresponding sulfoxide. One must keep in mind that reaction with oxygen in aqueous solution will involve the temperature dependence of oxygen solubility in water, which increases as the temperature is lowered (198). So, while Met oxidation typically follows Arrhenius behavior, reactivity may actually increase in refrigerated samples due to the higher solubility of oxygen than at room temperature. As these reactions are propagated by free radicals, it must be recognized that there are many potential sources of free radicals, including containers (199,200) and excipients (see below).

Even at the time of the 1989 review, it was known that different Met residues oxidized at different rates (201). However, little was known beyond that observation. What appears to be most important in controlling oxidation rates in proteins is the degree of solvent accessibility of the particular residue, allowing the oxidizing species to attack the side chain readily. Therefore, a Met residue that is fully solvent-exposed will exhibit a maximal rate of oxidation, while a buried side chain will oxidize very slowly (187,202–205). In other words, the ROS must be able to access the side chain easily for oxidation to proceed rapidly. This also means that while proteins have some ability to protect certain groups against Met oxidation because of their ability to bury the side chain in the interior of the protein, peptides do not have this ability to protect themselves against oxidation (206). Peptides lack higher order structures, causing the amino acids to be fully solvent-exposed at all times, resulting in maximum rate of oxidation for Met.

So, while solvent accessibility is key for controlling oxidation rates, it may not be the only important factor. Some evidence has also been presented that the rate of Met oxidation is linked to or correlated with conformational stability (207–209). Moreover, near the melting temperature of the protein, one can observe non-Arrhenius kinetics due to large-scale structural changes (208).

Oxidation of Met residues has been widely reported for MAbs, especially those in the Fc region (186,202,210). In one study, the exact distribution of oxidized residues differs whether the protein is stored for an extended period of time or subjected to t-BuOOH (186). This illustrates the fact that, while forced oxidation studies are valuable in formulation screening, they may not produce the exact distribution of products seen during long-term storage.

Metal-Catalyzed Oxidation (MCO)

Metal-catalyzed oxidation occurs when a redox active metal binds to a protein. The ligands are often Gly, Asp, His and Cys. Of these amino acids, His and Cys are sensitive to oxidative damage, as the ROS generated at the metal center does not have to diffuse very far before reacting with the protein (211). Mechanistic studies show that the metal ion and peroxides undergo a Fenton-type reaction, creating free radicals (212). The products of the oxidation of His are varied (213), but 2-oxo-His appears to be the major oxidation product. The 2-oxo-His product has been detected in human relaxin (214), prolastin (215) and human growth hormone (211,216).

Trp Oxidation

Oxidation of Trp residue can occur, even in the absence of light. The primary products are kynurenine derivatives (217), especially when iron-based oxidants are used. The oxidation of Trp has been seen in MAbs, leading to new peaks in both SEC and RP HPLC chromatograms (218).

Photooxidation

In 1989, little was known about photolytic degradation of proteins beyond some reports regarding heme proteins that

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absorbed in the visible wavelength region. Since then, exposure to light has been recognized as a potential source of chemical degradation, as reflected in the ICH Guidelines Q1B. Recently, a number of reviews have been published that describe sources of light exposure, degradation mechanisms and potential methods to reduce damage caused by light (183,185). When a protein is exposed to light, species are generated that will cause chemical oxidation to amino acids that are sensitive to the light-induced oxidation, which are Trp, Tyr and Phe. The light-induced oxidation reaction pathway starts when a photon is absorbed, causing an electron to enter an excited state (185). From the excited state, the amino acid has a number of degradation pathways resulting in different products. It now appears that photodegradation, especially photooxidation, is a common degradation pathway for many proteins, as exemplified by photodegradation on numerous proteins found in milk (219).

Trp is most sensitive to the light above 300 nm of the four amino acids, as it absorbs the bulk of the light at these wavelengths. Recently, a number of examples of photolytic damage to pharmaceutical proteins have been reported, primarily involving photooxidation of Trp. In IgG monoclonal antibodies, the oxidation of the Trp appears to cause loss of bioactivity and discoloration for high concentration formulations (192). The rate of oxidation for the high concentration antibody formulation is a pH-dependent process and, at basic conditions, can result in soluble aggregates (192). Another MAb, MEDI-493, showed a loss of binding and biological activity when irradiated with UV light, caused by oxidation of Trp (220). Another study measured the effect of exposing three different monoclonal antibodies to 254 nm; all three antibodies showed an increase in the percentage of aggregates over the course of the study (221). Structural changes were observed in recombinant human interferon-α_{2a} when exposed to UV radiation measured by absorbance, circular dichroism (CD) and fluorescence (222).

When Trp is photoexcited by absorbing near-UV light, it can affect neighboring amino acids and, in doing so, can reduce disulfide bonds (193,223). The photoionization of Trp can reduce disulfide bonds by electron transfer, resulting in chemical and physical degradation of the protein (193,223–225). Studies with different proteins have demonstrated the ability of photoexcited Trp to reduce disulfide bonds in both the liquid (223,224,226,227) and solid-state matrixes (225).

Please consider one final note on photooxidation. It is possible for photooxidation to be facilitated by polysorbate, that is, polysorbates (also known by their trade name as Tweens®) have been shown to be photoenhancers, leading to more facile production of singlet oxygen (228). Therefore, increased oxidation of proteins may not be solely due to oxidative impurities in the surfactant.

Cysteine Oxidation

While the primary oxidative process involving Cys residues is formation of disulfide linkages (see below), they are also subject to other oxidation processes as well. For example, they can form sulfenyl species (229), where an oxygen atom is added, in much the same way that Met is oxidized to a sulfoxide. Thiol oxidation has been shown to ablate the activity of alcohol dehydrogenase (87). In addition, there is a growing body of literature on formation of thiyl species, sulfur-based free radicals that can form from photolytic initiation or disulfide decomposition (230).

Protection Against Oxidation

A number of approaches can be used to limit oxidation of the proteins, which are dependent oxidation mechanism. Oxidative damage caused when a protein is exposed to UV radiation can be limited by adjusting the secondary packaging and addition of additives to the formulation (185,192). Otherwise, formulation strategies for mitigating oxidation are somewhat limited. Minimizing exposure to oxygen, by reducing the headspace in the vial, appears to be effective (225,228,231). This is particularly important for Met oxidation, where even less potent oxidants can cause damage.

Limiting the solvent accessibility of oxidation-sensitive side chains is one possible strategy, which has been shown to work for subtilisin (232) and alkaline protease (233). On the other hand, sucrose increases the oxidation rate of Factor VII (234), for reasons that are not yet known. While compounds, such as mannitol, have been reported to be free radical scavengers (235,236), there are no reports of them being effective at reducing oxidation in biopharmaceutical proteins.

However, it has been shown that sugars and polyols can complex metals at high concentrations, thereby reducing MCO-induced damage (237). Sacrificial additives can be employed, with the intention of having these compounds oxidized instead of the active ingredient. This has been shown to be an effective strategy for free Met (228,238,239), for N-Ac-Met (240), for thiosulfate (228), and for N-Ac-Trp (241).

Control of MCO can be accomplished, in some cases, by the addition of EDTA (242,243). Such chelating agents can reduce the reactivity of these metals. Keep in mind that the binding affinity of EDTA decreases significantly below pH 5, where the carboxylate side chains become protonated. Note that antioxidants, such as ascorbate, while effective at reducing lipid peroxidation, can actually increase the reactivity of transition metals and increase MCO-mediated damage (211,244–246). Otherwise, minimizing the levels in the bulk drug and excipients used is the other factor that will lead to improved storage stability with respect to oxidation. Many excipients carry oxidative impurities, including polysorbates (247–249) and PEG (242,250,251). A review of peroxide impurities in excipients has been published (252).

Disulfide Scrambling

The ability of Cys residues to form disulfide bonds has been known for decades. While they can play a significant role in aggregation, through covalent cross-linking (see below), they can also affect the overall conformation of a protein, as occurs during rearrangement of existing disulfides within a molecule. As discussed above, removal of free Cys residues (the reduced form), which can act as the starting point for disulfide scrambling or exchange, can retard this process significantly. Even though the number and type of chemical bonds in the rearranged forms are identical, because chemical bonds have been broken and formed, this should be considered a type of chemical instability.

A number of articles have now appeared about the formation of disulfide isoforms in IgG2 monoclonal anti-

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bodies. The preferred method for detecting and quantifying these disulfide species appears to be capillary electrophoresis (CE) in the presence of SDS (253–255), although RP-HPLC and LC-MS has also been reported to resolve them as well (128). There appears to be a functional difference associated with placement of the disulfides in IgG2s (256,257). This demonstrates how rapidly our understanding of the molecular details of these complex molecules is evolving.

PHYSICAL INSTABILITY

Physical instability refers to any process whereby the protein changes its physical state without any change in the chemical composition. In particular, this review, like the one in 1989, will focus on four processes: denaturation, surface adsorption, aggregation and precipitation. For the purposes of this review, aggregation is restricted to formation of soluble aggregates, where precipitation refers to a macroscopic event where the protein can be seen coming out of solution. As seen below, precipitation may or may not be connected with aggregation. It may simply be due to conditions whereby the protein has exceeded its solubility limit.

Each of these four topics is extensive and continues to evolve. Therefore, the purpose is not to provide a comprehensive overview of each topic. Rather, the intent is to demonstrate how our understanding of protein stability in each of these areas has increased over the past 20 years or so.

Denaturation

Denaturation denotes the loss of the globular or three-dimensional structure that most proteins adopt. This globular structure is referred to as the native state, although it is well understood that it is really a multiplicity of microstates (258). Consequently, upon unfolding or denaturation, the protein changes its physical state, but the chemical composition remains the same. Denaturation can involve the loss of secondary or tertiary structure (or both).

Thermal Denaturation

Probably the most common stress that causes the globular structure of proteins to be lost is elevated temperature. A plot of temperature *vs*. the fraction of unfolded protein is sigmoidal, with the midpoint being denoted as the T_m value (standing for the temperature of melting). In general, one can imagine that increasing T_m reflects an increase in conformational stability. Assuming that the thermal transition from the folded to the unfolded state has a similar degree of reversibility (see below), this is possibly true (259). However, in the past 20 years, it has been shown that reversibility may be an even better indicator of storage stability than T_m values (260,261). Consequently, other measures of conformational stability, as from chemical denaturation studies (see below), may be more reliable for guiding formulation decisions.

Most often, thermally induced denaturation is irreversible, as the unfolded protein molecules rapidly associate to form aggregates. This behavior is often observed during thermal denaturation studies using DSC. Even since the report by Sanchez-Ruiz *et al*. on using the scan rate dependence of T_m (262), there have been numerous reports of using DSC to examine aggregation rates by varying the scan rate. The problem has been that it assumes certain kinetics. More recently, efforts have been made to develop more general kinetic schemes (e.g., references 263,264). While they remove some of the limitations of previous approaches regarding the reaction order, the mathematics associated with these schemes can be quite involved.

Cold Denaturation

While the process of cold denaturation has been known since 1961 (265), there have been few reports of proteins undergoing cold denaturation (266). This is because the vast majority of proteins exhibit cold denaturation well below the freezing point of water. This would imply that it is not of great significance with respect to protein denaturation. However, one must realize that the glass transition temperature of the maximally freeze-concentrated state (T_g') is usually well below −20°C, even in the presence of common stabilizers, such as sugars. This means that proteins will have a mobility in the −20°C frozen state that is similar to that in fluid solution. Consequently, the potential for cold denaturation may be greater than previously believed. For example, a recent study on IL-1ra estimated that the cold denaturation temperature is ~−10°C (209), easily accessible in the frozen state unless the storage temperature is well below −30°C.

Chemical Denaturation

Another common method used to unfold proteins, and thereby determine the free energy of unfolding, is the addition of chaotropes, that is, compounds that cause the loss of the globular structure of proteins. The most common of these, by far, are urea and guanidinium hydrochloride (GnHCl). Analysis of these sigmoidal curves to determine the free energy of unfolding (ΔG_u) has been summarized quite well in reviews by Pace and coworkers (267–269).

There is a difference of opinion as to whether the free energy of unfolding indicated by thermally induced denaturation and chemically induced denaturation correlate. For example, one group finds a good correlation (270), while one group finds just the opposite (271). The differences might be due to the variability in protein size, slight differences in reversibility or even differences in the temperature dependence of the pre- and post-transition regions (272).

The mechanism by which these compounds disrupt and destabilize the globular structure is still under intense investigation. Even in the past year, a number of papers have appeared about whether a chaotrope destabilizes the native state or stabilizes the unfolded state (273–276). In addition, urea appears to impede the hydrophobic collapse associated with formation of the globular native state (275). One thing is clear. Unlike excluded solutes, chaotropes appear to bind to proteins, reducing their chemical potential. As the unfolded state has a much larger surface area than the native state (usually), the chemical potential of the unfolded state is lowered to a greater degree. When it falls below that of the native state, the protein unfolds. It has been reported that the addition of high concentrations of either urea or GnHCl can alter the pKa of amino acid side chains by 0.3 to 0.5 units

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(277). This alone could affect the conformational stability of the protein via increased electrostatic repulsion.

Pressure-Induced Denaturation

Another area that was virtually unknown in 1989 is the idea of using high pressure to unfold proteins. Since then, a number of good articles on the topic have appeared (278,279). Typically, pressures of greater than 2,000 bar (~2,000 atmospheres) are required, with up to 4,000 bar often needed (279). The molecular basis for pressure-induced denaturation was recently described (280). Also, the ability of osmolytes or excluded solutes to stabilize proteins appears to work for pressure-induced denaturation as well (281). In general, pressure-induced denaturation appears to be fully reversible, unlike other stresses that cause protein unfolding (e.g., reference 282). It should be noted that intermediate pressures, 1,000–1,500 bar, can be used to dissociate aggregates and allow for facile refolding of aggregated protein (283,284).

Denaturation in the Solid State

Proteins can denature at elevated temperatures, even when in the solid state, where one would imagine mobility is quite limited. For the most part, the reported denaturation temperatures for dried proteins are quite high, often above 150°C (285–287). The T_m value, like the T_g value, appears to scale with moisture content and with each other (287–289). A detailed discussion of how freeze-dried proteins denature has recently been published (288,290), as it relates to T_g and other glassy state behavior. For example, for hGH, the denaturation only occurs above T_g, is cooperative and is mostly irreversible (290).

Intrinsically Denatured Proteins

Over the past decade, there has been a realization that many proteins exist, under native conditions, as unfolded structures (i.e., so-called random coils). The current designation for these proteins is intrinsically denatured proteins (IDPs). More than 50 such proteins have now been identified, and the topic has been recently reviewed (291–293). IDPs include some proteins of pharmaceutical interest, especially those of the acidic fibroblast growth factor superfamily. Therefore, proteins can be functional without having a globular fold. In these systems, denaturation, in the usually sense, does not apply.

Aggregation

Since the 1989 review appeared, the subject of protein aggregation has become one of the most highly debated and researched areas in the field of protein stability. Consequently, there have been a large number of studies on the topic, and many excellent reviews have been published (e.g., references 294–299). Therefore, only a brief overview will be given here. While the number and classification of general aggregation mechanisms varies from review to review, it appears that there are five general mechanisms (298), as summarized in Table IV.

Table IV. General Mechanisms of Protein Aggregation (as Described in Reference 298)

Mechanism	Description
1	Association of native monomers (as with assembly of insulin hexamers)
2	Aggregation of conformationally altered monomers (conformational change is the rate-limiting step)
3	Aggregation of chemically-modified monomers (may follow behavior of mechanisms 2 or 4)
4	Nucleation-controlled aggregation (involves formation of critical nucleus or seed)
5	Surface-induced aggregation

Aside from its role in the pathogenesis of amyloid diseases, protein aggregation is a frequently cited challenge in the manufacturing and development of protein therapeutics. Non-native aggregation has received much attention from industry, academia, as well as regulatory agencies in recent years for a number of reasons. First, aggregates of therapeutic proteins can increase the likelihood of adverse immunogenic effects during therapy, which has been linked to increased patient morbidity or mortality (300,301). Second, the biological function of the molecule can be compromised in non-native aggregates, thus reducing its efficacy. Finally, aggregated protein can make a solution appear turbid or physically separate from the solution, thus reducing its pharmaceutical elegance and making the drug product unacceptable for use by health care professionals.

Protein aggregation is a term that can include many types of molecular assemblies. Aggregation can arise from non-covalent interactions or from covalently linked species, which can vary widely in terms of reversibility (295–297). One of the greatest challenges to the investigation of the causes of protein aggregation is that there is no single pathway by which proteins can form an aggregate (Table IV). Aggregation can have many causes, such as incorrect folding during protein expression, and perturbation of the native conformation during protein purification, formulation, freeze-thawing, freeze-drying, ultrafiltration/diafiltration, vial and syringe filling, pumping, transportation or storage (302–306). These processes can jeopardize product stability by exposing proteins to potentially damaging conditions, such as freezing, dehydration, extreme pH, air–liquid interfaces, solid–liquid interfaces, or high or low temperatures.

Despite the diversity of potential causes and aggregation pathways that are in existence, current paradigm is that, in order to better control protein aggregation during processing and storage, it is important to consider the roles of intrinsic conformational stability of a protein as well as protein–protein interaction. In general, conformational stability has been considered the most influential factor in modulating aggregation. This is because nonnative aggregation of protein molecules starts from a partially unfolded state; thus, the level of transient reactive species, sometimes referred to as N* (due to its structural similarity to the native state), is thought to be the rate-limiting factor in protein aggregation (307–310). For example, aggregation of human growth hormone and acidic fibroblast growth factor has been effectively suppressed by additional of stabilizers that thermodynamically favor the native state (311,312). In these cases the stabilizing agents exert their

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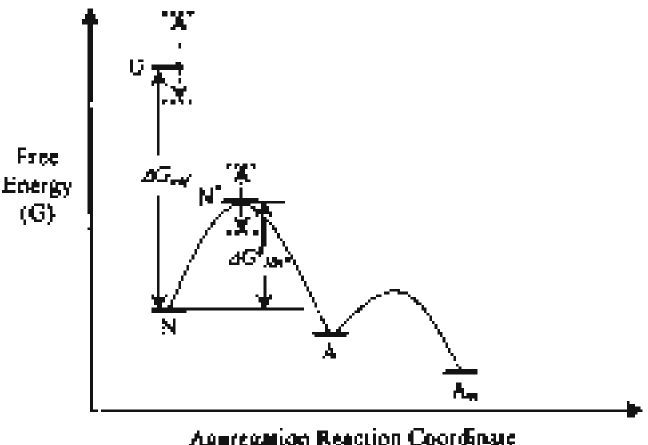

Fig. 4. Schematic reaction profile for aggregation of interferon-γ. A is the monomer aggregation intermediate. Dotted upward and downward arrows illustrate the shifts in free energy (relative to the native state, N) of the aggregation transition state, N*, and the unfolded state, U, when sucrose or GnHCl is added, respectively (taken from reference 296).

effect by preferentially binding to the native protein, thus reducing the equilibrium concentration of aggregation-competent species in solution. In another case, aggregation of recombinant human interferon-γ was inhibited by addition of sucrose, which increases the thermodynamic barrier between the native state and aggregation-prone state (Fig. 4). Similarly, the denaturant, guanidinium hydrochloride, lowers the barer, and aggregation rates increase. The stabilizing effect of sucrose occurs by way of the preferential exclusion mechanism pioneered by Timasheff and coworkers (308,313–315). In all of these cases, protein aggregation is reduced or inhibited under solution conditions that also increase the ΔG_u.

In order to understand the mechanism of aggregation and possibly design approaches to reduce aggregate formation, many measure aggregation kinetics. Some excellent overviews are available on the topic and make for excellent reading (296,316,317). The difficulty lies in that there are a multitude of possible kinetic schemes that can be envisioned (318). Distinguishing between them can be difficult, although a scheme proposed by Morris *et al*. appears to fit a number of data sets in the literature. In fact, many of the leading researchers in the field believe that unless elaborate kinetic studies are performed, it is nearly impossible to arrive at a unique mechanistic scheme (319,320). Therefore, it is expected that this area will continue to develop, as the need for fundamental understanding of the molecular details of aggregation is so great.

Precipitation/Particle Formation

The 1989 review listed precipitation as one of the four major physical instability pathways (1). It is important to specify what is meant by precipitation. On one hand, soluble aggregate formation can continue until the aggregates are so large that they can no longer remain soluble. This results in a macroscopic manifestation of aggregation that we observe as haziness or cloudiness. Often, this is now termed particle or particulate formation. This behavior is irreversible, and the protein is partially or completely unfolded. Formation of particulates has now become an important scientific and regulatory focus in the development of protein therapeutics.

There are compendial methods for measuring particulates in injectable products (321), such as USP method <788>. However, this method focuses only on particles above 10 and 25 μm. More recently, subvisible particulates have received a great deal of attention, both from regulatory agencies as well as researchers in the field (322). There is concern that these might be the most immunogenic of particulates found in protein products (301). Furthermore, new analytical methods, such as micro-flow imaging (MFI) allow one to not only quantify particles across this size range (323,324) but also capture images of the individual particles, making it possible to distinguish protein aggregates from foreign materials (325).

On the other hand, not all insoluble protein material is due to aggregation. One could have a protein that is salted-out, that is, the addition of an excluded solute has caused the chemical potential of the protein to exceed that of the solid phase (326,327). While our understanding of protein solubility is still imperfect, there have been significant advances in the past 20 years (326,328). Salted-out proteins still retain activity and native-like structure (327,329–331), and the precipitation is fully reversible upon dilution.

Surface Adsorption

Given the myriad of surfaces a protein may encounter during bioprocessing and in the final dosage form, interfacial stability is an important factor that cannot be underestimated. Adsorption itself is a physical instability, as it changes the physical state of the protein. However, even more problematic is the subsequent damage that can occur upon interfacial stress. Proteins in aqueous solution are known to adsorb to various surfaces. For example, G-CSF (332), a hydrophobic cytokine (174), and IL-2 (333) have been shown to adsorb to glass. The binding of IgG1 to plastic has been reported (334), and BSA, like other proteins, exhibits some propensity to bind to stainless steel (335). Consequently, many biophysical studies have been published on protein adsorption, especially as it applies to processing and protein instability (336–340).

Surface-induced protein instability begins with the adsorption of either native or partially unfolded protein on the surface; this interaction is usually more energetically favorable when a protein is partially unfolded due to their greater exposure of hydrophobic amino acid side chains, which are normally buried within the core of the protein. After initial adsorption of the protein, surface tension forces at various interfaces (i.e., air–liquid interface, solid–liquid interface) can drive aggregation by affecting structural integrity of protein molecules that populate the interfacial region (142,337,341,342). Structural perturbation at the surface combining with desorption of partially unfolded proteins from the surface can lead to nucleation and growth of aggregates in the bulk solution (142,343–346). Thus, interfacial stability of a protein is believed to be dependent on a number of key factors—surface tension, available surface area for adsorption, surface property of a protein molecule (i.e., hydrophobicity), and structural stability (342).

There are an increasing number of reports of proteins undergoing some type of degradation at solid interfaces. This is especially true for membrane interactions, where proteins

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undergo aggregation and fouling of membranes (347,348). It appears that the same approaches that stabilize protein against aggregation in solution will work for membrane-induced damage. This includes increasing conformational stability, reducing attractive protein–protein interactions, and using surfactants to limit adsorption of the protein to the surface.

In addition, there are a number of reports of foreign materials causing physical instability in proteins. For example, the shedding of glass nanoparticles during vial depyrogenation led to aggregation in PAFase (349). During filling, metallic particles can be introduced, forming nuclei for aggregate formation (304). Stainless steel nanoparticles can cause aggregation as well (350). A variety of leached materials from rubber, glass and metal components can cause instability in prefilled syringes (351–354), including issues with silicone oil. Silicone oil was implicated in insulin instability in the 1980s (355–357), but was rarely appreciated for causing instability problems for proteins. An extensive study on the effect of silicone oil on protein aggregation found that high concentrations were needed to have an effect (358). Yet, the problem persists. The data suggest that silicone oil may have little effect on a relatively stable protein, but may accelerate aggregation for a protein that is already compromised or marginally stable. This has been seen for an IgG1 where agitation accelerated aggregation in the presence of silicone oil, but silicone oil without the agitation stress did not cause aggregation (359).

Air–Water Interface

Of all interfacial damage, this is the one that appears to be most problematic. For one thing, it is a ubiquitous interface for any product during production. If the final formulation is an aqueous liquid, the opportunity for interfacial damage can occur during storage as well as shipping and handling. Thus, agitation studies are an important facet of screening excipients for a liquid formulation.

There have been a number of agitation studies performed on protein of pharmaceutical interest. The intent is to expose the protein to a sufficient interface to allow damage to occur, if it will. The usual formulation strategy to mitigate damage at the air–water interface is to add surfactants (see below). Agitation is usually accomplishing by stirring or shaking, although vortexing is sometimes reported. For example, extensive aggregation of porcine growth hormone (pGH) was observed upon vortexing for 1 min (360). Addition of 0.1% polysorbate 80 prevented vortexing-induced damage almost completely. Recently, the effect of shaking and stirring on an IgG1 was examined (361). Interestingly, stirring caused much more aggregation than shaking. In both cases, addition of polysorbate 20 was found to be quite effective at reducing interfacial damage. Agitation also caused damage of CNTF (362). In this case, increased stability was observed with the addition of PEG 3350 and propylene glycol as well as polysorbate 20. Therefore, surfactants are quite effective at diminishing interfacial damage of proteins in most systems. While this was well established 20 years ago, we now have many more examples and a greater mechanistic understanding of the stabilization afforded by surfactants. Further details about stabilization of proteins by surfactants are listed below.

Overall, surfactants are the most effective stabilizers with respect to minimizing interfacial damage. The effect of other classes of additives on damage at the air–water interface is less clear. For example, it is now known that increasing conformational stability can decrease the damage that occurs at an interface (363,364). On the other hand, addition of an excluded solute, like sucrose, increases the surface tension at the air–water interface, which can increase the likelihood of conformational rearrangement. Moreover, sucrose could enhance protein adsorption to the air–liquid interface (365) and promote unfolding at the interface (366,367). Therefore, it is not a simple matter to predict the effect of adding an excluded solute, like sucrose, on the interfacial stability of a protein in aqueous solution.

Agitation and Salt Effects

The effects of ions at interfaces have been studied, and such work has implications both for physical instability as well as for how ions affect the protein-solvent interface (368). Less is known about how ions affect protein behavior at the air–water interface. However, recent studies have begun to examine these effects. Enzyme deactivation in aqueous solution and at the gas–water interface in a bubble cell was found to depend on both bubble surface area size and salt concentration, with higher molarity of ammonium formate leading to increased deactivation (369). Salt-specific effects were investigated, and the enzyme was deactivated by chaotropes but not kosmotropes, providing some guidance regarding possible quantitative mechanisms of Hofmeister effects. In addition, formation of insoluble MAb aggregates due to agitation stress was examined in the presence of salts (370). Turbidity was found to increase with the chaotropicity of the added anion and ionic strength. So, there are an increasing number of examples demonstrating that salts can have both positive as well as deleterious effects on interfacial damage. Yet, our mechanistic understanding is still incomplete on this topic.

Ice–Water Interface

The first reports of damage at the ice–water interface appeared in the 1990s (142,371,372). In many cases, the addition of nonionic surfactants reduces the damage significantly (142,373). The extent of protein damage correlates with the surface area of the ice (374). Since then, there have been a number of reports of interfacial damage in frozen systems, including these listed above. One note is that the temperature dependence of the critical micelle concentration (cmc) is often overlooked. The cmc can increase by as much as five-fold upon cooling to near the freezing point of water (374), meaning that what might be a sufficient amount of surfactant for stabilization at room temperature may be insufficient at lower temperatures.

The detrimental effect of multiple freeze–thaw cycles is now well established (375,376). Consequently, nearly all freeze–thaw (F–T) studies are now conducted using multiple (three to ten) cycles. It should also be noted that it is important to use the same cooling and warming methods, as variations in each of these can affect the physical stability of the protein (e.g., references 371,377,378). Even proteins that are usually considered to be highly surface-active, such as IgGs, can undergo aggregation upon repeated F–T cycling (377,378).

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Reducing Interfacial Damage of Proteins

In the pharmaceutical industry, nonionic surfactants, especially polysorbate 20 and 80 (also known as Tween 20® and Tween 80®, respectively) are frequently added to protein solutions to prevent or reduce unwanted adsorption and aggregation during storage, filtration, purification, and transportation (142,343,379,380). The ability of surfactants, especially nonionic ones, to reduce interfacial damage is now well established (342). However, it should be noted that the use of nonionic surfactants might be associated with its own set of undesirable consequences. For example, although polysorbate 80 inhibited shaking-induced aggregation of IL-2 mutein, it increased both oxidation and aggregation of the protein during long-term storage (249). Additionally, depending on the temperature and nature of surfactant–protein interaction, nonionic surfactants may foster formation of protein aggregates in bulk solution in a concentration-dependent manner (381).

IMPROVING PROTEIN STABILITY

Our knowledge base on how to improve the stability of a protein is so much larger than in 1989. A few strategies and advances are examined here in some detail. Each topic could be the subject of its own review. However, they are worth noting in the context of protein instability, stabilization and formulation. They include

- ➢ Conformational Stabilization in Aqueous Solution
- ➢ Colloidal Instability
- ➢ Interfacial Instability
- ➢ Drying of Proteins
- ➢ Chemical Modification
- ➢ Site-Directed Mutagenesis

Conformational Stabilization in Aqueous Solution by Excluded Solutes (Osmolytes)

The ability of low-molecular-weight additives to increase the free energy of unfolding was well known in 1989, based on many publications by Timasheff and coworkers (313–315,382,383). However, the application of this approach as a general formulation strategy was only beginning to emerge at that time. Since then, numerous examples of conformational stabilization by excluded solutes have been reported. We now know that osmolytes operate by this general mechanism to provide increased conformational stability to proteins. Thus, nearly any sugar or polyol should increase the structural stability of a protein. In addition, it has also been shown that most amino acids act as excluded solutes (314,383–385), as do a number of salts (384,386) and many polymers, including gelatin (387) and even poloxamers (329).

Beyond increasing the conformational stability of proteins, another value of excluded solutes is to salt proteins out from solution. Such an approach has been widely used for enzymes, which are then sold as ammonium sulfate precipitates. The proteins that are salted out still retain native structure and activity (329,330). Recently, highly concentrated precipitates of native antibodies were prepared by this method (327).

Stabilization by Ligand Binding to the Native State

In contrast to the stabilization afforded by excluded solutes, it is possible to identify ligands that can bind selectively to the native state of a protein, resulting in net stabilization through the Wyman linkage function (388), a concept then elaborated by Tanford (389). Although this idea was known in 1989, it was not widely appreciated by pharmaceutical scientists at the time. Therefore, it is worth highlighting some of the reports that have been made in the last two decades, which demonstrate that many classes of excipients, including surfactants, buffers, polymers, and metal ions, all can improve conformational stability by this mechanism. At the same time, preferential binding to the denatured state can destabilize the conformation of a protein, as shown by Miyawaki (390,391). Consequently, while the Wyman linkage function can explain both conformational stabilization and destabilization, it affords another approach to increase structural stability of a protein. In turn, this should lead to reduced unfolding and subsequent aggregation during storage.

Buffers

The use of buffers to stabilize proteins has often been thought to be solely due to their ability to modulate changes in pH. However, for many protein formulations, especially when the protein concentration is relatively high, the protein, rather than buffer, provides the majority of the buffering capacity (392). In addition, other mechanisms for stabilization from buffers have now been reported (393). They can act as radical scavengers, a fact that goes back to original observations by Good *et al.* (394). These buffers are frequently referred to as Good's buffers. Even more important is the fact that some buffers appear to be able to bind directly to proteins, thereby increasing conformational stability.

Phosphate has been reported to confer some increased stabilization. One possible explanation for this, and many of the observed increases in stability with certain buffers, is direct binding of the buffer to the native state. According to Wyman, this should lead to net stabilization of the protein, provided there is no comparable binding to the unfolded state (388). In the case of phosphate, being highly charged, it is likely that the binding site will be at the N-terminus of an α-helix, where the helix dipole would favor interaction with a negatively charged ligand. Stabilization by phosphate has been reported (395,396).

More often, the stabilization seems to occur with nitrogen-based buffers. The preferential stabilization of an antibody by MES (397) over buffers like citrate has been reported. Similarly, histidine (His) has been shown to stabilize a monoclonal antibody (398), interferon-tau (399), and EPO (400). In the latter case, Tris buffer was also effective. In addition, some stabilization by citrate has been observed. In the case of interferon-alpha (IFN-α), citrate provides greater stability than phosphate or a phosphate–citrate mixture (401). Citrate also stabilizes antitrypsin (402) and IL-1ra (403) as well.

Surfactant

While surfactants mainly stabilize proteins by preventing access to and damage at interfaces (342), it has now been shown that polysorbates can bind to certain proteins, such as human growth hormone (311,376,404). Since those first

reports, polysorbates have been found to bind to fusion proteins as well (405). It is also worth noting that there have been reports of polysorbates not binding to certain proteins, especially antibodies (406,407). Pluronic F-107, another non-ionic surfactant, has been found to bind to G-CSF (408). Ionic surfactants also bind to proteins, conferring stabilization. Binding of anionic surfactants to proteins has been reported for a number of systems, including insulin (409), chymotrypsin (410), BSA (411), and TMV coat protein (412). In all of these cases, direct interaction with the native state of the protein leads to conformational stabilization.

Polymers

There have been a number of reports of anionic polymers (including biopolymers such as heparin) binding to basic proteins, resulting in stabilization. This has been seen repeatedly for aFGF and bFGF proteins (413,414). In these proteins, there is a highly positively charged cleft where the anionic polymer can bind. Other examples of anionic polymers (polyanions) binding to increase protein stability have been reported for insulin (415) and cytochrome c (416).

Cyclodextrins

Cyclodextrins (CDs) are annulated, bowl-shaped sugars that are known to bind small molecules, and a number are now found in approved pharmaceutical products (417). The binding occurs within the relatively hydrophobic interior of the CD, allowing the CD to present a more hydrophilic surface on the outside. In doing so, hydrophobic compounds can be solubilized (418). In the case of proteins, binding to the native state should result in net conformational stabilization, resulting in improved physical stability.

Addition of β-CDs has been reported to increase the physical stability of hGH with respect to aggregation (419,420). In both cases, the CD was shown to bind to the native state of the protein with a millimolar binding constant. In the latter study, it was also shown that the CDs did not improve chemical stability (420). Similar results of improved stability in the presence of CDs have been reported for insulin (421). On the other hand, there has also been a report that CDs decrease protein stability (422). In addition, there is some evidence that CDs may not stabilize simply by increasing conformational stability but play some role as a surface-active agent. It is known that hydroxypropyl-β-CD protects porcine GH from agitation-induced damage (356).

Metal Ions

Many proteins contain metal binding sites. Even a small cluster of acidic amino acids can allow metal binding. Keep in mind that even millimolar binding constants can produce an increase of 1 kcal/mole or more in the free energy of unfolding. For example, it has been known for some time that the four-helix bundle protein, human growth hormone (hGH) can bind a variety of divalent cations (423,424). On the other hand, too much zinc can lead to aggregation of hGH (425).

Similarly, calcium has been shown to provide significant stabilization for the marketed enzyme, DNase (426). The enzyme, fibrolase, contains one mole of zinc. It is not necessary for catalysis, but the zinc atom is critical for the conformational stability of the protein (427). Metal ions, such as calcium, are essential for bridging the two polypeptides chains of Factor VIII (428).

Anion Binding

Stabilization of proteins by direct binding of anions has been observed as well. For example, sulfate appears to bind to endostatin (429) and ribonuclease (430). Ribonuclease has also been shown to bind two moles of chloride ion, resulting in significant structural stabilization (~2–3 kJ/mol) (431). Stabilization of HSA has been observed from binding chloride (432) and carboxylates, such as formate and acetate (433).

Stabilization of Proteins by Ions

The effects of adding salts to a protein solution are many. Some of the effects of are due to specific interactions, such as the binding of cations or anions directly to the protein, as described above. Some of the effects are due to altering colloidal stability through charge screening. At the same time, there has continued to be investigation of the basis for the Hofmeister effect, which first described how the solubility and stability of proteins could be affected by ionic species.

Hofmeister Effects

There has continued to be investigation of the basis for the Hofmeister effect, which first described how the solubility and stability of proteins could be affected by ionic species (434). All in all, our understanding of this behavior has increased since 1989. Therefore, it is worth summarizing what is known about this important aspect of protein stabilization.

Several models have been devised to explain Hofmeister effects on proteins, including those based on the hydrophobic effect (435) and protein-solvent surface tension increments (436). Recently, Broering and Bommarius have challenged the validity of using surface tension increments to predict Hofmeister effects on proteins (437). Instead, the authors suggest that the B-viscosity coefficient from the Jones-Dole equation serves as a more accurate predictor of Hofmeister effects. The B-viscosity term describes the contribution of ion-solvent effects to solution viscosity (438). For three model enzymes, the authors demonstrate a strong correlation of kinetic deactivation constants with B in the presence of anionic chaotropes, whereas a similar correlation using surface tension increments was not found. However, a second study found that a similar correlation did not exist when using chaotropic cations, supporting the claim that anions dominate salt effects in solution (439,440). The most recent work from this group has focused on development of a mathematical model for B-dependent Hofmeister effects (441). In another interesting publication, Sedlak *et al.* report a correlation between change in thermal unfolding midpoint with ion concentration and water surface/bulk ion partition coefficients (442). Two proteins with very different net charges at pH 7.0 (−19 and +17) were examined, and both showed similar behavior under the authors' experimental conditions. This suggests that Hofmeister effects are not based on ion–protein electrostatic interaction.

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While various predictive models have been described in the literature, recent studies on the fundamental mechanisms behind Hofmeister effects question commonly held views on the behavior of these ions in solution (443). Bulk properties of aqueous solutions, such as boiling point and surface tension, are affected by the presence of ions. Likewise, ionic kosmotropes ("water structure makers") and chaotropes ("water structure breakers") have traditionally been viewed as exerting their effects on proteins through changes in bulk solution properties. To this end, much attention has been directed towards ion solvation by water. Hribar *et al.* modeled ion solvation in a two-dimensional plane using statistical mechanics and Monte Carlo simulations (444). The model was in agreement with Hofmeister series effects and Jones-Dole *B*-viscosity terms. Collins has argued that ionic charge density determines the degree and strength of hydration of an ion and that ion solvation in bulk solution indirectly influences protein solvation (445,446). Theories about long-range water-ordering properties of ions were challenged when Omta *et al.* used femtosecond pump-probe spectroscopy to show that dissolved ions do not affect water structure beyond the first hydrated shell (447). Structuring of water by ions was further investigated by Batchelor *et al.*, who used "pressure perturbation" calorimetry to demonstrate a lack of correlation between protein stability and ion hydration (448). While the body of literature devoted to understanding the Hofmeister effect has expanded greatly in the last two decades, a consensus on the mechanisms of this process has yet to emerge.

Colloidal Stability

Protein solutions can be characterized as a population or ensemble of colloidal particles suspended in an aqueous environment. Thus, colloidal stability is a reflection of the energetics of protein–protein interaction that can be manifested in solution properties, such as solubility, viscosity, crystallization and aggregation (449–451). Importantly, the nature of interaction between protein molecules in solution (i.e., attractive *versus* repulsive) can influence the rate of aggregation and the size of aggregates formed (309,452,453). In these studies, osmotic second virial coefficient (B_{22}), a thermodynamic parameter derived from McMillan-Mayer theory, was utilized to provide a quantitative measure of colloidal stability (454). There is an increasing amount of evidence which suggests that, in situations where the structural stability is comparable, reducing the attractive forces between protein molecules (e.g., through charge–charge repulsion) leads to greater physical stability with respect to aggregation (309,450,453,455) For example, Chi *et al.* demonstrated that independent of conformational stability, rhGCSF, a four-helix bundle protein, was maximally stabilized against aggregation when it is in solution conditions that have optimized colloidal stability (455).

Stabilization by Drying

One means of improving protein stability is lyophilization, also called freeze-drying, although other drying methods for proteins have been reported (see below). Despite the increased cost of production of lyophilization, this process can often provide advantages for shipping, long-term storage stability, and improved stability to temperature excursions. However, as with any protein formulation, there must be a good rationale for the additives that are selected. Since 1989, our understanding about developing lyophilized formulations has increased tremendously, so that, by 1997, the rules for rational design of stable lyophilized protein formulations were published (456) and then later expanded (457).

Freeze-drying (lyophilization) can produce instability in proteins at each step of the process. These include cooling, freezing, primary drying, and secondary drying. Details of each of these stages of freeze-drying can be found elsewhere (458–460). In short, lyophilization can be considered a combination of two distinct stresses: freezing and drying.

During freezing, the protein can be damaged by a variety of mechanisms, emphasizing the importance of controlling the freezing stage during lyophilization (461). There is the possibility that a pH shift can occur during freezing as a result of selective crystallization of buffer salts. While this was known for sodium phosphate since 1959, a number of newer studies have illustrated further how various buffers tend to crystallize during freezing (462–465), including phosphates, succinate, and tartrate (466). Citrate has also been shown to acidify to as low as pH 3 during freezing (467). This acidification arises due to selective crystallization of one of the buffer components. In general, the effect is minimized or eliminated in the presence of additional solutes or by increasing the protein concentration.

Also, freeze concentration can occur during the freezing process, especially for larger sample volumes. This is particularly problematic for large volumes of bulk drug (468,469). Deleterious effects of freeze concentration can result from the enormous increase in protein concentration and ionic strength in the non-ice phase of the formulation. This freeze concentration will lead to an increase in protein interactions, potentially leading to an increase in protein aggregation. This can often be inhibited by use of a preferentially excluded solute such as sucrose. Sucrose or other disaccharides can improve the stability of the protein during freeze-concentration by means of preferential exclusion (295,315,470).

Third, during freezing, proteins are exposed to a large ice–water interface. This presents a challenge for proteins that are surface labile (371,372). Therefore, many excipients, such as non-ionic surfactants, are added to the formulation to prevent surface-induced degradation. Polysorbate 20 and polysorbate 80 are commonly used for this purpose (142,295,375,462). Fourth, for many formulations cold denaturation could occur, provided the denaturation temperature is near −25°C or above. This instability was discussed above.

During the subsequent stages of lyophilization are the water removal processes of primary and secondary drying. Briefly, during primary drying, the water is removed in the form of ice in a process of sublimation under vacuum. In secondary drying, the bound residual water is removed under vacuum. This requires a higher temperature than for primary drying, which may be why the stress placed upon a protein is greater during secondary drying than during primary drying (373). Nevertheless, during the drying stages of this process, water is removed from the formulation. These drying stresses can often lead to dehydration-induced structural changes. However, these dehydration-induced structural perturbations can be often minimized by use of formulation additives, such

as disaccharides (470–472). Many labile proteins are protected from dehydration-induced degradation by a mechanism referred to as the water replacement mechanism, in which disaccharides are able to hydrogen bond to the protein, thus preserving the secondary structure of the protein (470–472). Hydrogen bonding of additives to polar and charged groups has been shown to protect proteins in cases of lysozyme (470), α-lactalbumin (473), and many other examples (470–472). The maintenance of the secondary structure of the protein has been shown to be a critical parameter for the successful development of a freeze-dried formulation, as it seems to correlate well with storage stability (474–477).

Stability during long-term storage typically requires a lyoprotectant to replace the hydrogen binding lost with removal of water and to provide a glassy matrix that limits mobility. Usually, disaccharides are used, such as sucrose, trehalose, or maltose. Even though these sugars are similar in size, they display very different solid-state properties in terms of molecular mobility and glass transition temperature (T_g) (478,479). Simply having a higher T_g is not sufficient to provide increased storage stability (480), although many studies have found a correlation between T_g and storage stability (474,477,481). Increasing the T_g by adding high-molecular-weight polymeric additives is usually ineffective due to phase separation of the protein and polymer (482).

Numerous studies have examined the differences between sucrose and trehalose in the stabilization of proteins in the solid state (483–485). Many reports on lyophilized myoglobin have shown trehalose to be superior due to greater coupling between protein and matrix relative to sucrose or maltose (483,486,487). This may be due to stronger water-mediated hydrogen bonding between protein and sugar (483) or lack of nanophase separation (484). More likely, it is due to modulation of β-relaxation processes in the solid state. In recent years, Cicerone and coworkers have demonstrated that these higher frequency relaxation processes appear to be critical in controlling long-term stability (488–490). Interestingly, there does not appear to be a correlation between lower frequency modes in the solid state (termed α-relaxation) and storage stability (491,492).

These concepts of solid-state mobility and structural relaxation are connected to other emerging ideas for stabilization of lyophilized proteins. First, the work of Cicerone and coworkers (488), along with observations from other laboratories, has demonstrated that small amounts of low molecular weight compounds, called plasticizers for their ability to lower T_g, can provide improved stability despite compromising T_g. This has been shown for water (115,492), glycerol (488), and sorbitol (492). The degree to which plasticization will improve stability may depend on properties of the protein, such as lack of disulfides and the percentage of polar groups on the surface (493). Second, it appears annealing the frozen matrix prior to primary drying can improve stability as well (494,495). Annealing provides a number of benefits, including reducing intervial heterogeneity and possibly reducing primary drying times (496). Overall, our understanding of the relationship between solid-state properties, protein stability and structure have increased in the last 20 years.

A couple other aspects of lyophilization development should be mentioned. First, it is useful to consider that the formulation and lyophilization cycle are well matched to ensure maximal product quality. This means having a cycle that is not only efficient, but also produces a pharmaceutically elegant cake, as well as a stable product. Elegant cake structure is often achieved by the use of bulking agents, which are additives that crystallize to provide mechanical rigidity to the cake. These include compounds such as mannitol or glycine (142,497,498). Amorphous bulking agents, such as hydroxyethyl starch, have also been reported (499). Recently, it has been found that bulking agents also have an impact on stability (477), presumably by having a small amount remain amorphous and plasticizing the matrix. Second, there are recent reports of material other than sugars yielding glassy matrices that might be used for embedding proteins and achieving stable dosage forms. These include many of the naturally occurring amino acids (500–502). In addition, combinations of compounds provide glassy matrices that have properties superior to the individual components. These include amino acids and polycarboxylic acids (503), LiCl and trehalose (504), and Arg in the presence of organic acids (505). This approach appears to work if there are strong interactions between the two components, such as electrostatic attraction or extensive hydrogen bonding (506).

Other Drying Methods

Several other drying methods have been examined for their ability to stabilize proteins. For bulk powders, there have been a number of reports using spray drying (507–510). These include formation of stable powders of IgGs (511,512) and hGH (513). Another approach is called spray freeze-drying, where droplets of the protein solution are frozen in liquid nitrogen and the subsequent particles are freeze-dried to remove the water (514–518). This approach uses a standard lyophilizer, but also requires equipment for conducting the spray-freezing process.

Both air drying (470) and vacuum drying (500,519) have also been reported for proteins. For example, IFN-α has been vacuum dried and reconstituted with nearly complete recovery of structure and activity (520). On the other hand, film drying (air drying of a protein solution, forming a film) has been reported for hGH (521). Also, supercritical fluid drying has been widely reported (522–525). A comparison of the various drying methods that have been used for protein formulation has been published (526).

Site-Directed Mutagenesis

Current recombinant DNA technology enables scientists to make specific and rational changes to the primary sequence by means of site-directed mutagenesis (527,528). Certainly, mutagenesis can be used to improve the solubility of the protein. For example, using portions of the hGH sequence, the solubility properties of bovine growth hormone (bGH) were improved by site-directed mutagenesis (529,530). Another example of site-directed mutagenesis to enhance the physical stability of a protein is the in the case of leptin. In these studies, Ricci and coworkers examined several mutations aimed at minimizing aggregation and precipitation occurring near neutral pH (531). One of the driving forces for the aggregation of leptin is believed to be one or both of

the surface-exposed tryptophans. By making numerous mutations to enhance the physical stability of leptin, making mutants with increased neutral pH solubility was achieved. It has also been shown that improving the stability of beta-turns through mutagenesis increased the conformational stability of proteins (532).

Mutagenesis has been used to improve the chemical stability of proteins as well. The IgG-binding domain of streptococcal protein G was modified by mutagenesis. Here, the investigators were able to enhance the protein's stability in alkaline conditions by replacing amino acids susceptible to high pH, asparagines and glutamine, with amino acids that are less susceptible to degradation under basic conditions (79). As with the mutation of a labile residue such as Asn for a protein under alkaline conditions, similar mutations could be considered for the enhancement of stability of a protein susceptible to oxidation. Lu *et al*. elegantly demonstrated the use of site-directed mutagenesis of methionine residues of GCSF to investigate the impact of oxidation on the stability and activity of G-CSF (204). In addition, Kim *et al*. reported an example where mutations were made to staphylococcal nuclease in order to decrease its lability to oxidation (207). However, these investigators not only studied the effects of methionine mutations on protein oxidation, but also to the conformational stability determined by guanidine unfolding between oxidized, non-oxidized, and the various mutations.

Finally, mutagenesis has been widely employed to improve the physical stability of proteins, especially with respect to aggregation. A few examples will suffice to demonstrate this point. Numerous studies on the use of mutagenesis to stabilize antibodies have appeared. Using an aggregation propensity mapping algorithm, a number of more stable mutants of full-length antibodies were designed and prepared (533–536).

Computational Methods and Protein Stability

In the past two decades, numerous algorithms have been developed to predict the aggregation behavior of proteins. A recent book chapter summarizes these methods (537) and the current advances in this area. In general, there are numerous algorithms available to predict aggregation propensity from the primary sequence. There are also methods to make predictions based on the overall properties of the protein (e.g., hydrophobicity, pI, *etc*.) (e.g., references 538 and 539).

In addition, researchers are continuing to advance our knowledge of protein stability by combining tools from computational chemistry and structural biology to design proteins that have specific characteristics, such as decreased immunogenicity (540,541), improved activity (542), and enhanced stability (534,543). Additionally, computational methods have the potential to play a large role in protein stabilization, such as optimizing the electrostatic field on the surface (544). In addition, Dahiyat has eloquently described other such examples of *in silico* design of protein surfaces and mutations occurring in the boundary between the core and surface (545). Space limitations do not permit a full description of all of the activities in this field. The use of computational methods is certain to improve the design of stabilization studies as well as provide greater mechanistic insight into the stability of proteins.

Chemical Modification

In many cases, the specific protein may not be amenable to changes in the primary sequence due to decreased activity or other significant manifestations of the behavior of the protein. Moreover, post-translational modifications, many of which occur *in vivo*, can alter the properties of proteins in profound ways (546). Therefore, controlling the extent of modification, such as with glycosylation, can provide a means of modulating the chemical and physical stability of a protein. These modifications can be accomplished *in vivo* (by controlling fermentation and using molecular biology methods) or *in vitro*.

Among synthetic methods, the most common method to modify proteins is by the addition of polyethylene glycol (PEG) groups (547) in a process referred to as pegylation. While the initial intent was to extend the half-life of a protein *in vivo*, it is now known that pegylation can improve the conformational and physical stability of a protein. For example, interferon-α_{1b} was stabilized by the addition of PEG groups in a site-specific manner (548). Similarly, pegylation has been found to stabilize trypsin (549), chymotrypsin (550), endostatin (551), and single chain antibody fragments (552).

There is certainly a strong rationale for employing glycosylation of proteins based upon lessons learned from nature. Glycosylated proteins are certainly very prevalent in biological systems. It has been reported that close to half of all proteins are glycosylated (553). The role of glycosylation in the stabilization of proteins was recently reviewed by Solá and Griebenow (554). Their review contains a detailed summary of how protein glycosylation affects the physical stability of proteins, many of pharmaceutical relevance. In the case of protein solubility, it has been proposed that the addition of glycans to the protein via chemical glycosylation can enhance the solubility by increasing the degree of glycosylation and by increasing the surface-accessible surface area (555,556). Glycosylation can also improve chemical stability. In the case of erythropoietin (557), the glycosylated form displayed an enhanced stability with respect to tryptophan degradation compared to non-glycosylated erythropoietin. Even the attachment of a sugar via glycation can improve the physical stability of a protein. It has been shown that the glycation of proteases (trypsin, chymotrypsin) improves its thermostability (558).

A variety of approaches for stabilization of enzymes have been summarized by Fagain (559). This included a number of cross-linking studies and even chemical modification of Lys residues (560), which presumably increased solvation and colloidal stability. The crosslinking could involve introduction of a disulfide bond or could be accomplished by chemical reaction of surface acidic and basic groups using linkers.

INTERRELATIONSHIP BETWEEN CHEMICAL AND PHYSICAL INSTABILITY

Although it is convenient to distinguish chemical from physical instabilities for purposes of discussion and mechanism, the fact remains that chemical and physical instabilities are interrelated. Our understanding of this has continued to improve. This section describes how certain chemical degradation processes make a protein more prone to aggregation. Likewise, there are examples of denaturation increasing the chemical reactivity of a protein.

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Connection Between Deamidation and Aggregation

Deamidation has been found to produce species that are more prone to aggregate than the unmodified protein. For example, deamidation of βA3-cystallin leads to destabilization of the native structure and increased aggregation (561,562). Deamidation has also been linked with decreased solubility of lens crystallins (563) and to a lower kinetic barrier for unfolding (100). The collagen-to-gelatin transition appears to be affected by deamidation (564).

Aggregation of βB1-crystallin is accelerated by deamidation at both Asn and Gln residues (565). The stability in urea, with respect to deamidation, is decreased as well (566,567). Deamidation appears to lead to increased fibril formation in Aβ peptides (568,569). Also, deamidation may lead to increased amyloid propensity in β2-microglobulin (570).

Connection Between Glycation and Physical Stability

Recently, it has been shown that glycation can increase thermostability (558). Whether this is a general phenomenon is not clear. Rearrangement of the initial Schiff base leads to what are referred to as advanced glycation end products (AGEs). These materials have been shown to exhibit increased aggregation propensity in some cases (571).

Correlation Between Oxidation and Physical Stability

It is important to note that oxidation can lead to increased propensity for physical instability, such as aggregation. This has been seen for an IgG1 (210), α-synuclein (572), calcitonin (573), trypsin inhibitor (574) and fumarase (575). Moreover, it has been shown that oxidation can reduce the conformational stability of a protein, as with glutamine synthetase (576), calmodulin (577), and a Fc fragment from an IgG1 (210).

SUMMARY

The field of protein stabilization and formulation has made tremendous progress in the past 20 years, and this review only contains a portion of the relevant studies. Our increased understanding of chemical instability pathways now allows us to adjust solution conditions to minimize degradation. Stabilization by excipients seems to be due to the mechanisms first espoused by Timasheff and Wyman. Now we have a large number of examples of how proteins can be stabilized in aqueous solution using these general approaches. Our understanding of stabilization of proteins in dried solids has evolved as well, with attention now turning to the molecular details of interactions in the solid state. Interfacial damage continues to be a challenge, and we are just beginning to appreciate how various excipients can modulate behavior at interfaces. Finally, it is clear that chemical and physical instability are linked in many systems. Overall, this field has advanced so that rational design of dried and liquid formulations is more possible than ever before. Yet, we continue to find new facets of protein behavior as we apply the tools of computational chemistry, biophysics, and molecular biology. This suggests that the next 20 years will continue to provide improved insight and knowledge regarding the stability of pharmaceutical proteins.

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Abstract □ Recombinant humanized monoclonal antibody HER2, rhuMAb HER2, in liquid formulations undergoes oxidation when exposed to intense light and elevated temperatures (30 & 40 °C). Met-255 in the heavy chain of the Fc region of the antibody is the primary site of oxidation. Met-431 of the Fc fragment can also be oxidized under extreme conditions. The amount of oxidation was determined by cleaving the Fab and Fc fragments by papain digestion, and the oxidized Fc fragment was detected by hydrophobic interaction chromatography. Oxidation of rhuMAb HER2 was also formulation dependent. The presence of NaCl in the rhuMAb HER2 formulation caused an increase in oxidation at higher temperatures after contact with stainless steel containers or stainless steel components in the filling process. The corrosion of stainless steel by chloride ions at the low pH of the formulation buffer generated iron ions that catalyzed methionine oxidation in rhuMAb HER2. Temperature-induced oxidation of rhuMAb HER2 occurred by the formation of free radicals, and light-induced oxidation of rhuMAb HER2 occurred via singlet oxygen pathway. Antioxidants, such as methionine, sodium thiosulfate, catalase, or platinum, prevented Met oxidation in rhuMAb HER2, presumably as free radicals or oxygen scavengers. The minimum effective levels (molar ratios of protein to antioxidant) required to inhibit temperature-induced oxidation were 1:5 and 1:25 for methionine and thiosulfate, respectively. A thiosulfate adduct of rhuMAb HER2 was observed by cation-exchange chromatography. These studies demonstrate that stoichiometric amounts of methionine and thiosulfate are sufficient to eliminate temperature-induced oxidation of rhuMAb HER2 caused by free radicals that were generated by the presence of metal ions and peroxide impurities in the formulation.

Introduction

Oxidation of methionine is one of the major degradation pathways in many protein pharmaceuticals, including interleukin-2, relaxin, parathyroid hormone, and human growth hormone.[1−7] Methionine residues in proteins are susceptible to oxidation, resulting in the formation of methionine sulfoxide and, under extreme conditions, sulfones. Methionine sulfoxide formation can occur during synthesis, purification, formulation, manufacturing, and storage of protein pharmaceuticals. During formulation and storage, oxidation of methionine-containing proteins can be caused by the presence of certain formulation excipients, including polyethylene glycols and nonionic polyether surfactants; these compounds can undergo autooxidation to form peroxides.[8] In fact, peroxides such as hydrogen peroxide have been widely used for studying the kinetics and mechanisms of methionine oxidation in proteins.[9−11] Peroxides react with metal ions to form free radicals that can initiate oxidation of proteins.[12,13] Methionine can also be photooxidized by a free radical pathway,[14] or via singlet oxygen intermediate formation.[15] For instance, photosensitizers can absorb radiation energy to form excited species that initiate the formation of free radicals for methionine oxidation. The excited species can also react with oxygen to form singlet oxygen, which in turn oxidizes methionine to yield methionine sulfoxide. Photolytic degradation of protein drug products can be influenced by many factors, including the buffer and its concentration, excipients, and formulation pH. Storage conditions, such as radiation intensity and duration and temperature, can also affect the rate of photolytic degradation.

The use of antioxidants in pharmaceutical products is one of the common ways to protect the drugs from oxidative degradation.[16,17] The unknown toxicity of many antioxidants and their incompatibility with proteins and/or excipients in parenteral formulations has hampered their use in protein pharmaceuticals. Nevertheless, several antioxidants have been identified for the prevention of methionine oxidation in recombinant human proteins,[18] including chelating agents, reducing agents, oxygen scavengers, and chain terminators.[19] Chelating agents bind to metal ions that catalyze oxidative reactions. Metal ions can react with peroxide impurities or the protein itself in the formulation to form free radicals that initiate oxidative degradation. Reducing agents reduce an oxidized drug product. For instance, the oxidized form of methionine, methionine sulfoxide, can be reduced to methionine by a reducing agent. Oxygen scavengers are substances that are more susceptible to oxidation than the drugs they are protecting. These substances are reducing agents that can react with oxygen by preferential oxidation, and thus remove the source of oxidation. Chain terminators are substances that can react with free radicals to produce intermediates that terminate the oxidation reactions via free radical pathways.

The overexpression of a 185 kDa glycoprotein ($p185^{HER2}$) has been found in tumor cells of breast and ovarian cancer, and rhuMAb HER2 binds to the extracellular domain of the glycoprotein to inhibit the growth of human breast carcinoma cells.[20,21] In the initial development of a liquid formulation for rhuMAb HER2 used in the treatment of breast cancer patients, methionine sulfoxide formation was detected using *tert*-butylhydroperoxide oxidant.[22] The goals of this study were to investigate the effect of temperature and light on oxidation of rhuMAb HER2 in liquid formulations, to determine the mechanisms of methionine oxidation, and to identify antioxidants for their prevention.

Experimental Section

Materials— rhuMAb HER2, expressed in Chinese hamster ovary cells, was purified by Process Recovery Operations at Genentech, Inc. All chemicals and reagents used were reagent grade. Solvents used for hydrophobic interaction and cation-exchange chromatography (HPLC) assays were filtered before use. *L*-Methionine, sodium thiosulfate, and catalase were purchased from Sigma Chemical Company (St. Louis, MO). Platinum was purchased from Aldrich Chemical Company (Milwaukee, WI). Carboxypetidase B and papain (from Carcia papaya) were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN).

rhuMAb HER2 Formulations—The thermal stability and photostability studies of rhuMAb HER2 were assessed for the single and multiple dose formulations listed in Table 1. Formulation A was designed for use in single dosing, and Formulations B−D were

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Table 1—List of rhuMAb HER2 Liquid Formulations

Formulation	Usage	Protein Concentration, mg/mL	Buffer Excipients
A	Single dose	5	5 mM sodium acetate, pH 5.0 147 mM NaCl 0.01% polysorbate 20
B	Multiple dose	21	5 mM sodium acetate, pH 5.0 4% mannitol 0.01% polysorbate 20 1% benzyl alcohol
C	Multiple dose	21	5 mM sodium acetate, pH 5.0 4% mannitol 0.01% polysorbate 20 1% benzyl alcohol 14.5 mM methionine
D	Multiple dose	21	5 mM sodium acetate, pH 5.0 4% mannitol 0.01% polysorbate 20 0.01% benzethonium chloride 14.5 mM methionine

designed for multiple does and therefore contain preservatives. All formulations were filled under aseptic conditions into 10-mL glass vials with a fill volume of 10 mL.

HPLC Assays—A Hewelett Packard HP-1090L HPLC instrument equipped with a diode array detector and solvent delivery system was used to assess the stability of rhuMAb HER2 by hydrophobic interaction, size exclusion, and cation-exchange chromatography methods.

Hydrophobic interaction HPLC (HIC) was employed to quantify the amount of oxidized Fc in rhuMAb HER2. For separating the Fc domain from the Fab fragment of the monoclonal antibody, rhuMAb HER2 samples were first digested with carboxypeptidase B at a concentration of 1:100 (w/w; carboxypeptidase B: rhuMAb HER2) at 37 °C for 20 min, followed by papain digestion at a concentration of 1:200 (w/w; papain: rhuMAb HER2) at 37 °C for 2 h. Digested samples were injected directly onto a TSK Butyl-NPR column (4.6 × 35 mm, Tosohaas) and eluted at 0.5 mL/min with a mobile phase consisting of buffer A (20 mM Tris at pH 7.0) and buffer B (2 M ammonium sulfate in buffer A). A linear gradient from 10 to 100% buffer A was performed over 37 min at ambient temperature. Peak detection was performed at 214 nm.

Native size exclusion chromatography (SEC) was used to determine the amount of soluble aggregates and monomer present in the rhuMAb HER2 formulations. Samples were eluted isocratically at ambient temperature using a TSK G3000SWXL column (7.8 × 300 mm, Tosohaas) at 1 mL/min with phosphate buffered saline (PBS) at pH 7.2 as mobile phase. The runtime was 20 min, and peaks were detected at 280 nm.

Ion exchange HPLC (IEC) was employed with a Bakerbond WP Carboxy Sulfon column (4.6 × 250 mm, J. T. Baker) to characterize deamidation of rhuMAb HER2 in the formulations. Samples were eluted at 1 mL/min with mobile phase consisting of buffer A (20 mM sodium phosphate at pH 6.9) and buffer B (0.2 M sodium chloride in buffer A). A linear gradient from 10 to 45% buffer B was performed over 55 min. The column was maintained at 40 °C, and peaks were detected at 214 nm.

Extracellular Domain (ECD) Plate Binding Assay—This assay assessed the ability of rhuMAb HER2 to bind to the ECD of the $p185^{HER2}$ glycoprotein. rhuMAb HER2 samples were diluted into assay buffer to concentrations within the linear range (10−70 ng/mL) of the standard curve for the assay. Diluted samples were incubated with recombinant $p185^{HER2}$ ECD protein in 96 wells of a microtiter plate for 1 h at ambient temperature. After incubation, the wells were washed with assay buffer, and a HRP-conjugated anti-human Fc goat antibody was added. After additional washing, a substrate of *o*-phenylenediamine was added for a 10 min incubation at ambient temperature in the dark. Absorbances at 490−492 nm were read, and sample concentrations were determined from a standard curve using a four-parameter logistic curve fitting program.

Bioassay—This assay quantitated the bioactivity of rhuMAb HER2 by measuring the antiproliferative effects of the antibody on BT-474 cells derived from human breast ductal carcinoma. rhuMAb HER2 samples were diluted into assay buffer to a concentration of 0.25 μg/mL. Diluted samples (100 μL) were incubated with BT-474 cells in a 96-well tissue culture plate at 37 °C for 96 h. After incubation, medium was removed and the plate was stained with Crystal Violet stain. Bioactivity of rhuMAb HER2 was quantitated by measuring the absorbance at 540 nm. Sample concentrations were determined from a four-parameter fit equation generated from the standard curve data. The range for sample quantitation was 20% from the low and high asymptote of the curve generated.

Thermal Stability Studies—The effect of temperature on oxidation of rhuMAb HER2 was studied by incubating samples of Formulation A (single dose) and Formulation B (multiple dose) at 5, 30, and 40 °C for 2 weeks. At each timepoint, samples were analyzed for oxidation by HIC, aggregation by SEC, deamidation by IEC, and activity by ECD plate binding assay and bioassay.

Photostability Studies—Two vials of each liquid formulation (A−D) were stored in a light box (Forma Scientific, model 3890) under high intensity fluorescent light maintained at 20 000 lux, which is ∼15−20 times that of indoor fluorescent light. Another two vials wrapped with aluminum foil were also stored in the light box as controls for the same period of time. The temperature of the light box was at 27 °C. After 2 weeks, samples were assayed by the same analytical methods as those used for the thermal stability studies.

Antioxidant Studies—To study the inhibitory effect of antioxidants on light-induced oxidation of rhuMAb HER2, methionine and sodium thiosulfate were added to formulation A at final concentrations of 3.5 mM (0.05% w/v) and 6.3 mM (0.1% w/v), respectively. The antioxidant-containing formulations were filtered into 10-mL glass vials with a fill volume of 10 mL, and two vials from each formulation were kept in the light box. As controls, two other vials were shielded from light by wrapping with aluminum foil and stored in the same light box. At 2 weeks, samples were analyzed for oxidation by the HIC assay.

The inhibitory effect of antioxidants on temperature-induced oxidation of rhuMAb HER2 was evaluated by adding 3.5 mM methionine (0.05%), 6.3 mM sodium thiosulfate (0.1%), catalase (0.002%), and platinum (0.005%) to Formulation A before filling into 10-mL glass vials. Samples were stored at 40 °C for 2 weeks and then assayed by the HIC method. The effect of molecular oxygen on oxidation of rhuMAb HER2 was also examined by replacing headspace oxygen from two 10-mL filled vials of Formulation A with nitrogen before they were stored at 40 °C for 2 weeks. Samples were analyzed by the HIC assay.

To determine the minimum effective levels required to inhibit rhuMAb HER2 oxidation, methionine and sodium thiosulfate at molar ratios between 1:1 and 1:180 (protein: antioxidant) were added to Formulation A. Antioxidant-containing rhuMAb HER2 material was then filled into 10-mL glass vials. Samples were stored at 40 °C for 2 weeks, and then assayed for oxidation by the HIC method.

Results and Discussion

Thermal Stability of rhuMAb HER2—The effect of temperature on oxidation of rhuMAb HER2 was studied in Formulation A (single dose) and Formulation B (multiple dose) at 5, 30, and 40 °C. As shown in Figure 1, the increase in oxidation of rhuMAb HER2 was temperature and formulation dependent. After incubation for 2 weeks, the rhuMAb HER2 in Formulation A (5 mg/mL protein in 5 mM sodium acetate, 147 mM NaCl, 0.01% polysorbate 20, pH 5.0) had 10, 17, and 52% oxidized Fc at 5, 30, and 40 °C, respectively. The percentage of oxidized Fc analyzed by HIC at each timepoint was defined as the sum of the peak areas of the two oxidized peaks (1 & 2) divided by the total peak areas of the Fc peaks (Figure 2). Previous studies demonstrated that peak 1 contained oxidized Met-255 and peak 2 consisted of rhuMAb HER2 oxidized at both Met-255 and Met-431 in the Fc.[22] Although these two methionine residues located on the surface

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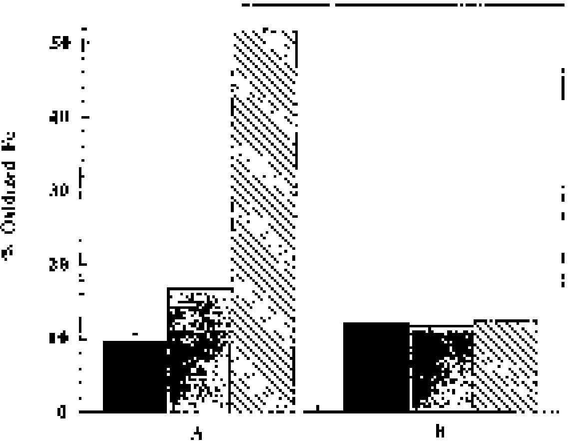

Figure 1—Effect of temperature on oxidation of rhuMAb HER2 in Formulations A and B. Samples were analyzed for methionine oxidation by HIC after 2 weeks of incubation at 5 °C (black), 30 °C (gray), and 40 °C (striped).

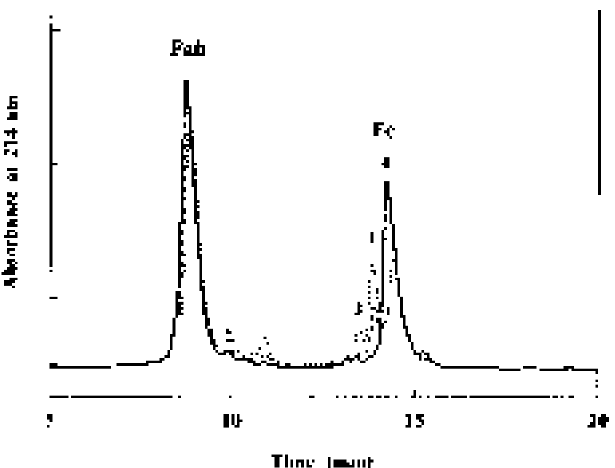

Figure 2—HIC chromatograms showing the effect of temperature on oxidation of the two methionine residues on the Fc domain of rhuMAb HER2 (Formulation A). Samples were incubated at 5 °C (solid line), 30 °C (dashed line), and 40 °C (dotted line) for 2 weeks. The three main peaks of the rhuMAb HER2 Fc domain are non-oxidized Fc (peak 0), Fc fragment with oxidized Met-255 (peak 1), and Fc fragment with oxidized Met-255 and Met-431 (peak 2). After incubation, the 5, 30, and 40 °C samples contained 10, 17, and 52% oxidized Fc (peaks 1 & 2), respectively.

of the antibody are more susceptible to oxidation than other methionine sites that are buried in the variable domains, they are neither within the Fcγ receptor region nor the complement C1q epitopes in the Fc of the heavy chain. In Formulation A, oxidation of these two methionine residues increased with temperature. In contrast, Formulation B (multiple dose), which consisted of different excipients, did not exhibit an increase in methionine oxidation at high temperatures (30 & 40 °C) after 2 weeks of incubation. In addition, when the surfactant (polysorbate 20) was not added in Formulaiton A, <10% oxidized Fc was detected in the samples that were strored at 30−40 °C. These results indicate that oxidation of rhuMAb HER2 was also formulation dependent.

The difference in the degree of oxidation of rhuMAb HER2 in Formulations A and B at high temperatures was also process related. The NaCl-containing rhuMAb HER2 Formulation A had 52% oxidized Fc at 40 °C for 2 weeks when it was filled into 10-mL glass vials by a stainless steel filler, as compared with 18% oxidized Fc when filled by a nonstainless steel filler. The replacement of NaCl by mannitol as a tonicifier yielded Formulation B that did not induce rhuMAb HER2 oxidation at high temperatures, even though it was filled by the same stainless steel filler. These results reveal that the excipient, NaCl, in Formulation A played an important role in rhuMAb HER2 oxidation. One hypothesis for the role of salt in the oxidation of rhuMAb HER2 was the corrosion of the stainless steel components of the filler by chloride ions at low pH, generating metal ions such as iron that catalyzed oxidation. When rhuMAb HER2 in Formulation A was tested for metal ions by induced coupled plasma (ICP) spectrophotometry, the amount of iron increased from <0.1 ppm in the control sample (glass vial) to 3.1 ppm in a sample stored at 5 °C for 3 months in a 30-mL stainless steel container (Type 316L, Fluid Line Technology). Furthermore, when the NaCl-containing rhuMAb HER2 Formulation A was prepared by diluting its concentrated bulk with a formulation buffer that had been made in a stainless steel tank, the protein had a 26% increase in oxidation after 2 weeks at 40 °C. No increase in oxidation was observed for the formulation buffer prepared in glass container. These results further reveal that the presence of NaCl in a formulation and contact with stainless steel both contributed to the increase in rhuMAb HER2 oxidation, probably through the generation of metal ions.

To further confirm that the formation of iron ions as a result of corrosion of stainless steel by the salt was the major cause for the oxidation of rhuMAb HER2 at high temperatures, a metal chelating agent (EDTA) was added to the sample vials filled by stainless steel filler prior to incubation. After incubation at 40 °C for 2 weeks, samples containing either 0.02 or 0.05 mM EDTA did not show an increase in methionine oxidation, as compared with a 42% increase in the control sample without the chelating agent. This result suggests that the formation of iron ions in the rhuMAb HER2 Formulation A due to the contact with stainless steel was responsible for the oxidation of the protein at high temperatures.

The oxidation of methionine residues in peptides and proteins is often initiated through the generation of reactive oxygen species,[23] and the rate and amount of methionine sulfoxide formation are affected by exogenous factors such as metal ions.[24] Although many oxidative pathways are possible for methionine, the mechanism for the temperature-induced oxidation of methionine residues of rhuMAb HER2 in the NaCl-containing Formulation A may involve metal-ion-catalyzed free radical formation. The formulation contained polysorbate 20, a nonionic polyether surfactant that can undergo autooxidation upon storage to form alkyl hydroperoxides. These peroxide impurities can be further decomposed by the presence of heavy metal ions, such as iron formed by the contact between stainless steel, and chloride ion at low pH to initiate alkoxyl free radical (RO•). This alkoxyl free radical can then react with methionine residues of rhuMAb HER2 to form a positively charged methionine free radical that can further react with the hydroxide ion to generate hydroxyl methionine free radical in the propagation phase. The alkoxyl free radical can also react with molecular oxygen to generate hydroxyl radical ($HO_2^\bullet$), which can be terminated by reacting with the hydroxyl methionine free radical to form methionine sulfoxide, as shown in the following proposed mechanism:

Initiation $$ROOH + Fe^{2+} \rightarrow RO^\bullet + Fe^{3+} + OH^- \quad (1)$$

Propagation $$RO^\bullet + R'\text{-}S\text{-}CH_3 \rightarrow R'\text{-}S^{\bullet}_{+}\text{-}CH_3 + RO^-$$
$$R'\text{-}S^{\bullet}_{+}\text{-}CH_3 + OH^- \rightarrow R\text{-}S^\bullet(OH)\text{-}CH_3 \quad (2)$$
$$RO^\bullet + O_2 + H_2O \rightarrow HO_2^\bullet + ROOH$$

Termination $$R\text{-}S^\bullet(OH)\text{-}CH_3 + HO_2^\bullet \rightarrow R\text{-}S\,(O)\text{-}CH_3 + H_2O_2 \quad (3)$$

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Table 2—Effect of Temperature on Stability of rhuMAb HER2 Liquid Formulations

Temp (°C)	Days	Formulation	% Monomer (SEC)	% Deamidation (IEC)	% Specific Activity (ECD Assay)	% Specific Activity (Bioasay)
5	0	A	100	9.4	104.6 ± 2.4	94.9 ± 10.6
		B	100	12.0	94.8 ± 2.1	113.0 ± 18.1
30	14	A	99.4	8.6	98.0 ± 3.1	93.5 ± 6.1
		B	99.4	8.3	107.7 ± 3.2	104.0 ± 4.5
40	14	A	99.0	8.4	91.9 ± 3.9	96.8 ± 3.0
		B	99.6	6.7	98.5 ± 0.1	97.5 ± 8.3

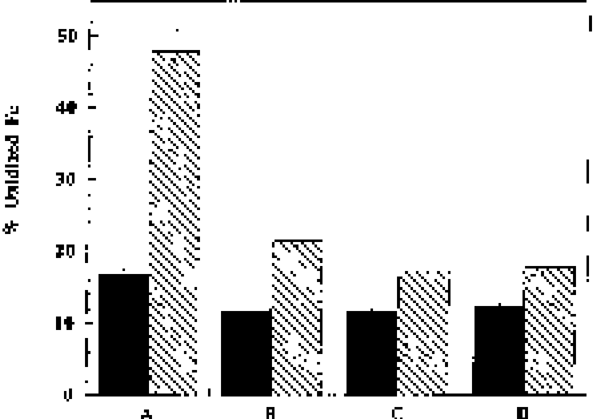

Figure 3—Effect of light on oxidation of rhuMAb HER2 in various formulations. Wrapped (black) and unwrapped (striped) samples were stored in a ligth box with light intensity of 20 000 lux for 2 weeks. The light box temperature was 27 °C. After light exposure, samples were analyzed for methionine oxidation by HIC.

The polysorbate 20 was necessary to prevent agitation-induced aggregation of the protein, so it could not be excluded so as to eliminate oxidation by the pathways described in eqs 1−3. Although temperature and excipient in the formulation could cause methionine oxidation of rhuMAb HER2, they had no significant effect on aggregation, deamidation, and biological activity of the protein (Table 2). In fact, rhuMAb HER2 in Formulation A, which contained 52% oxidized Fc, still retained >90% of its binding activity and bioactivity, as determined by the plate binding assay and bioassay, respectively. This result is probably due to the fact that the potential oxidation sites are neither within the complementarity-determining regions (CDRs) nor the Fc receptor binding sites of the antibody. The major concern about methionine oxidation in rhuMAb HER2 is related to regulatory issue. To fulfill the FDA requirement for a stable formulation, one must demonstrate that the pharmaceutical product is not >10% degraded and that the degradation products do not have any adverse effects on the safety and efficacy of the drug.

Photostability Studies—To study an alternative oxidation pathway for rhuMAb HER2, the effect of light on methionine oxidation in rhuMAb HER2 was assessed in various formulations. The results are shown in Figure 3. After 2 weeks of storage in the light box, the unwrapped vials of rhuMAb HER2 in formulations B, C, and D all had a slight increase in oxidized Fc (17−22%) as compared with ~12% in their wrapped control vials. However, the amount of oxidation induced by light in these formulations was still much less than that observed in Formulation A (48% oxidized Fc in the unwrapped vials and 16.5% in the wrapped control vials). These results suggest that rhuMAb HER2 was susceptible to photooxidation in all formulations tested, and that the greater extent of methionine oxidation in Formulation A was probably due to the combined effects of light and the increase in temperature of the light box (27 °C).

Photolytic oxidation of rhuMAb HER2 can occur when the protein itself absorbs energy from the radiation source (fluorescence light in the light box). This absorbed energy can be dissipated in the form of thermal energy, which produces an increase in temperature for inducing methionine oxidation of rhuMAb HER2. This sequence may also be an explanation for the greater increase in methionine oxidation in Formulation A than in Formulations B, C, and D after light exposure, because Formulation A was shown to be temperature sensitive (Figure 1). Another mechanism for photolytic degradation of rhuMAb HER2 in these formulations may involve the formation of singlet oxygen, which has shown to be one of the photooxidation pathways for methionine-containing compounds.[15] The formation of singlet oxygen was probably due to the presence of polysorbate 20, a photosensitive agent, in the rhuMAb HER2 formulations. After the absorption of radiation energy from the light box, polysorbate may dissipate its energy by reacting with molecular oxygen to generate singlet oxygen. This singlet oxygen can react with methionine to form an intermediate that oxidizes a second methionine molecule to form sulfoxide, as shown in the following equations:

$$\text{Polysorbate} \xrightarrow{h\nu} \text{Polysorbate}^* \quad (4)$$

$$\text{Polysorbate}^* + O_2 \rightarrow \text{Polysorbate} + {}^1O_2 \quad (5)$$

$$\text{R-S-CH}_3 + {}^1O_2 \rightarrow \text{R-S(OO)-CH}_3 \quad (6)$$

$$\text{R-S(OO)-CH}_3 + \text{R-S-CH}_3 \rightarrow \text{R-S(O)-CH}_3 + \text{R-S(O)-CH}_3 \quad (7)$$

The photo-induced oxidation of rhuMAb HER2 was prevented by the presence of methionine in the formulation. As shown in Figure 3, Formulation B (21 mg/mL rhuMAb HER2 in 5 mM sodium acetate, pH 5.0, 4% mannitol, 0.01% polysorbate 20, and 1% benzyl alcohol) contained 22% oxidized Fc after 2 weeks of light exposure as compared with 17% in Formulation C, which consisted of the excipients used in Formulation B plus 14.5 mM methionine. Formulation D, which contained 0.01% benzethonium chloride as preservative, photo-oxidized at a similar rate as Formulation C, which contained 1% benzyl alcohol. Thus, the preservative did not affect the rate or extent of oxidation. These results suggest that the presence of a reducing agent such as methionine can act as an antioxidant to reduce photooxidation of methionine in the protein. In fact, methionine has been shown to be effective in reducing methionine sulfoxide formation in peroxide-mediated oxidation of recombinant human ciliary neurotrophic factor and nerve growth factor.[18]

In summary, methionine oxidation was the major degradation pathway of rhuMAb HER2 caused by light. The oxidized rhuMAb HER2 (light-induced) appeared to retain its full binding activity and bioactivity in the plate binding assay and bioassay, respectively. Light had no significant effect on

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Table 3—Effect of Light on Stability of rhuMAb HER2 Liquid Formulations

Condition	Days	Formulation	% Monomer (SEC)	% Deamidation (IEC)	% Specific Activity (ECD Assay)	% Specific Activity (Bioasay)
Dark	14	A	99.4	8.3	118.0 ± 6.8	NT[a]
		B	100	9.4	93.1 ± 9.5	104.7 ± 4.5
		C	100	9.8	88.7 ± 5.4	106.4 ± 4.7
		D	100	9.6	90.1 ± 0.9	111.7 ± 5.8
Light	14	A	98.6	8.0	113.7 ± 5.2	NT
		B	100	8.2	95.0 ± 7.6	93.2± 6.3
		C	100	8.2	100.6 ± 1.4	98.8 ± 4.6
		D	100	8.2	98.6 ± 6.9	95.0 ± 0.7

[a] NT = not tested.

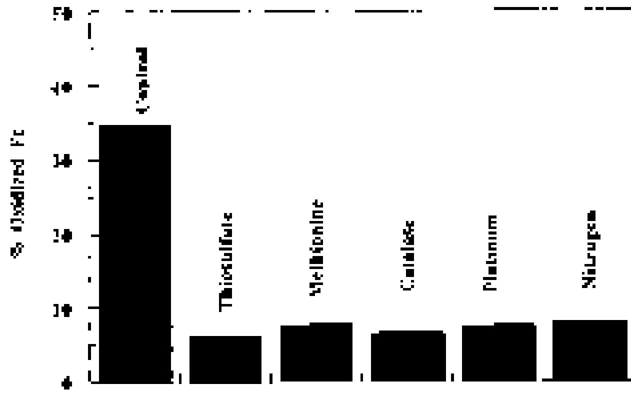


Figure 4—Methods to prevent temperature-induced oxidation of rhuMAb HER2 in Formulation A. Antioxidants were added to the formulation before filling into sample vials for incubation at 40 °C for 2 weeks. After incubation, samples were assayed by HIC to assess methionine oxidation. The amount of oxidation was compared with a control sample containing no antioxidant stored under the same conditions.

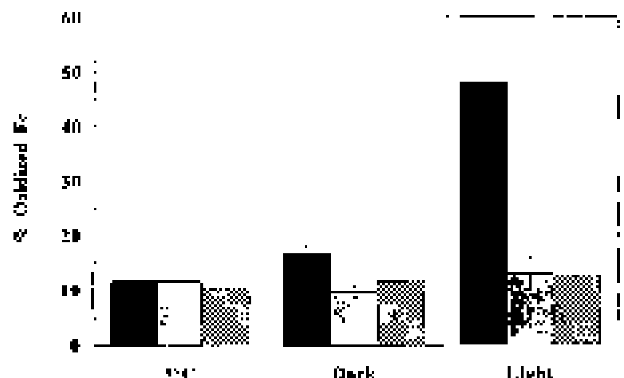
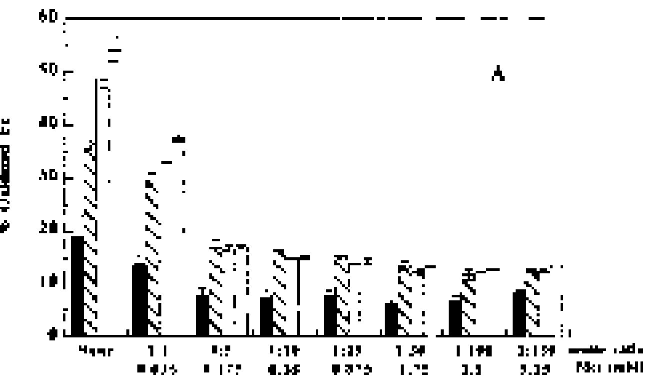


Figure 5—Effect of antioxidants on light-induced oxidation of rhuMAb HER2 Formulation A. Antioxidants were added to the formulation before filling into sample vials. Sample containing no antioxidant (black), 6.3 mM sodium thiosulfate (gray), and 3.5 mM methionine (striped) were stored wrapped (Dark) and unwrapped (Light) in a light box with light intensity of 20 000 lux for 2 weeks. The light box temperature was 27 °C. After light exposure, samples were assessed for methionine oxidation of rhuMAb HER2 by HIC. Results were also compared with the control samples stored in the dark at 5 °C for 2 week.



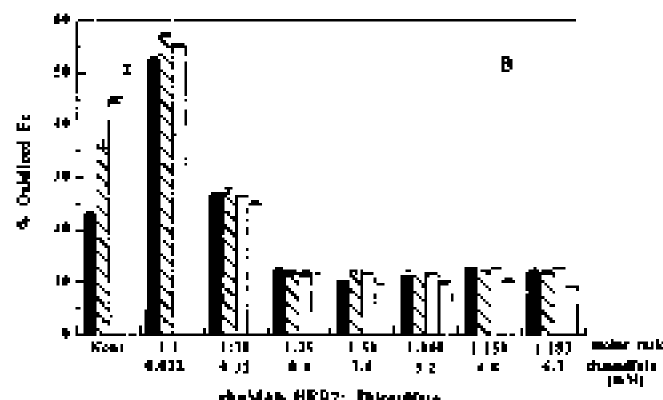


Figure 6—(A) Determination of the minimum effective level of methionine required to prevent oxidation of rhuMAb HER2. (B) Determination of the minimum effective level of sodium thiosulfate required to prevent oxidation of rhuMAb HER2. Methionine or thiosulfate was added to the rhuMAb HER2 Formulation A at different protein-to-antioxidant molar ratios before filling. Samples were stored at 40 °C for 1 week (black), 2 weeks (striped), 3 weeks (gray), and 4 weeks (white) and analyzed for oxidation of rhuMAb HER2 by HIC at the end of each storage period.

aggregate formation and deamidation of the protein as determined by native SEC and IEC (Table 3).

Mechanisms for Prevention of Methionine Oxidation by Antioxidants—Although methionine oxidation did not affect the activity of rhuMAb HER2, it may impact its immunogenicity or shelf-life. Potential methods to reduce temperature-induced methionine oxidation of rhuMAb HER2 were investigated to select inhibitors of oxidation and understand the mechanism of oxidation. As shown in Figure 4, rhuMAb HER2 in Formulation A containing added antioxidants, such as methionine, sodium thiosulfate, catalase, or platinum, did not oxidize after 2 weeks at 40 °C. In contrast, the control sample without antioxidant consisted of 52% oxidized Fc under the same storage conditions. Interestingly, removal of oxygen in the sample vials by repeated pulling vacuum and replacing with nitrogen was also effective in reducing rhuMAb HER2 oxidation. These results support the proposed mechanism that temperature-induced methionine oxidation of rhuMAb HER2 in the NaCl-containing Formulation A occurred by free radical formation in the presence of molecular oxygen. Methionine and thiosulfate can either inhibit free radical-induced oxidation by terminating the chain reaction or simply by competing with the methionine residues in rhuMAb HER2 for reaction with the free hydroxyl radicals. The free radical scavengers, catalase and platinum, also prevented methionine oxidation in rhuMAb HER2.

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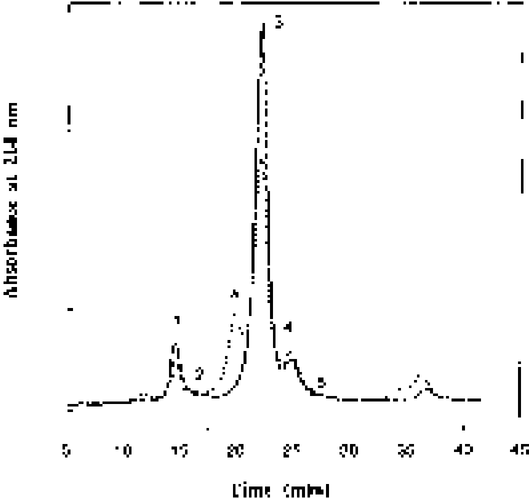

Figure 7—Interaction of sodium thiosulfate with rhuMAb HER2 detected by cation-exchange chromatography (IEC). After storage in a light box for 2 weeks, a degradation species (peak a) was observed in samples containing 6.3 mM thiosulfate (dotted line) as compared with the samples containing 3.5 mM methionine (dashed line) or no antioxidant (solid line). Peaks 1–5 are the five major isoforms of rhuMAb HER2 analyzed by IEC.

The effect of antioxidants on light-induced methionine oxidation in rhuMAb HER2 Formulation A was also studied (Figure 5). Unwrapped sample vials, containing either 6.3 mM sodium thiosulfate or 3.5 mM methionine (concentrations commonly used in parenteral pharmaceuticals), did not show an increase in oxidation when stored in a light box for 2 weeks. In contrast, oxidized Fc was increased to 48% for the unwrapped sample without antioxidants. These results suggest that methionine and sodium thiosulfate can be used for inhibiting photolytic oxidation in addition to temperature-induced oxidation of rhuMAb HER2. These antioxidants may prevent photolytic oxidation of rhuMAb HER2 by competing with methionine residues on the protein to react with molecular oxygen or singlet oxygen.

Different concentrations of methionine and thiosulfate were added to the rhuMAb HER2 Formulation A to determine the minimum effective level required to inhibit protein oxidation. Methionine at a final concentration of 0.175 mM, equivalent to a protein-to-methionine molar ratio of 1:5, was the minimum effective level for reduction of rhuMAb HER2 oxidation caused by temperature (Figure 6a). Thiosulfate at a minimum concentration of 0.8 mM, protein-to-thiosulfate molar ratio of 1:25, was required to inhibit rhuMAb HER2 oxidation in Formulation A (Figure 6b). Unlike methionine, thiosulfate reacted with rhuMAb HER2 as detected by cation-exchange chromatography (Figure 7). At a 1:100 molar ratio of protein-to-thiosulfate, an unknown degradation species (peak a) was observed in the sample. This peak may be an adduct between rhuMAb HER2 and thiosulfate yielding a new charged species. This species was more acidic than the native rhuMAb HER2 and eluted before the main peak of the native protein (peak 3). These data demonstrate that methionine is the preferred antioxidant for preventing rhuMAb HER2 oxidation.

Conclusions—Based on these studies, we conclude that temperature and light induce methionine oxidation of rhuMAb HER2. The rate of oxidation increases with temperature and is formulation dependent. The corrosion of stainless steel by sodium chloride present in rhuMAb HER2 liquid formulation to generate iron ions was the major catalyst for the protein oxidation at high temperatures. Temperature-induced oxidation of rhuMAb HER2 may occur by the formation of free radicals, and photo-induced oxidation may occur via singlet oxygen pathway. Methionine oxidation of rhuMAb HER2 either caused by temperature or light can be reduced by adding a stoichiometric amount of methionine or thiosulfate (antioxidants) to the formulation, but thiosulfate reacted with the protein. A protein-to-methionine molar ratio of 1:5 can inhibit rhuMAb HER2 oxidation.

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BIOTECHNOLOGY

Methionine, Tryptophan, and Histidine Oxidation in a Model Protein, PTH: Mechanisms and Stabilization

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ABSTRACT: Recent oxidation events on monoclonal antibody candidates prompted us to investigate the mechanism of oxidation of Met, Trp, and His residues and to search for suitable stabilizers. By using parathyroid hormone (1–34), PTH, as a model protein and various oxidants, aided by liquid chromatography, peptide mapping, and mass spectrometry, we identified and quantified the oxidation of these vulnerable residues. Whereas H_2O_2 and *t*-butyl hydroperoxide (*t*-BHP) primarily oxidized the two Met residues, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), and $H_2O_2 + Fe(II)$ oxidized Met and Trp residues, with AAPH more capable of generating oxidized Trp species than the latter. $H_2O_2 + Fe(III)$ generated results comparable to those with $H_2O_2 + Fe(II)$, except that there was a lesser amount of hydroxylated Phe. Oxidation of the His residue in PTH occurred when copper was used instead of iron. AAPH, a free-radical generator, produced alkylperoxides, which simulated the oxidizing species from degraded polysorbate, commonly found in protein formulations. It is prudent to screen stabilizers by using H_2O_2, $H_2O_2 + Fe(II)$, and AAPH because these agents represent potential assaults from the H_2O_2 commonly present in degraded polysorbate, the residue of aseptic agents and the metal from stainless steel surfaces, and alkylperoxides from degraded polysorbate, respectively. Free Met protected the Met residues in PTH from oxidation by H_2O_2 and $H_2O_2 + Fe(II)$. Mannitol and EDTA were effective against $H_2O_2 + Fe(II)$. Free Trp protected only the Trp residue in PTH from oxidation by AAPH, the combination of Trp and Met was effective against all three oxidant conditions. By using AAPH to generate oxidant, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and pyridoxine were also found to exhibit good free-radical scavenging activity and thus protected Trp in PTH against oxidation. © 2009 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 98:4485–4500, 2009

Keywords: proteins; oxidation; HPLC; mass spectrometry; free radicals; stabilization; excipients; PTH; tryptophan; Trolox; pyridoxine

INTRODUCTION

Oxidation is one of the major chemical degradation pathways for proteins in pharmaceutical products. This topic has been reviewed by

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Nguyen,[1] Li et al.,[2] and Hovorka and Schöneich.[3] Side chains of cysteine (Cys), methionine (Met), tryptophan (Trp), histidine (His), and tyrosine (Tyr) residues are prone to oxidation, in that order. The thiol group in Cys is the most reactive functional group; hence, very few pharmaceutical proteins contain free Cys. In this report, Cys oxidation will not be addressed; instead, we intend to focus on the oxidation of Met, His, and Trp. Another aspect of oxidation, photo-oxidation, will also be excluded from this discussion.

Met Oxidation

Met oxidation forms Met sulfoxide (Met[O]) and, under extreme conditions, sulfone. Examples of pharmaceutical proteins exhibiting Met oxidation are listed below, along with the associated studies (in chronological order) and the oxidants used:

- Growth hormone (Teh et al.,[4] using H_2O_2; Pearlman and Bewley,[5] and Zhao et al.,[6] using ascorbate/Cu(II)/O_2 [Asc/Cu(II)/O_2]).
- Interleukin-2 (Sasaoki et al.,[7] using 100× H_2O_2; Cadée et al.,[8] using peroxodisulfate; and Ha et al.,[9] using polysorbate).
- Murine antibody (Orthoclone OKT3) (Kroon et al.,[10] using no oxidant).
- Relaxin (Nguyen et al.[11] and Nguyen and Shire,[12] using 2000× H_2O_2; Li et al.,[13,14] using Asc/Cu(II) or Fe(III)).
- Small peptides (Li et al.[15]).
- Insulin-like growth factor I (Fransson,[16] using dissolved O_2, Fe(III), and EDTA).
- Recombinant human ciliary neurotrophic factor and recombinant human nerve growth factor (Knepp et al.,[17] using H_2O_2).
- Herceptin® (trastuzumab) (Shen et al.,[18] using *t*-butyl hydroperoxide [*t*-BHP]; Lam et al.,[19] using heat, light, and stainless steel).
- Recombinant interferon gamma (Actimmune) and recombinant tissue plasminogen activator (alteplase, Activase®) (Keck,[20] using *t*-BHP).
- Recombinant human granulocyte colony-stimulating factor (Herman et al.[21] and Yin et al.,[22,23] using H_2O_2; Lu et al.,[24] using H_2O_2 and *t*-BHP).
- Recombinant human leptin (Liu et al.,[25] using *t*-BHP and H_2O_2).
- Brain-derived neurotrophic factor (Jensen et al.,[26] using Asc/Cu(II)/O_2).
- Recombinant human vascular endothelial growth factor (rhVEGF) (Duenas et al.,[27] using H_2O_2 and *t*-BHP).
- Parathyroid hormone (PTH) (Yin et al.[22] and Chu et al.,[28,29] using H_2O_2).
- Two different monoclonal antibodies (Wei et al.,[30] using *t*-BHP, UV irradiation, and ozone, Chumsae et al.,[31] using *t*-BHP and heat).

It is noteworthy that in the past 20 years, a great variety of oxidants have been used to study the oxidation of proteins, with *t*-BHP and H_2O_2 used predominantly. These peroxides were tested without addition of metal. Only when ascorbate was used, either copper or iron was included. Methionine oxidation certainly drew the greatest attention in the past by various investigators as it is most easily oxidized except cysteine, and readily detected.

His Oxidation

His oxidation predominantly forms oxo-histidine but also forms a variety of other oxidation products, depending on the oxidation conditions. By using Asc/Cu(II)/O_2, Li et al.[13,14] observed oxidation of the His residues in relaxin. With human growth hormone, Zhao et al.[6] observed oxo-histidine when the same oxidizing system was used to simulate metal-catalyzed oxidation at the metal-binding site. Aspartic acid and asparagine as oxidation products of His were also detected in β-amyloid peptide in the presence of Cu(II)/H_2O_2.[32] Better understanding of His oxidation in a pharmaceutical preparation is needed.

Trp Oxidation

Multiple products are formed by way of Trp oxidation. Stability studies of Trp alone in aqueous solution[33] and Trp residues in small peptides and lysozyme[34] and in bovine α-crystallin[35] clearly identified the main degradants as 5-hydroxy-Trp, oxy-indole alanine, kynurenine, and *N*-formylkynurenine. There are very few articles on the oxidation of Trp in pharmaceutical proteins. Davies et al.[36] oxidized bovine serum albumin with oxygen radicals generated from cobalt radiation; Uchida et al.[37] stressed albumin with Fe(II)/EDTA/Asc and detected selective oxidation of Trp and His. Trp oxidation in monoclonal antibodies was reported recently

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by Yang et al.[38] and Wei et al.,[30] who used ozone and UV irradiation as stress conditions). The reason that Trp oxidation has not been studied in depth perhaps was due in fact that no model oxidizing condition has been adopted and system that promotes Trp oxidation is not easy to handle or reproduce. In the case of Met oxidation, *t*-BHP, and H_2O_2 are easy to handle.

Comparison of Met, His, and Trp Oxidation

The fact that Met[O] is readily detected in numerous pharmaceutical proteins may be attributed to its susceptibility to various oxidizing agents in addition to H_2O_2 alone. Light, *t*-BHP, and peroxodisulfate have been used by various laboratories to generate Met[O]. The oxidation of Trp or His in pharmaceutical proteins under normal storage conditions can be very slow. To expedite oxidation, one or more stress models are needed. Trp or His oxidation is considered metal-catalyzed or free-radical-mediated oxidation.[36,39]

Model Stress Conditions: H_2O_2

Many protein formulations contain polysorbate 20 or polysorbate 80. It has been reported that the oxidants present in aged polysorbate consist predominantly of H_2O_2 (up to 75%).[40] Ha et al.[9] reported increased oxidation of an interleukin-2 mutant by aged polysorbate. Since polysorbate is the source of oxidant in protein drug product, the use of H_2O_2 may be a way to simulate the oxidative reaction in surfactant-containing formulations. In addition, H_2O_2 has been used as an aseptic agent for the isolators used in the filling of sterile products; consequently, residual H_2O_2 can be found in the drug product. For this reason, it is important to determine the sensitivity of the protein to oxidation by H_2O_2.

Model Stress Conditions: $H_2O_2 + Fe(II)$

Theoretically, metal is ubiquitous and should be included in experimental stress conditions; however, it may be difficult to select a metal, whether iron or copper, and to determine whether to use a chelating agent (e.g., EDTA). These factors have a profound effect on the outcome of the experimental results. The following examples illustrate the complicated results of oxidation involving metal. With relaxin, two Met and one His residues, but neither of two Trp residues, were oxidized when metal was used in the oxidizing system, such as Asc/Cu(II)/O_2. With bovine serum albumin, free radicals generated from an Fe(II)/EDTA/Asc system preferred Trp, whereas Cu(II)/Asc (without EDTA) preferred His.[37] The use of ascorbate has frequently been cited in the literature; however, since ascorbate will not be included in our formulation, it will not be considered. H_2O_2/Fe(II)/EDTA and H_2O_2/Cu(II) generated different patterns of albumin degradation.[41] Metal-catalyzed oxidation of Trp and Met residues in α-crystallin was observed with H_2O_2/Fe(II)/EDTA.[35] Whether EDTA promotes or inhibits metal catalyzed reaction, or what would be the preferred oxidation site, Trp or His, no clear trend emerges from these results.

During pharmaceutical production, recombinant proteins are necessarily exposed to stainless steel; thus, protein solutions may contain trace amounts of iron or other metals. In addition, common excipients such as sucrose, amino acids, and buffers, and leachables from plastic or glass containers may also contribute minute quantity of metals. Therefore, we chose H_2O_2 with Fe(II)—the commonly known Fenton reaction—as a stress condition to evaluate the oxidation potential of our drug candidates. The Fenton reaction involves several oxidizing species, the best characterized being hydroxyl radicals which is the most reactive free radical. Fe(III), as added species or derived from oxidation of Fe(II) by H_2O_2, might be able to react further with H_2O_2 to generate superoxide radical which is far less reactive than hydroxyl radical.

Model Stress Conditions: AAPH

As discussed above, the presence of metal increases the complexity of oxidation studies. 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) is a metal-ion-independent, reactive-oxygen-species-generating system.[42] At a defined rate, it decomposes in aqueous, aerobic solutions to yield alkyl radicals that immediately add oxygen to yield alkylperoxides. The chemical structure and generation of alkylperoxides are shown in Scheme 1. Strictly speaking, AAPH is not an oxidant; rather, it reacts with oxygen to generate the oxidant, an alkyperoxyl radical. In this report we group it with *t*-BHP and H_2O_2 and refer to them together as oxidants. Treatment with AAPH led to the oxidation of Met, Tyr, and

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Scheme 1. Structure of AAPH. Upon heating, AAPH, an azo compound, releases nitrogen gas and generates alkyl radical, which combines with oxygen to form alkylperoxide.

Trp residues in liver proteins.[43] In the same study, another amino acid derivative from oxidation, dityrosine, was also detected. When glutamine synthetase was exposed to AAPH for 4 h, both Trp residues, 2 of 16 His residues, 6 of 17 Tyr residues, and 5 of 16 Met residues were lost.[44] Anraku et al.[45] used AAPH on albumin; the degree of oxidation was determined by the number of carbonyl groups formed. These reports indicated that AAPH led to the oxidation of a wide range of amino acids in addition to Met. More recently, with respect to small molecule drugs, 2,2′-azobis-isobutyronitrile and 4,4′-azobis-4-cyanovaleric acid, both azo compounds similar to AAPH, were evaluated for use in oxidative forced-degradation studies.[46] Because azo compounds can generate reproducible amounts of radicals, independently of metal, AAPH was used as a model oxidant for its ability specifically to generate Trp-oxidized protein.

Selection of a Model Protein

PTH (1–34) was chosen because of its minimal tertiary structure[47] and its sequence, which contains all three desirable amino acids (one Trp, two Met, and three His); the ease with which it can be assayed by reversed-phase high-performance liquid chromatography (rp-HPLC); and its availability. When Chu et al.[28,29] studied Met oxidation in PTH stressed only by H_2O_2, the different oxidation rates of Met8 and Met18 were found to correlate to the 2-shell water coordination number. The difference, <1.5-fold, was not sufficiently significant to influence the conclusion that we would draw from our study. The oxidation rates of different Met residues in growth hormone (Teh et al.[4]) and rhVEGF (Duenas et al.[27]) were attributed primarily to different degrees of solvent exposure. We would expect the oxidation rate of the fully solvent-exposed Met in PTH, growth hormone, and rhVEGF to be comparable. Therefore, the two Met residues on PTH can simulate solvent-exposed Met in all proteins. Ease of analysis by LC/MS because of the presence of only one Trp made PTH a good model protein for our study. One should recognize that the results from PTH cannot fully simulate the oxidation in a complex protein such as a MAb where protein structure exerts influence on the rate and extent of residues. This study differs from the prior work on PTH,[28,29] in the use of oxidizing conditions. Prior studies employed only hydrogen peroxide whereas the conditions used in this study allowed the Trp, His, and Phe to be oxidized and observed.

Antioxidants Used in Protein Products

A key objective of this study was to screen stabilizers. It is anticipated that the information generated by these stress studies might lead us to a novel stabilizer. Prior to the screening study, however, it is useful to review anti-oxidants that have already been incorporated into marketed protein or peptide products. As an antioxidant, free Met was cited in US Patent 5849700 (by Takruri[48]) and subsequently in a patent application from Amgen (by Li et al.[49]). Met was described as a potential stabilizer for relaxin gel (by Nguyen[1]). As cited in package insert of the following parenteral products, free Met can be found in depo-subQ Provera, Follistim AQ, Gonal-f RFF, and lutropin alfa. Other agents that may control the oxidation of protein include EDTA (a metal-chelating agent) and mannitol (a free-radical scavenger and metal-binding reagent), which have been widely cited (e.g., by Wang and Hanson[50] and Nema et al.[51]). *N*-Acetyl tryptophan has been used along with octanoates as ligands that bind to specific sites to stabilize human serum albumin during pasteurization (by Anraku et al.[45] and Peters[52]).

Most of prior literature focused on the oxidation of one, and seldom two or more amino acids. We believe our study is among selected few that studied and compared the oxidation of all potentially oxidizable amino acids, Met, Trp, His, and Phe in one protein. In prior studies, typically one oxidizing condition was employed; we compared all three different conditions side-by-side. Results from this study will be valuable in comparing the vulnerability of these amino acids under various conditions.

MATERIALS AND METHODS

Materials

PTH (1–34) (SVSEIQLMHNLGKHLNSMERVEWLRKKLQDVHNF, mol wt: 4117.72 Da) was

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purchased from American Peptide Company (Sunnyvale, CA). In this report, this 1–34 version is simply referred to as PTH. AAPH was purchased from Calbiochem (La Jolla, CA).

L-Methionine and EDTA disodium were purchased from J.T. Baker (Phillipsburg, NJ). Sodium acetate, ammonium acetate, H_2O_2, *t*-BHP, copper acetate, and ferric chloride hexahydrate were purchased from Sigma–Aldrich (St. Louis, MO). Ferrous chloride tetrahydrate was purchased from EMD (Gibbstown, NJ). Mannitol and sucrose were obtained from Ferro Pfanstiehl. L-Tryptophan and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, a water soluble vitamin E) were obtained from Fluka (Buchs, Switzerland). Pyridoxine was obtained from Spectrum (Gardena, CA). Trypsin (sequencing grade, *N*-tosyl-L-phenylalanine chloromethyl ketone-treated) was purchased from Promega (Madison, WI). HPLC-grade acetonitrile and water were purchased from Fisher Scientific (Fairlawn, NJ). Water used in sample-preparation experiments was obtained from a Milli-Q Plus purification system (Millipore, Bedford, MA).

Sample Preparation

PTH (0.1 mg/mL, 0.024 mM) was mixed with H_2O_2, H_2O_2/Fe(II), *t*-BHP, *t*-BHP/Fe(II), or AAPH. In each case the oxidant concentration was 1 mM, at a molar ratio of 1:42 (protein:oxidant) in 20 mM ammonium acetate buffer at pH 5.0. The concentration of Fe(II) was 0.2 mM. The compositions are presented in detail in Table 1. Mannitol (15%, w/v), sucrose (6%), EDTA (0.04%, w/v), Trp, Met, Trolox, and pyridoxine (where the final concentration in the test samples is shown in parentheses) were added to the reaction solution at 2 mg/mL as stabilizers at their respective concentrations. After incubation at 40°C for 6 and 24 h, all without exposure to light, aliquots of the samples were mixed with methanol and Met to quench the reaction prior to rp-HPLC analysis, peptide mapping, and liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS). All experiments and analyses were done without replication.

rp-HPLC

Analysis of oxidized and intact PTH was carried out on a Waters HPLC instrument using a C4 (Vydac, 214TP, 5 μm, 2.1 mm × 250 mm) column. Solvent A was 0.1% trifluoracetic acid (TFA) in H_2O, and solvent B was 0.08% TFA in acetonitrile. The samples were analyzed with a linear gradient from 20% B to 80% B at a flow rate of 0.2 mL/min in 45 min. The column temperature was set at 30°C. UV detection was set at 214 nm.

Trypsin Digestion

The pH of the samples was adjusted to 7.5 by adding 1 M ammonium bicarbonate. Five microliters of 0.5 mg/mL trypsin was added to 200 μL samples, which were then incubated at 37°C for 3–4 h. The digestion was quenched with 0.1% TFA.

LC/MS/MS Characterization of the Tryptic Peptide Map

PTH samples after tryptic digestion were separated with an Agilent 1200 Series HPLC system, and the masses and sequences of the peptides were determined with an online-coupled LTQ linear ion-trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA). A Jupiter Proteo 1.0 mm × 150 mm column (particle size 4 μm, pore size 90 Å; Phenomenex, Torrance, CA) was used; its temperature was controlled at 30°C, and the column effluent was monitored at 214 nm. The flow rate was controlled at 150 μL/min, and the mobile phases used were 0.1% TFA in water (A) and 0.1% TFA in acetonitrile (B). A 100 μL volume of the sample was injected. The optimized gradients (expressed as min/per %B) were 0/2%, 3/2%, 10/8%, 15/8%, 60/40%, 61/95%, 65/95%, 66/2%, and 76/2%. The effluent from the HPLC was directly infused into the LTQ electrospray ionization source. Electrospray ionization in positive-ion mode was achieved by using a needle spray voltage of 4.5 kV and a capillary voltage of 44 V. In the LC/MS/MS experiments, nine scan events, including a full scan in the range of 300–2000 m/z, were followed by four cycles of zoom scans and MS/MS scans on the four most intense ions.

MS/MS spectra interpretation and peptide assignments were accomplished with an automatic database search with a SEQUEST algorithm using BioWorks Browser version 3.2 software (Thermo Fisher Scientific) and manual investigation of each matched product ion spectrum. A FASTA single-protein database of PTH was created and used as the searching target. For the identification of oxidation products, oxidation-

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related modifications were defined as variable ones (+4, +16, and +32 Da for Trp; +16 and +32 Da for Met; and +16, −22, and −23 Da for His). Peptide matches with satisfied correlation-factor (Xc) values ($Xc \geq 1.5$ for singly charged, ≥ 2.0 for doubly charged, and ≥ 2.5 for triply charged peptide ions) were selected as potentially significant matches for an oxidation-modified peptide. Subsequently, manual investigation of zoom-scan mass spectra and MS/MS spectra of the matched peptide ions was performed to eliminate false positive identifications. Zoom-scan MS profiles were examined to confirm the charge state and monoisotopic mass of matched peptides. To estimate the oxidation level for each oxidation site, the extracted ion chromatograms of corresponding peptides were manually integrated using an Xcalibur Qual Browser. The relative percentage of oxidation was subsequently calculated by dividing the peak area of the oxidized peptide ion by the sum of the peak areas of oxidized and nonoxidized peptides.

RESULTS AND DISCUSSION

Met and Trp Oxidation in PTH

Figure 1 shows the rp-HPLC chromatograms of PTH reacted with H_2O_2, in which Met18[O]-PTH, Met8[O]-PTH, and doubly oxidized PTH species were detected; their identities were confirmed by LC/MS. Reaction conditions, pH 5 and 40°C were chosen because most peptide and protein formulations are in the pH range of 5–6, thus pH 5 is a representative pH for this range, and most of the stability studies carried out in the industry were using 40°C as the highest temperature tested. This trend is consistent with data generated by Chu et al.,[28,29] who reported the three Met-oxidized species detected by rp-HPLC, with Met18 oxidized more than Met8, followed by the doubly oxidized species. Figure 2 shows the rp-HPLC chromatograms of PTH reacted with AAPH and reveals a pattern very different from that shown in Figure 1. Two sets of triplet peaks appeared at retention times between the PTH and Met[O] peaks. Although the individual peaks were not fully characterized, it was later confirmed by tryptic digestion, followed by LC/MS/MS, that these new peaks were Trp[O]-modified PTH species. Tryptic peptide mapping of the PTH digests showed that, in addition to the Met oxidation products, three tryptic peptide species with molecular masses of M+4, M+32, and M+16 (where M is the mass of tryptic peptide VGWLR of PTH) were produced when PTH was treated with AAPH. Analysis of MS/MS spectra of the peptide species resulted in their assignment as three Trp oxidation derivatives—namely, kynurenine (M+4), *N*-formylkynurenine (M+32), and 5-hydroxytryptophan, or ox-indole alanine (M+16). Their chemical structures are shown in Figure 3.

PTH was oxidized by three model oxidants (H_2O_2, H_2O_2 + Fe(II), and AAPH). Table 1 summarizes the overall oxidation of Met8 and Trp23 of PTH in these degraded samples. Altogether, 43% and 84% of the Trp residues of PTH were

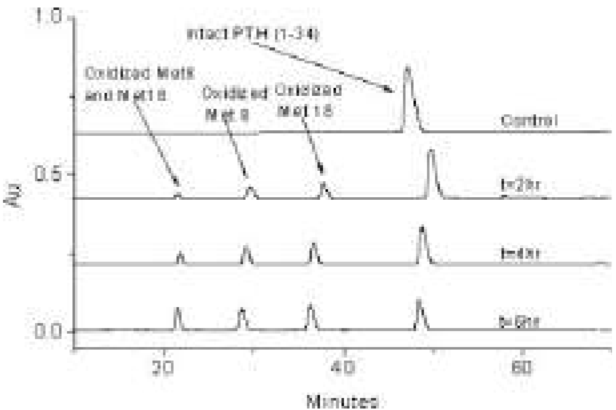

Figure 1. rp-HPLC chromatogram of 0.1 mg/mL PTH in 20 mM ammonium acetate buffer at pH 5.0, degraded by 34 ppm H_2O_2. The reaction was conducted at 40°C, and samples were removed at 2, 4, and 6 h. The increased peak height of mono-oxidized PTH and dioxidized PTH is shown.

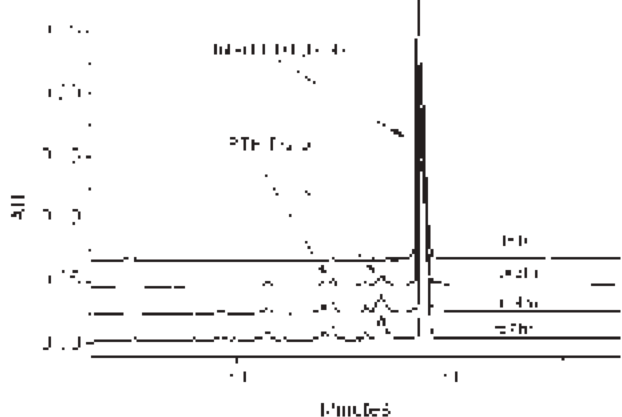

Figure 2. rp-HPLC chromatograms of 0.1 mg/mL PTH in 20 mM ammonium acetate buffer at pH 5.0, treated with AAPH. Trp[O]-PTH peaks were predominantly observed. The reaction was conducted at 40°C, and samples were removed at 2, 4, and 6 h.

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Table 1. Quantitation of PTH Oxidation (Trp23 and Met8) in 20 mM Ammonium Acetate Buffer at pH 5.0, by Peptide Mapping

Oxidants/Reaction Time	% Residue Oxidized				
	Met8[O]	Trp[O] Total	Trp+16	Trp+32	Trp+4
AAPH					
6 h	29	42	35	6	1
24 h	58	84	61	20	3
H_2O_2					
6 h	41	1	0.6	0.5	0.1
24 h	83	2	0.8	0.5	0.2
$H_2O_2 + Fe(II)$					
6 h	55	17	11	5	1
24 h	91	35	22	11	2
$H_2O_2 + Fe(III)$					
6 h	47	19	11	6	2
24 h	97	50	21	24	5
$H_2O_2 + Cu$					
6 h	99	69	16	39	14
24 h	100	96	15	65	16
t-BHP					
6 h	9	0	0	0	0
24 h	18	0	0	0	0
t-BHP + Fe(II)					
6 h	10	3	2	0.8	0.2
24 h	22	3	2	0.6	0.2
Control					
6 h	0	0	0	0	0
24 h	1	0	0	0	0

AAPH is more specific toward Trp than is the Fenton reaction. His residues were not affected by these oxidants. The reaction was conducted at 40°C for 6 and 24 h. PTH was at 0.1 mg/mL, all oxidants (AAPH and peroxides) were at 1 mM; Fe (II), Fe(III), and Cu (II) were at 0.2 mM.

oxidized by APPH when treated for 6 and 24 h, respectively. Hence, the new peaks shown in Figure 2 were identified as Trp[O]-modified PTH species. Oxidized Met18 containing tryptic peptide was not retained on the reversed-phase column; therefore, no data related to Met18 oxidation are provided.

The key observations are as follows:

1. Three conditions—*t*-BHP with iron, *t*-BHP without iron, and H_2O_2—resulted in a minimal amount of Trp oxidation.
2. None of the three His residues was affected.
3. Only AAPH and the Fenton reaction, $H_2O_2 + Fe(II)$, generated Trp oxidation.
4. More M+16 Trp[O] than other species (M+4 and M+32) was generated.
5. To reach a comparable degree of Trp oxidation, AAPH treatment for 6 h generated 43% Trp[O] and 29% Met[O] at Met8, whereas H_2O_2/Fe(II) treatment for 24 h generated 35% Trp[O] but a much larger amount (91%) of Met[O] at Met8. This comparison shows that AAPH treatment is more specific for Trp oxidation than is the Fenton reaction.

The mechanism of thioether (Met) oxidation by peroxides (H_2O_2, *t*-BHP, or other ROOH species) is a one-step nucleophilic attack of sulfide on a peroxide–protic solvent complex, followed by a series of concerted electronic displacements that leads to the transfer of oxygen to the sulfur atom, resulting in Met[O].[2] The fact that the oxidation level of Met was not affected by the addition of Fe(II) to the H_2O_2, as shown in Table 1, is evidence that Met oxidation is a nucleophilic reaction and that it does not involve free radical. This reaction mechanism implies that the opposite reactant, peroxide oxygen, is electrophilic. Thus, electron-donating groups such as *t*-butyl decelerate the reaction by decreasing the electrophilicity of oxygen. For this reason, the fact that *t*-BHP

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Figure 3. Chemical structures of degraded (oxidized) Trp. The additional masses of these products are noted (+4, +16, and +32 Da).

was associated with less oxidation (Tab. 1) than H_2O_2 is not surprising. It should be pointed out that *t*-BHP offers the advantage of oxidizing only the exposed Met, as Keck[20] reported when recombinant interferon gamma (Actimmune) and recombinant tissue plasminogen activator (alteplase, Activase®) were investigated. Since there is little tertiary structure in PTH, we do not expect any Met in PTH to be selectively oxidized by *t*-BHP.

Although the mechanism of nucleophilic attack predicts a specific acid catalysis component, the reaction rate does not vary significantly within a pH range of 2–8, as shown with PTH.[28] For this reason, data generated using pH 5 in acetate buffer would be applicable to the typical pH range, pH 5–7, found in protein formulations.

Only AAPH and the Fenton reaction resulted in Trp oxidation. This finding supports the notion that nucleophilic reaction of H_2O_2 alone cannot cause oxidation of Trp.

Fe(II) Versus Fe(III) and Hydroxylation of Phe

During pharmaceutical manufacturing, dissolved iron is probably in a Fe(III) state. A separate experiment using the same molar concentrations of PTH, metal, and H_2O_2 was conducted with Fe(III) added instead of Fe(II). The results are shown in Table 1. For both Trp and Met, the extent of oxidation with Fe(III) was similar to that with Fe(II). The similarity in the extent of oxidation by Fe(II) or Fe(III) suggests that the added Fe(II) was quickly oxidized to Fe(III) by the fivefold molar excess of H_2O_2. Equilibrium between Fe(II) and Fe(III) was rapidly established, and the amount of reactive oxygen species generated within the 6 and 24 h intervals are comparable.

Automatic data mining of the tandem mass spectra of the tryptic digested peptides of PTH revealed the presence of a modified LQDVHNF with +16 Da on Phe at the C-terminus. Further manual investigation of the MS^2 spectrum, shown in Figure 4, confirmed that the +16 Da increase in mass, probably hydroxylation caused by the hydroxyl radical, is on the Phe residue: the fragment ions of y_1 to y_6 have masses of +16 Da, whereas the b_2 to b_6 series do not. About 2.2% and 1.1% of the Phe was hydroxylated at the 24-h time point by $H_2O_2 + Fe(II)$ and $H_2O_2 + Fe(III)$, respectively. Although the percent modification is small relative to that exhibited by Trp and Met, this hydroxylation is direct evidence that a highly reactive hydroxyl free radical was generated by the Fenton reaction. The difference in the amount of hydroxylation suggests that $H_2O_2 + Fe(II)$ was a more effective hydroxyl radical generator than was $H_2O_2 + Fe(III)$. Harmon et al.[53] studied the oxidation of a model compound using 10% polysorbate solution (a source of H_2O_2) and iron. They observed that within 4 h after the start of the reaction, the oxidation rate with Fe(II) was about twice that with Fe(III). The results of the current study are similar to theirs. On the contrary, PTH exposed to AAPH showed no hydroxylation on the C-terminal Phe. The absence of hydroxylation by AAPH further supports the idea that AAPH generates only peroxyl radicals and no hydroxyl free radicals.

$H_2O_2 + Cu(II)$ and His Oxidation

Under the current experimental conditions for AAPH and $H_2O_2 + Fe(II)$, we observed no His oxidation. When bovine serum albumin reacted with Fe(II)/EDTA/Asc or Cu(II)/Asc, the former caused more oxidation of Trp, whereas the latter caused more His oxidation.[37] In another study,

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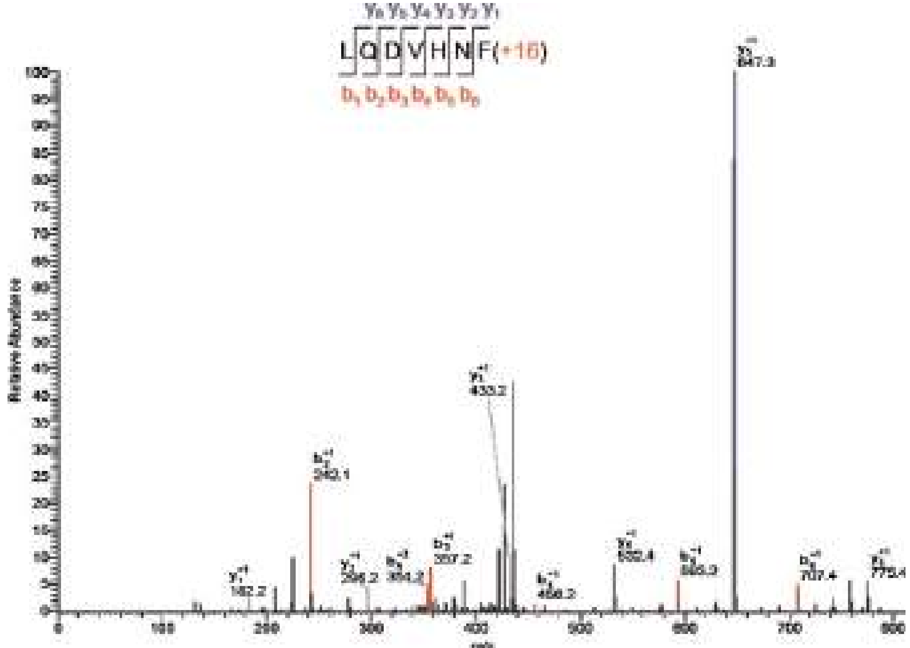

Figure 4. MS2 spectrum of precursor ion 444.61 at a retention time of 24.6 min on the tryptic peptide map. An automatic SEQUEST search suggested that this is the product ion mass spectrum of modified LQDVHNF peptide with hydroxylation (+16 Da) on the Phe residue at the C-terminus. The MS2 spectrum was acquired on an LTQ mass spectrometer.

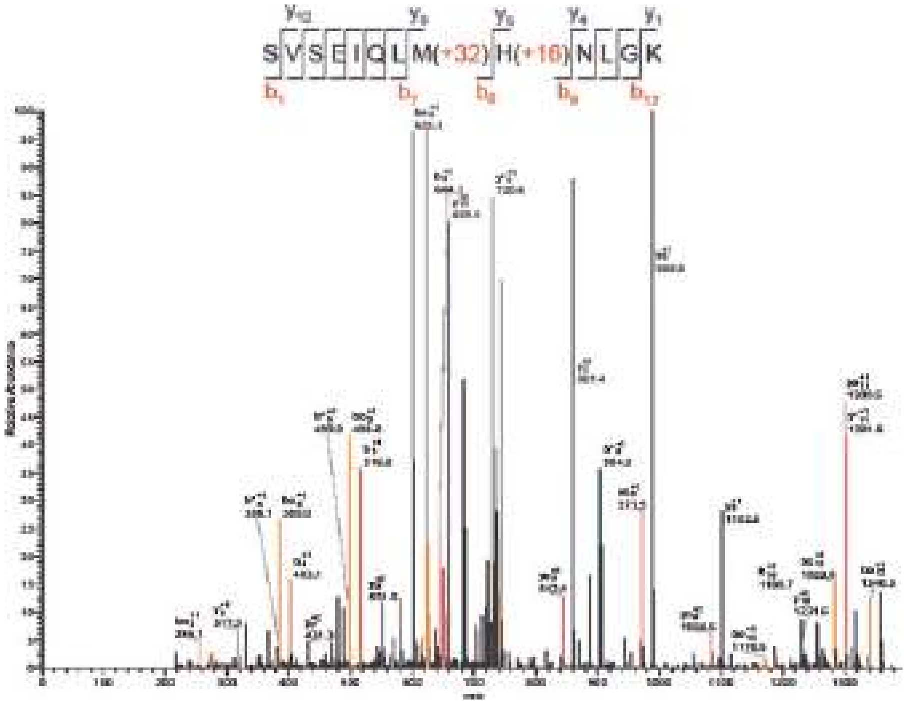

Figure 5. MS2 spectrum of precursor ion 752.74 at a retention time of 35.6 min on the tryptic peptide map. An automatic SEQUEST search suggested this is the product ion mass spectrum of modified SVSEIQLMHNLGK peptide with double oxidation (+32 Da) on the Met and single oxidation (+16 Da) on the adjacent His residue. The MS2 spectrum was acquired on an LTQ mass spectrometer.

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relaxin oxidation by AscA/Cu(II) resulted in a significant amount, and AscA/Fe(III) in a small amount, of His oxidation, whereas Trp oxidation was not observed.[13,14] Both sets of results differ from our observation of a total absence of His oxidation in PTH when $H_2O_2 + Fe(II)$ was used. It is possible that His oxidation depends on the presence of copper. Inclusion of imidozole favors the coordination of the copper binding and subsequently facilitates His oxidation.[54] It is prudent to examine the influence of copper on the oxidation of the three His residues in PTH.

The same molar ratios of PTH, H_2O_2, and Cu(II) as those in the aforementioned experiment with Fe(II) were used—namely, 0.1 mg/mL, 1 mM (34 ppm), and 0.2 mM, respectively. Higher amounts of Trp and Met were oxidized, with greater amount of sulfones, when compared with the amounts generated with Fe(II), as shown in Table 1. With respect to the hydroxylation of Phe, about 3.2% and 5.0% of Phe residue were hydroxylated at the 6 and 24 h time points (data not shown). At the same molar ratio of oxidants to metal, Cu(II) appeared to cause more oxidation than iron.

Among the three His residues in PTH molecule, only His9 oxidation was observed in the presence of H_2O_2/Cu (II) by LC-MS/MS analysis. Figure 5 shows the His9 oxidation in the tryptic peptide of SVSEIQLMHNLGK. It is noteworthy that this His residue is adjacent to Met8, which was almost completely oxidized by H_2O_2 and Cu(II), yielding both Met sulfoxide and sulfone. Two other His residues, without adjacent Met or Trp, were not oxidized at all. This preferential oxidation of His9 and extensive oxidation of the Met8 can be attributed to the formation of the Cu(II) coordination sphere on PTH protein in the vicinity of imidozole and thio moieties. The catalysis by coordination sphere is consistent with the observation by Kowalik-Jankowska et al.[55] of site-directed oxidation of alpha-synuclein fragments by a Cu(II)/H_2O_2 system.

Stabilizer Screening Using PTH as a Model Protein

Based on the analysis described above, we propose that protein may be susceptible to oxidative attack via any or all three of the degradation mechanisms shown in Scheme 2. A nucleophilic reaction with H_2O_2 (and no metal) may be the oxidation reaction observed when the protein product is exposed to the vapor H_2O_2 used as an aseptic

Stress Conditions	Reaction Mechanism	(Oxidant Sources)
[illegible]	[illegible]	(VHP)
H_2O_2 + Fe [illegible]	[illegible]	(SS)
AAPH [illegible]	[illegible]	(polysorbate)

Scheme 2. The three possible routes of oxidation of Met, Trp, and His. VHP, vapor hydrogen peroxide; SS, stainless steel.

agent in an isolator When trace metal (iron, copper, or chromium) is introduced into the formulation solution as a result of contact with stainless steel, the Fenton reaction—H_2O_2 with Fe(II)—is operative. The third mechanism is via alkylperoxides, which could come from degraded polysorbate.[40] AAPH, capable of generating alkylperoxyl radicals, can be a good model system to simulate degraded polysorbate. In the current study, we used AAPH to generate reactive oxygen species, such as alkylperoxides (Scheme 1).

Methionine

Free Met neutralized the effect of the oxidant H_2O_2, as expected, whether iron was present or not. Free Met significantly reduced the oxidation of Met residues in PTH, as the peaks corresponding to Met[O]-PTH did not appear (Fig. 6). Free Met had no effect on the oxidation of Trp, as the Trp[O] peak persisted.

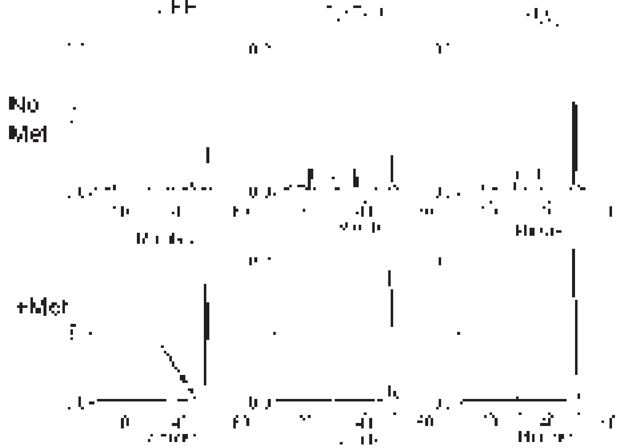

Figure 6. rp-HPLC chromatograms of PTH solution in 20 mM ammonium acetate buffer at pH 5.0 oxidized by AAPH, H_2O_2 + iron, or H_2O_2. The reaction was conducted at 40°C for 6 h. Samples were prepared either with or without the addition of free Met (2 mg/mL). The arrow is pointing the Trp[O]-PTH species.

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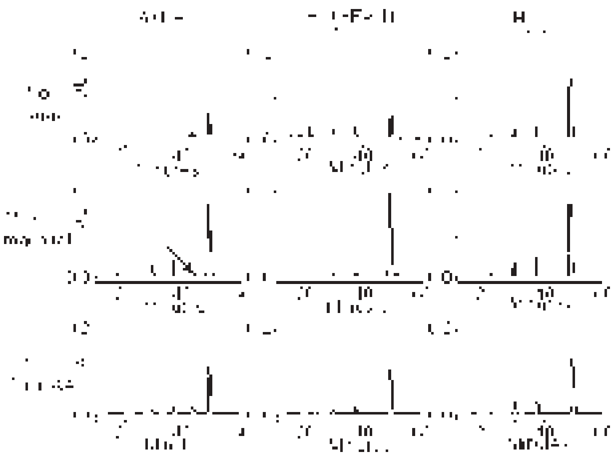

Figure 7. rp-HPLC chromatograms of PTH solution in 20 mM ammonium acetate buffer at pH 5.0, oxidized by AAPH, H_2O_2 + iron, or H_2O_2. The reaction was conducted at 40°C for 6 h. Samples were prepared either without or with the addition of 15% mannitol or 6% sucrose. The arrow is pointing the Trp[O]-PTH species.

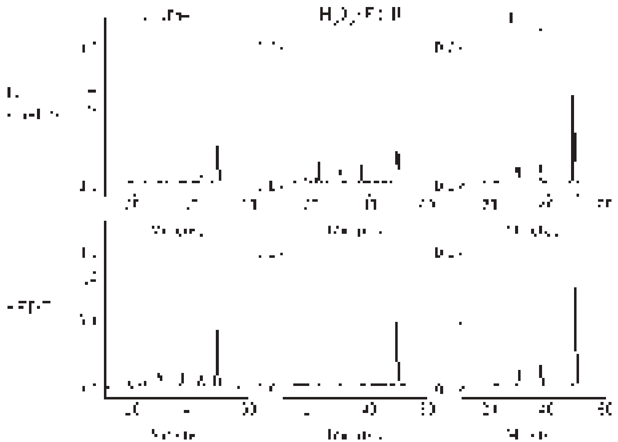

Figure 8. rp-HPLC chromatograms of PTH solution in 20 mM ammonium acetate buffer at pH 5.0, oxidized by AAPH, H_2O_2 + iron, or H_2O_2. The reaction was conducted at 40°C for 6 h. The samples were prepared either with or without the addition of EDTA (0.4 mg/mL).

Mannitol and Sucrose

Mannitol is a well-known hydroxyl free-radical scavenger. Figure 7 shows complete protection from the Fenton reaction by mannitol, as evidenced by the absence of any Met[O]- or Trp[O]-derived PTH when it was stressed with H_2O_2/Fe(II). However, when stressed with AAPH or H_2O_2, PTH was not protected by mannitol at all, because mannitol does not react with alkylperoxides or H_2O_2. Sucrose generated similar results, except it was less effective than mannitol when protecting PTH against a hydroxyl free radical. Polyols are considered a universal stabilizer against both physical and chemical degradation. As noted in a review by Li et al.,[2] hemoglobin can be lyophilized without oxidation with the use of certain sugars. Results from our model using AAPH suggest that protein with polyols may be left unprotected when faced with alkylperoxides.

EDTA

As shown in Figure 8, EDTA completely protected PTH when it was stressed with H_2O_2/Fe(II). In this instance, EDTA mitigated not just the generation of free radical, but also the oxidative effect of the H_2O_2. EDTA did not protect PTH when it was stressed with H_2O_2 alone. EDTA seemed to exacerbate AAPH oxidation, given that there were abundant Met[O]- and Trp[O]-PTH peaks. Reports of the effect of EDTA or other metal chelators (such as EGTA) have been mixed. The metal chelators may enhance[41] or inhibit[50] a metal-catalyzed reaction. One may generalize and say that EDTA, because it sequesters copper effectively, inhibits copper-catalyzed reactions.[41] Because it cannot cover all five valences on iron, the EDTA–iron complex sometimes is very reactive. It is unknown why more oxidation was observed when EDTA was added to a reaction mixture of PTH and AAPH, but such an investigation is beyond the scope of this study.

Frequently described in the literature, the oxidation of Met, Trp, or His residues *in vivo* has been attributed to metal-catalyzed oxidation. In our experiment, PTH oxidized by AAPH with no added metal generated significant amounts of Met and Trp, suggesting that neither Met nor Trp oxidation depends solely on metal catalysis.

Free Trp

In the literature, many substances have been cited as scavengers for free radicals. Thiourea, methanol, and uric acid are examples; however, they are not suitable for use in protein formulations. In addition, butylated hydroxyl-anisol (BHA) and butylated hydroxyl-toluene (BHT) are radical chain-reaction terminators that are effective in quenching radicals from lipids. Because of their low water solubility, they are not suitable for aqueous formulation of proteins.

To our knowledge, the use of free Trp as an antioxidant in parenteral formulations has not been

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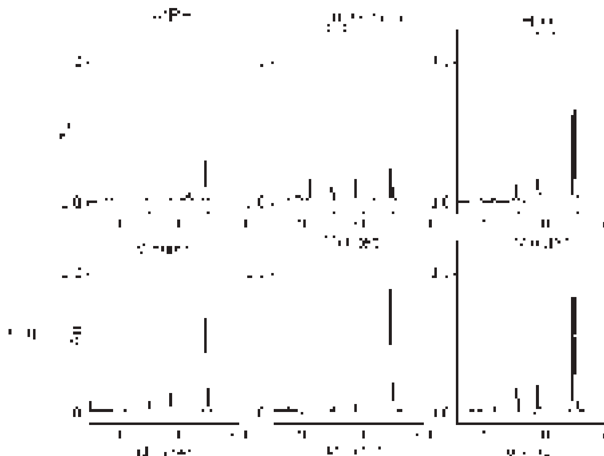

Figure 9. rp-HPLC chromatograms of PTH solution in 20 mM ammonium acetate buffer at pH 5.0, oxidized by AAPH, H_2O_2 + iron, or H_2O_2. The reaction was conducted at 40°C for 6 h. Samples were prepared either with or without the addition of free Trp (2 mg/mL).

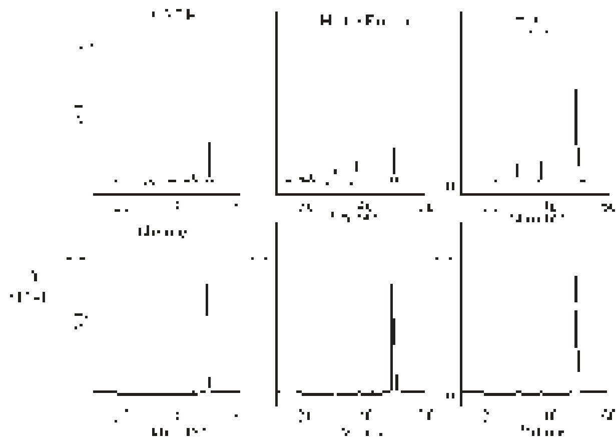

Figure 10. rp-HPLC chromatograms of PTH solution in 20 mM ammonium acetate buffer at pH 5.0, oxidized by AAPH, H_2O_2 + iron, or H_2O_2. The reaction was conducted at 40°C for 6 h. Samples were prepared either with or without the addition of a combination of free Trp and Met (both 2 mg/mL).

mentioned in the literature. Akin to the use of free Met, it may protect PTH against oxidation. Figure 9 shows that free Trp offers good protection from oxidation of the Trp residue in PTH when PTH is stressed with AAPH or the Fenton reaction. Met[O]-PTH peaks were prominent in the case of AAPH stress and much less so in the case of the Fenton reaction. Free Trp offered no protection against oxidative stress by H_2O_2.

Combination of Trp and Met

This combination provided nearly complete protection of PTH under all three oxidative conditions (Fig. 10). One can surmise that free Trp and Met counteract the effect of alkylperoxides and H_2O_2, respectively. When Trp and Met are used in combination, a protein formulation should be able to withstand assault by all three mechanisms shown in Scheme 2.

The analysis described above was derived from qualitative examination of the peaks on the respective rp-HPLC chromatograms of oxidized PTH. Samples oxidized by AAPH and the Fenton reaction was subjected to quantitative, residue-specific analysis by tryptic peptide mapping and LC/MS/MS characterization. According to the relative quantification results obtained by integrating the corresponding extracted ion chromatogram of mass signals, free Met alone suppressed Met oxidation, and free Trp alone suppressed Trp oxidation. The combination of Trp and Met provided the most effective protection against oxidation by AAPH or the Fenton reaction (Figs. 11 and 12).

Other Free Radical Scavengers as Anti-Oxidants

Although free Trp as a formulation component is an excellent and safe stabilizer, there may be other candidates that function similarly. Review

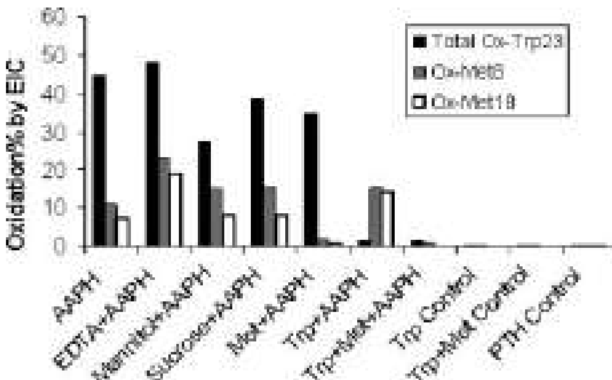

Figure 11. Site-specific oxidation of PTH in 20 mM ammonium acetate buffer at pH 5.0, by AAPH and the protective roles of Trp and Met as compared with other reagents. The identification of individual oxidized Trp23, Met8, and Met18 residues was based on the MS/MS fragmentation spectra of their corresponding tryptic peptides. The relative oxidation level was quantified according to the integrated extracted ion chromatograms (EIC) in mass spectrometric analysis of oxidized and nonoxidized peptides.

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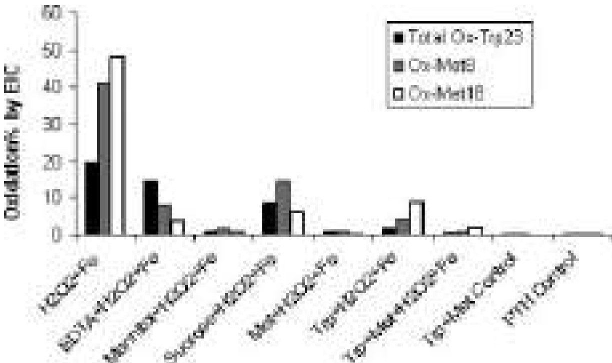

Figure 12. Site-specific oxidation of PTH in 20 mM ammonium acetate buffer at pH 5.0, by H_2O_2/Fe (II) and the protective roles of Trp and Met as compared with other reagents. The identification of individual oxidized Trp23, Met8, and Met18 residues was based on the MS/MS fragmentation spectra of their corresponding tryptic peptides. The relative oxidation level was quantified according to the integrated extracted ion chromatograms (EIC) in mass spectrometric analysis of oxidized and nonoxidized peptides.

articles by Wang and Kowal,[56] Wang and Hanson,[50] and Nema et al.[51] cite several anti-oxidants used in market products. However, these agents are unsuitable for protein formulation for the following reasons: BHA and BHT are limited to lipid- or surfactant-containing formulations; thio compounds (thioglycerol, Cys, *N*-acetyl cysteine, and glutathione) may cause disulfide exchange; and reducing agents (sulfites) and ascorbic acid have been shown to cause undesirable reactions

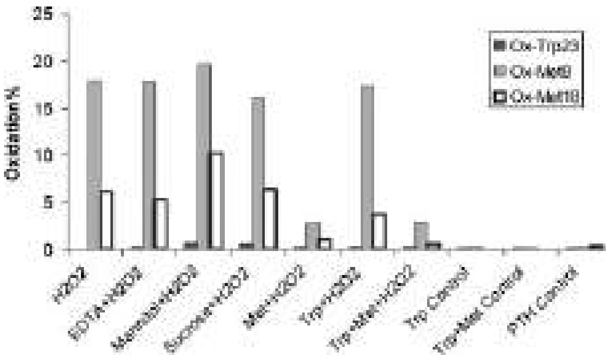

Figure 13. Site-specific oxidation of PTH in 20 mM ammonium acetate buffer at pH 5.0, by H_2O_2 and the protective roles of Trp and Met as compared with other reagents. The identification of individual oxidized Trp23, Met8, and Met18 residues was based on the MS/MS fragmentation spectra of their corresponding tryptic peptides. The relative oxidation level was quantified according to the integrated extracted ion chromatograms (EIC) in mass spectrometric analysis of oxidized and nonoxidized peptides.

among protein residues. Families of vitamins and amino acids that have been given to patients intravenously as hyperalimentary solutions are suitable candidates to screen.

Free His and Tyr were each added at a concentration of 2 mg/mL to PTH stressed with AAPH. When comparing the Trp[O] peaks in rp-HPLC analyses of PTH stressed with AAPH, the formulation with added His or Tyr showed a slight decrease in peak height as compared with the formulation without added His or Tyr (data not shown). His and Tyr both exhibited slight protection of the Trp residue in PTH. This result is in agreement with the fact that His needs stronger oxidizing conditions than Met or Trp.

Of the vitamins, because of the necessity of water solubility in aqueous formulation, vitamin Bs, and a water-soluble vitamin E (Trolox) were tested. Trolox and pyridoxine (vitamin B_6) showed excellent protection of PTH against AAPH oxidization, as evidenced by rp-HPLC analyses in which Trp[O] peaks were not present when stabilizer containing PTH was stressed with AAPH (data not shown). The free-radical-scavenging efficacy of Trolox could be anticipated, as the phenolic benzopyran moiety of tocopherol is preserved in Trolox, and this moiety resembles the well-known oil-soluble free-radical scavenger BHA or BHT. Pyridoxine has not been referred to as an antioxidant in the pharmaceutical literature. It has been reported to protect red blood cells from hemolysis induced by AAPH.[57] According to the density functional theory calculation, the free-radical-scavenging property was attributed to a hydrogen abstraction reaction, where $^{\cdot}H$ is removed from the CH_2OH groups or the ring-bound OH group in pyridoxine.[58]

The use of Trp and pyridoxine as free radical scavengers is not limited to protein formulations. Many small molecule drugs are susceptible to free-radical-mediated degradation.[46,53] Currently there is no safe, effective, water-soluble free-radical scavenger that can be used as an excipient in a parenteral formulation. The utility of Trp and pyridoxine as stabilizers should be further explored in various dosage forms with drug candidates that are susceptible to free-radical degradation.

CONCLUSIONS

To our knowledge, this is the first report of a study comparing the degree of oxidation among vulner-

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able residues such as Met, Trp, His, and Phe on a single protein by various oxidants such as H_2O_2 and t-BHP in the absence or presence of transition metals (Fe(II), Fe(III), and Cu(II)) and the free-radical generator AAPH. In brief, Met is the most readily oxidized of the residues evaluated. Trp cannot be oxidized by H_2O_2 or t-BHP alone; a transition metal is required as a catalyst to generate free radical to oxidize Trp. The oxidation of His requires the presence of copper. The hydroxylation of Phe, in relatively small amounts, occurs only when highly reactive hydroxyl free radical is generated.

In this report, AAPH is demonstrated to be a predictive and reliable model reagent that can oxidize, without the complication of metal catalysis, the Trp residues in a protein. AAPH reliably causes Trp oxidation when the proper oxidant-to-protein ratio is used. It is prudent to use H_2O_2, $H_2O_2 +$ iron, and AAPH (Scheme 2) together to screen stabilizers against the oxidation of Met and Trp residues.

The addition of free Met and Trp can effectively curtail the oxidation of Met and Trp residues, respectively. It was surprising to observe that pyridoxine and Trolox, when added to the study formulations effectively blocked the oxidation of the Trp residue in PTH. It is apparent that Met and Trp are being oxidized by two separate mechanisms. Met is oxidized by H_2O_2 through a nucleophilic reaction, whereas Trp is oxidized by a free-radical mechanism. If authentic samples with oxidation only on Met or Trp residues are needed for analytical method development, free Met may be added to generate Trp[O] species, and free Trp may be added to generate Met[O] species.

To ensure that all vulnerable amino acid residues such as Met, Trp, and possibly His are protected, a combination of Met and a free-radical scavenger such as Trp, pyridoxine, or Trolox, may be considered. The amount of antioxidants required for any given product depends on the potential amount of oxidant that protein product may encounter during its manufacturing and storage.

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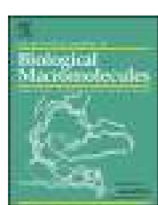

Evaluation of antioxidants in protein formulation against oxidative stress using various biophysical methods

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ABSTRACT

To evaluate the biophysical stability of protein against oxidative stress, hydrogen peroxide (H_2O_2) was used to induce non-site-specific protein oxidation. Various biophysical methods were utilized including RP-HPLC, DSC, DLS, and CD. Lysozyme was chosen as a model protein and three different antioxidants (ascorbic acid, N-acetyl-L-cysteine, and L-methionine) were selected to observe their effect. Significant increase in hydrodynamic size, decrease in α-helix propensity, and increase in β-sheet content evident with increasing H_2O_2 concentration and temperature suggested methionine residues as the most probable site of oxidation. Among the three anti-oxidants, methionine proved superior in suppressing protein oxidation with its increasing concentration. Methionine reacted with H_2O_2 to form methionine sulfoxide, which aided in decreasing the oxidant concentration to react with the protein. The hydrodynamic size of methionine containing protein was retained when incubated at 40 °C after 14 days with unchanged transition temperature (T_m). In contrast, RP-HPLC revealed oxidation alterations when the same samples were stored at 40 °C, highlighting the significant impact of temperature on kinetics. N-acetyl-L-cysteine and ascorbic acid were relatively less protective. Their hydrodynamic size was increased with decreasing T_m compared to the reference. In summary, methionine was a superior antioxidant, implicating a promising component in the protein formulation for suppressing oxidation.

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1. Introduction

Therapeutic proteins are highly specific and efficacious in many diseases. Protein-based drug development have increased the demand for more stable protein formulations; unlike low molecular weight drugs, the physicochemical properties of proteins can render them physically and chemically instable [1–3]. Physical instability involves the change in the physical state of proteins, such as denaturation, aggregation, precipitation, and adsorption. Chemical instability includes processes that form or break covalent bonds resulting in new chemical entities with different physicochemical properties. Common chemical degradations may include oxidation, deamidation, racemization, and hydrolysis [4].

Oxidation is a major chemical degradation pathway of amino acid residues in protein pharmaceuticals [5]; mechanisms of oxidation include photo-oxidation, free radical cascade oxidation, and metal-catalyzed oxidation [4,6]. Amino acids, such as histidine (His), methionine (Met), cysteine (Cys), tyrosine (Tyr), and tryptophan (Trp), are typically susceptible amino acids for oxidation by reactive oxygen species (ROS) [4–6]. Oxidation at these sites may occur during production, purification, formulation, and storage [7–9]. Protein oxidation can be site-specific or non-site specific. Site-specific oxidation involves metal catalyzed reactions. Non-site specific oxidation involves light or presence of any oxidants in the protein formulations [4]. Oxidation in protein formulation should be minimized by adequate processes with stable formulations. Hence, excipients to stabilize the protein need to be investigated systematically.

Different types of excipients have been used to improve the physical stability of protein pharmaceutical formulations. Excipients like polysorbate 20 and 80, which have been used in formulations to reduce the loss of proteins due to adsorption and interface induced adsorption, are potent oxidants [10]. Polysorbate degrades over time in aqueous solution by hydrolysis and auto-oxidation [11]. Hydrogen peroxide (H_2O_2) is a predominant (up to 75%) oxidant in aged polysorbates [12,13].

Protein structure is a crucial factor affecting immunogenicity [14]. A small change may induce a significant change in the physical and chemical properties of the protein. Oxidation may lead to

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protein misfolding, which might have a great influence in the overall stability of the formulation and in immunogenicity [15,16]. Strategies with regard to stable formulation development may need to be adopted during various development processes.

Various techniques have been applied to prevent the oxidation in formulations. Use of antioxidants can be a fast and efficient means to achieve sufficient stability. Antioxidants may be a chelating agent that binds to metal ions that catalyze oxidation, a reducing agent that reduces an oxidized drug, an oxygen scavenger, or a chain terminator [17,18]. The efficacy of these antioxidants in formulations varies with different factors including pH, presence or absence of metal ions, and light [18,19]. Some antioxidants, such as ascorbic acid and N-acetyl-L-cysteine, tend to act as both an oxidant and pro-oxidant [18,20,21].

In this study, chicken egg white lysozyme was chosen as a model protein to evaluate its sensitivity to oxidative stress and evaluate the efficacy of typical antioxidants. Lysozyme is a single chain polypeptide of 129 amino acids cross-linked with four disulfide bridges [21]. It has been used in pharmaceutical and food products, and has been used as a model protein [1,22]. H_2O_2 was used to induce non-site specific oxidation in proteins. The antioxidants chosen for this study were ascorbic acid, N-acetyl-L-cysteine, and L-methionine. Biophysical methods of examination were differential scanning calorimetry (DSC), reverse-phase high performance liquid chromatography (RP-HPLC), circular dichroism (CD), and dynamic light scattering (DLS). Thermodynamic properties of the protein were investigated by DSC for the conformational stability and folding of biomolecules with their transition temperature (T_m). Thermal stress as proteins are exposed to high temperature can affect the conformational stability and diminish protein efficacy. CD was utilized to evaluate the secondary structure of the protein.

2. Material and methods

2.1. Materials

Chicken egg white lysozyme (3× crystallized; molecular weight 14.3 kDa) was purchased from Sigma–Aldrich (St. Louis, MO, USA) and used as received without further purification. L-methionine, sodium phosphate monobasic dihydrate, and sodium Phosphate dibasic dihydrate were also purchased from Sigma–Aldrich. N-acetyl-L-cysteine was purchased from Daejung Chemicals & Metals (Gyeonggi-do, South Korea). L-(+)-ascorbic acid and 30% (w/w) H_2O_2 were purchased from Junsei Chemical (Tokyo, Japan). All other reagents were of analytical grade and were used as received.

2.2. Sample preparation

2.2.1. Oxidative degradation

Lysozyme was dissolved in 10 mM sodium phosphate buffer (pH 6.2) with the protein concentration adjusted to 10 mg/mL. Lysozyme solutions with 0.15%, 0.30%, 1.0%, 2.0%, and 3.0% (w/w) H_2O_2 were also prepared and incubated at 4 °C, 25 °C, and 40 °C. The protein samples were taken at predetermined time intervals for further analysis.

2.2.2. Effect of antioxidants on oxidative degradation

Lysozyme solution (10 mg/mL) in 10 mM sodium phosphate buffer (pH 6.2) was prepared and the solutions of antioxidants (10 mM and 100 mM N-acetyl-L-cysteine, L-methionine, and L-(+)-ascorbic acid) were also prepared in the buffer. Samples were dialyzed in antioxidant containing buffer for 24 h at 4 °C in a Cellu Sep® F2 cellulose membrane having a molecular weight cut-off of 6000–8000 Da (Membrane Filtration Products, Seguin, TX, USA). Following the dialysis, 3.0% (w/w) H_2O_2 was added. All the prepared samples were filtered through DISMIC®-13_{HP} disposable syringe filter units with a pore size of 0.45 μm (Toyo Roshi Kaisha, Tokyo, Japan) and incubated at 4 °C, 25 °C, and 40 °C.

2.3. DLS

Particle size measurement was performed using a Zetasizer Nano ZS90 apparatus (Malvern Instruments, Worcestershire, UK). The samples were equilibrated at a measuring temperature of 25 °C. Each sample of 1 mL was analyzed for hydrodynamic radius in a disposable sizing cuvette (Sarstedt, Numbrecht, Germany). All the samples were measured for five times with fixed angle of 90°. The hydrodynamic size was calculated from the correlation function using the auto-correlated function in Zetasizer software version 7.11 provided with the equipment (Malvern Instruments).

2.4. DSC

Thermodynamic properties of the protein samples were evaluated using a Microcal VP-DSC (Northampton, MA, USA) having 0.51471 cm^3 twin cells for the reference and sample solutions. Prior to the DSC measurements, samples and references were degassed under vacuum while being stirred. Respective buffer prepared for the dialysis was used as a reference to obtain the baseline. Measurements were repeated three times at a scanning rate of 1 °C/min from 25 °C to 90 °C for every sample. The final thermogram of prepared sample was obtained by subtracting the baseline from the sample thermogram. The obtained thermo compensation curves were evaluated using the Microcal LLC DSC plug-in for the Origin 7.0 software package provided with the equipment. The results were further processed to calculate transition melting point (T_m).

2.5. RP-HPLC

The prepared samples were investigated using a HPLC 1100 system (Agilent, Santa Clara, CA, USA) with a diode array detector at an UV wavelength of 280 nm. A TSK-GEL Protein C_4-300 3.5 μm (4.6 mm × 150 mm) column (TOSOH Bioscience, King of Prussia, PA, USA) was used as a stationary phase and maintained at 40 °C. Mobile phase A was composed of 95% H_2O, 5% acetonitrile, and 0.1% trifluoroacetic acid (TFA). Mobile phase B was composed of 5% H_2O, 95% acetonitrile, and 0.1% TFA. The samples were analyzed with a linear gradient of A:B of 85:15 to 45:55 for 30 min at a flow rate of 0.75 mL/min. The injection volume was 10 μL. The relative percentage areas of the non-oxidized samples were calculated using the following equation:

$$\%\,\text{Non-oxidized} = \left(\frac{A_t}{A_0}\right) \times 100$$

where A_t is the area of non-oxidized lysozyme at time 't' and A_0 is the total area of all the peaks at the same time. The percentage of the peak area of non-oxidized lysozyme on day zero was considered to be 100%.

2.6. Far-UV CD

The secondary structural contents of the prepared samples were investigated by a Chirascan-plus spectrometer (Applied Photophysics, Surrey, UK) with a peltier-type temperature controller TC125 (Quantum Northwest, Spokane, WA, USA). For the accelerated stability studies by CD, all the samples were incubated at 55 °C for 24 h. The samples were diluted to 0.5 mg/mL with the respective buffers before the analysis. The samples and the references were degassed prior to the measurements. After the calibration to remove the noise from the device itself, buffer was scanned before every sample measurement. Rectangular quartz cuvette with light path lengths of 0.2 mm (Hellma Analytics, Müllheim, Germany)

was used for the scanning of CD spectra. Respective buffer of the samples was used as a reference to obtain the baseline. After the buffer scanning, the samples were scanned at 25 °C from 190 nm to 260 nm far-UV wavelength region with a resolution of 1 nm. Every scan is carried out three times. The temperature was regulated by a temperature controller (Quantum Northwest). Spectrums obtained were processed to obtain specific CD spectra and secondary structure contents of the protein using a Pro-Data viewer and CDNN software provided with the equipment.

3. Results

3.1. Effect of oxidation on hydrodynamic size

DLS is a powerful technique for the qualitative analysis of protein particle size in a solution. It measures the time-dependent fluctuations in the scattering intensity that arise from particles undergoing Brownian motion [23,24]. The technique was applied in this study to analyze the aggregation and changes in size of the protein molecules along with the oxidative stress, and to assess the prowess of the antioxidants in minimizing the change. To evaluate the influence of temperature on protein oxidation, the oxidized samples were incubated at 4 °C, 25 °C, and 40 °C for up to 7 days. All the measurements were carried out according to the volume of distribution.

Table 1
Hydrodynamic size (diameter, nm) of lysozyme with different concentrations of H_2O_2 at days 0 and 7 after incubation at 4 °C, 25 °C, and 40 °C.[a]

Sample	0 day	7th day		
		4 °C	25 °C	40 °C
Control	2.47 ± 0.12	2.47 ± 0.04	2.47 ± 0.02	2.47 ± 0.06
0.15% H_2O_2	2.46 ± 0.10	2.62 ± 0.06	2.74 ± 0.05	2.56 ± 0.04
0.30% H_2O_2	2.53 ± 0.07	2.63 ± 0.05	2.52 ± 0.01	2.67 ± 0.02
1.00% H_2O_2	2.53 ± 0.07	2.58 ± 0.09	2.56 ± 0.17	2.59 ± 0.02
2.00% H_2O_2	2.50 ± 0.08	2.52 ± 0.07	2.54 ± 0.07	2.80 ± 0.12
3.00% H_2O_2	2.47 ± 0.11	2.29 ± 0.10	2.68 ± 0.06	3.02 ± 0.03

[a] All values are expressed as mean ± SD (n = 5).

The hydrodynamic size of the control was constant as 2.47 nm throughout the experiment irrespective of temperature (Table 1, Fig. 1a). However, the monomer size increased depending on the concentration of H_2O_2 and storage temperature, indicating the effects of oxidation and temperature on the hydrodynamic size of protein. The size increased from 2.50 nm to 2.80 nm on day 7 in the sample with 2.0% H_2O_2 and from 2.47 nm to 3.02 nm in sample with 3.0% H_2O_2 after incubation at 40 °C for 7 days (Table 1). The percentage of monomer was decreased up to 99.61% in the sample with 3% of H_2O_2 at 40 °C. The overall data on the changes in the hydrodynamic size are summarized in Table 1. These results suggest that along with the chemical changes that occur during

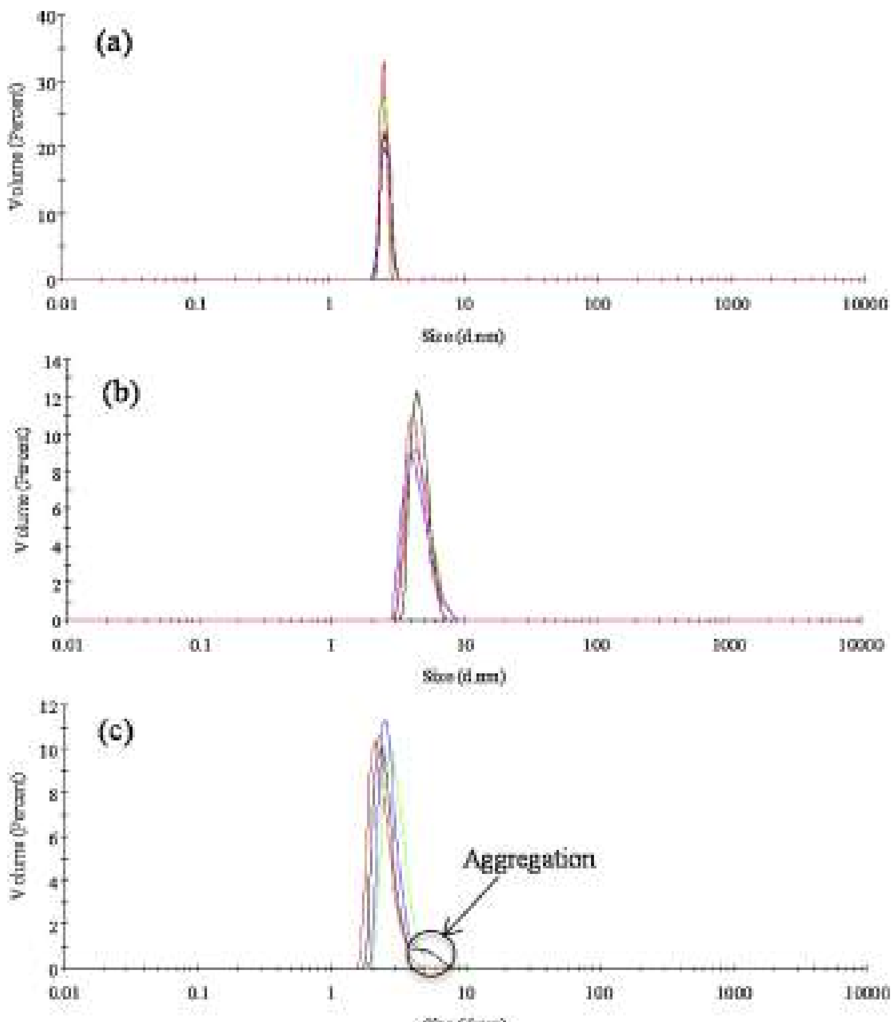

Fig. 1. Hydrodynamic size of lysozyme (distributed by volume) incubated at 40 °C for 14 days: (a) control, (b) oxidized lysozyme, and (c) oxidized lysozyme with 100 mM of methionine.

Table 2
Hydrodynamic size (diameter, nm) of lysozyme in presence of different antioxidants on days 0 and 14 after incubation at 4 °C, 25 °C, and 40 °C.[a]

Sample	0 day	14th day		
		4 °C	25 °C	40 °C
Control	2.56 ± 0.04	2.53 ± 0.05	2.55 ± 0.06	2.61 ± 0.04
Lysozyme + H_2O_2	2.44 ± 0.10	2.53 ± 0.10	2.72 ± 0.05	4.56 ± 0.15
10 mM methionine	2.61 ± 0.03	2.64 ± 0.05	2.75 ± 0.02	3.87 ± 0.20
100 mM methionine	2.60 ± 0.04	2.47 ± 0.13	2.86 ± 0.21	2.81 ± 0.24
10 mM N-acetyl-L-cysteine	2.52 ± 0.04	2.62 ± 0.21	3.04 ± 0.05	4.20 ± 0.14
100 mM N-acetyl-L-cysteine	2.73 ± 0.06	3.02 ± 0.03	4.72 ± 0.05	4.56 ± 0.04
10 mM ascorbic acid	2.81 ± 0.11	2.79 ± 0.15	2.95 ± 0.14	4.06 ± 0.18
100 mM ascorbic acid	3.91 ± 0.34	4.31 ± 0.16	4.55 ± 0.34	4.03 ± 0.20

[a] All values are expressed as mean ± SD (n = 5).

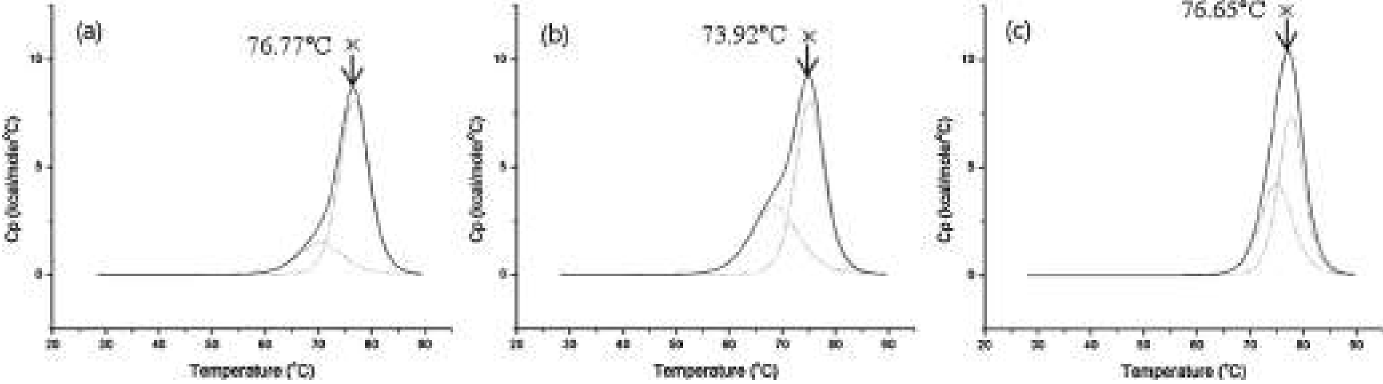

Fig. 2. (a) Typical DSC thermogram of control sample showing two domains, (b) DSC thermogram of oxidized lysozyme, and (c) DSC thermogram of oxidized lysozyme with 100 mM of methionine. *Midpoint unfolding transition at temperature of maximum heat flux.

oxidation, physical modification also follows in the protein molecules that manifested through the change in the hydrodynamic size. After the analysis on the effects of oxidation, antioxidants were added in two different concentrations to evaluate their efficacy.

Table 2 summarizes the hydrodynamic size of the protein in the presence of H_2O_2 and different antioxidants. Initially, the size of the control was 2.56 nm. The size gradually increased to 2.61 nm by day 14 when incubated at 40 °C. The size of monomer in the samples was retained when stored at 4 °C until day 14, except for the samples with 100 mM N-acetyl-L-cysteine and ascorbic acid (3.02 nm and 4.31 nm, respectively; Table 2). As the storage temperature was increased the size also increased. The size of the protein was 2.47 nm, 2.86 nm, and 2.81 nm after incubation at 4 °C, 25 °C, and 40 °C, respectively on day 14 in samples with 100 mM methionine. This showed that there was little or no change in the monomer size in samples with methionine even at high temperature. However, aggregation was noticed on day 14 in the same samples and the percentage of the monomer was also 97.12% after incubation at 40 °C (Fig. 1). Of the three antioxidants, methionine was more efficient in conserving the hydrodynamic size of the protein in solution.

3.2. Thermodynamic analysis by DSC

DSC determines the temperature and heat flow of biomolecules associated with material transitions as a function of time and temperature. It is capable of elucidating the factors that contribute to the folding and stability of biomolecules [25,26]. Three different aspects were considered: midpoint unfolding temperature at the temperature of maximum heat flux (T_m), calorimetric enthalpy (ΔH), and van't Hoff enthalpy (ΔH_v) to determine the thermal stability of the protein.

Fig. 2a shows a typical DSC thermogram of lysozyme. Following the curve fitting deconvolution, the thermogram revealed two domains. This result is consistent with the previous work where the thermodynamic analysis of lysozyme revealed two distinct domains upon the curve fitting deconvolution [27]. These two domains might indicate that there are two different regions in the protein structure having different sensitivity to the temperature, revealing two unfolding steps during the thermal degradation process. Upon the oxidative stress, the two domains separated significantly with decreasing T_m (Fig. 2b). In the sample with 100 mM of methionine, although the domains seemed to be separated more than the control, the peak shapes were broader and shifted to higher T_m than that without any antioxidant, suggesting a slower thermal degradation (Fig. 2c). On the other hand, samples containing N-acetyl-L-cysteine and ascorbic acid showed largely exothermic peak (data not shown) with decreasing T_m than those with methionine.

T_m is the temperature at which 50% of the protein has native conformation and 50% is denatured. Higher values of T_m indicate greater stability of proteins against thermal degradation [25]. The T_m of control was 76.77 °C which decreased to 73.92 °C with oxidative stress. Samples containing 100 mM methionine exhibited significant increase in T_m up to 76.65 °C, suggesting an increase in stability against the thermal unfolding of the protein. However, N-acetyl-L-cysteine and ascorbic acid decreased the T_m of the protein.

The ratio of van't Hoff enthalpy (ΔH_v) and calorimetric enthalpy (ΔH) can indicate whether the denaturation involves two state or non-two transition state [25,28]. $\Delta H_v/\Delta H$ was more than one, indicating that the denaturation process involved significant intermediate states. The ΔH and ΔH_v of control was 8.01×10^4 cal/mol and 1.25×10^5 cal/mol, respectively. Along with the oxidative stress, ΔH and ΔH_v decreased to 5.77×10^4 cal/mol and 1.11×10^5 cal/mol, respectively. However, methionine showed an increase in both ΔH and ΔH_v as 7.35×10^4 cal/mol and 1.15×10^5 cal/mol, respectively. Samples containing N-acetyl-L-cysteine and ascorbic acid showed a sharp decrease in enthalpy

Table 3
Midpoint transition temperature (T_m), calorimetric enthalpy (ΔH), and van't Hoff enthalpy (ΔH_v) at maximum heat flux of lysozyme in presence of antioxidants.[a]

Samples	T_m (°C)	ΔH (×10^4 cal/mol)	ΔH_v (×10^5 cal/mol)
Control	76.77 ± 0.02	8.01 ± 0.5	1.25 ± 0.1
Lysozyme + H_2O_2	73.92 ± 0.08	5.77 ± 0.7	1.11 ± 0.5
10 mM methionine	74.16 ± 0.06	7.24 ± 0.2	1.20 ± 0.9
100 mM methionine	76.65 ± 0.03	7.35 ± 0.1	1.15 ± 0.2
10 mM N-acetyl-L-cysteine	70.29 ± 0.02	3.79 ± 0.2	1.01 ± 0.1
100 mM N-acetyl-L-cysteine	60.40 ± 0.01	2.41 ± 0.1	1.07 ± 0.8
10 mM ascorbic acid	59.62 ± 0.04	1.92 ± 0.1	0.91 ± 0.2
100 mM ascorbic acid	NA[b]	NA	NA

[a] All values are expressed as mean ± SD (n = 3).
[b] Not available.

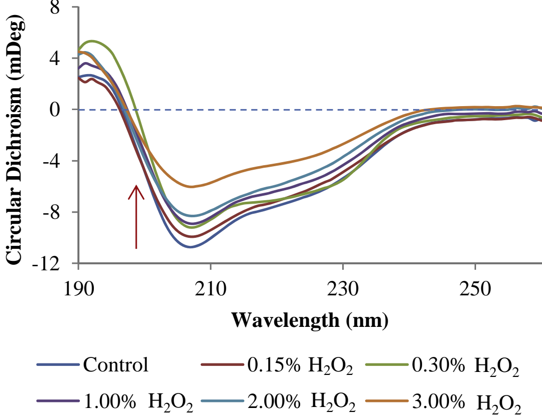

Fig. 3. CD spectra of oxidized lysozyme obtained after the incubation at 55 °C for 24 h. The depth of the peaks decreased at 208 nm and 222 nm as the concentration of H_2O_2 increased.

compared to methionine. The overall DSC data is summarized in Table 3. T_m, ΔH, and ΔH_v results suggested the superiority of methionine compared to the other two antioxidants.

3.3. Changes in secondary structure with CD

DLS and DSC revealed that the conformational stability of the protein decreased due to the oxidative stress at high thermal conditions, which might also involve a side-by-side influence in the structural stability of the proteins. CD is a good tool for the quantification of protein secondary structure [29,30]. CD was used presently to analyze the correlation between the chemical and structural instability of protein molecules, and to assess if antioxidants could enhance the stability.

Fig. 3 shows the CD spectra of lysozyme (190–260 nm) with different concentrations of H_2O_2. A positive band near 193 nm and two negative bands near 208 nm and 222 nm wavelengths were observed in the control sample, indicating α-helix as a dominant structure. As the concentration of H_2O_2 increased, the depth of the two negative bands decreased, indicating decreased propensity of the α-helix. The relative percentages of all the secondary structures changed throughout the experiment (Table 4). However, significant variations were seen on the percentages of α-helix and antiparallel β-sheet with the addition of H_2O_2. Following the incubation, the control sample had 24.83% α-helix and 15.11% antiparallel β-sheet contents. As the concentration of the H_2O_2 increased the percentage of α-helix decreased and antiparallel β-sheet increased. The percentage changed to 18.14% and 19.20%, respectively, with the addition of 3.0% H_2O_2. The result indicated the presence of two factors governing the change in secondary structural content of proteins: temperature and presence of an oxidant. The overall percentage of all the secondary structures is summarized in Table 4. The percentage of α-helix and antiparallel β-sheet was plotted in the graph against the concentration of H_2O_2 (Fig. 4a) and clearly demonstrated a decrease in the propensity of α-helix and increase in antiparallel β-sheet of the protein with sharp changes at high concentrations of 2.0% and 3.0% H_2O_2.

Methionine was screened to investigate its efficacy in minimizing the changes in the secondary structures. The results were consistent with the DLS and DSC. After incubation at 55 °C, control sample had 24.83% and 15.11% of α-helix and antiparallel β-sheet, respectively. This percentage changed to 18.14% and 19.20%, respectively, with oxidative stress. However, the percentage of α-helix and antiparallel β-sheet was 22.84% and 17.25%, respectively, in samples containing 100 mM methionine. Thus, methionine might have decreased the contact between the protein and H_2O_2, suppressing the change. The overall percentage of the secondary structures in the presence of methionine has been summarized in Table 5. Fig. 4b shows the change in the percentage of α-helix and antiparallel β-sheet of samples in the presence of different concentration of methionine. The graph shows that the percentage of α-helix increased and antiparallel β-sheet decreased with the increasing concentration of methionine, showing its efficacy in protecting against oxidation with increasing concentration. Hence, along with its role in increasing the conformational stability of protein, methionine also has a significant role in increasing the structural stability of the protein during the oxidative degradation.

3.4. Oxidative degradation and effects of antioxidants by RP-HPLC

The qualitative study on the stability of the protein molecule under the oxidative stress was performed using DLS and DSC.

Table 4
Percentage of secondary structures of lysozyme in presence of different concentrations of H_2O_2.

Samples	Control	H_2O_2				
		0.15%	0.30%	1.00%	2.00%	3.00%
α-Helix	24.83	25.56	24.08	22.92	21.55	18.14
Antiparallel β-sheet	15.11	16.16	16.86	16.86	17.91	19.20
Parallel β-sheet	9.72	9.50	9.73	10.04	10.17	10.88
β-Turn	17.90	18.32	18.21	18.09	18.00	17.61
Random coil	32.34	30.56	31.12	32.20	32.37	34.16
Total	100.00	100.00	100.00	100.00	100.00	100.00

Table 5
Percentage of secondary structures of lysozyme in presence of different concentration of methionine.

Samples	Control	Lysozyme + H_2O_2	10 mM methionine	50 mM methionine	100 mM methionine
α-Helix	24.83	18.14	19.26	22.75	22.84
Antiparallel β-sheet	15.11	19.20	20.16	17.54	17.25
Parallel β-sheet	9.72	10.88	10.45	9.95	9.95
β-Turn	17.90	17.61	18.07	18.20	18.10
Random Coil	32.34	34.16	32.06	31.66	31.85
Total	100.00	100.00	100.00	100.00	100.00

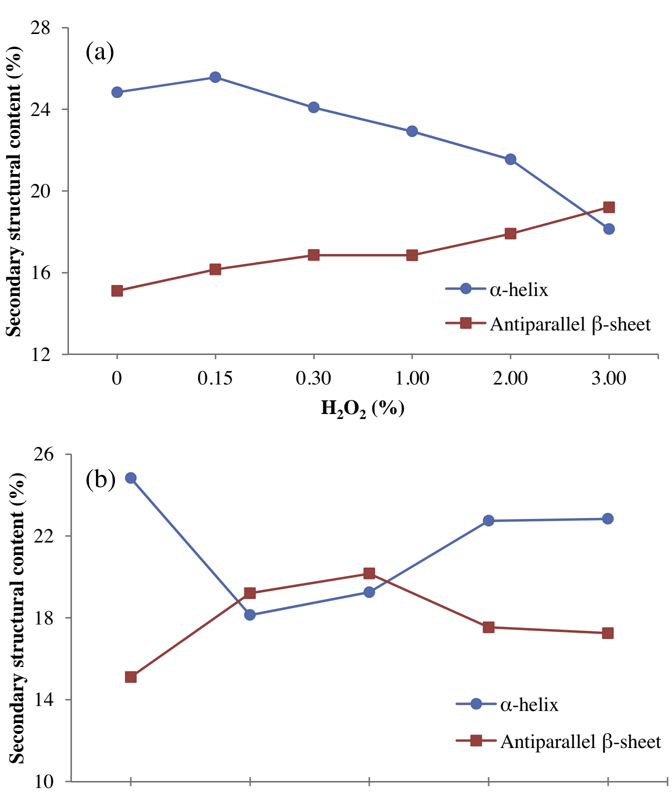

Fig. 4. Changes in the secondary structural content in (a) oxidized lysozyme and (b) oxidized lysozyme in presence of different concentration of methionine incubated at 55 °C for 24 h.

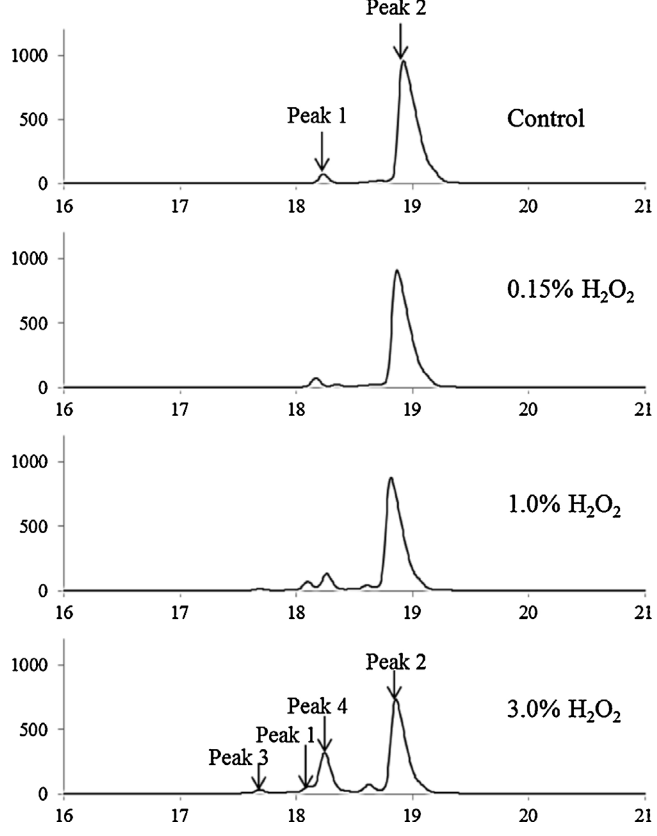

Fig. 5. RP-HPLC chromatogram of lysozyme (control and oxidized) obtained after incubation at 4 °C for 7 days. Two new oxidized peaks (peaks 3 and 4) appeared in front of the respective non-oxidized lysozyme peaks (peaks 1 and 2) that increased with the concentration of H_2O_2.

For further quantification of the degradation kinetics, the protein solution was incubated at 4 °C, 25 °C, and 40 °C and analyzed through RP-HPLC at different times. Fig. 5 shows the RP-HPLC chromatograms of non-oxidized and oxidized lysozyme after the incubation at 4 °C for 7 days. Control chromatogram showed two distinct peaks: a small peak (Peak 1) at around 18.2 min retention time and a large peak (Peak 2) at around 18.9 min. These two peaks represent different domains of the protein with different hydrophilicity. Peak 1 seemed to increase even in control as the storage temperature and time increased. Therefore, peak 1 might represent a misfolded hydrophobic domain of the protein formed due to the thermal degradation. These two hydrophobic domains may be correlated with the two thermal domains of lysozyme in the DSC thermogram. Further study is needed to discern the correlation of the domains. Moreover, in the oxidative stress condition, two peaks (peak 3 and peak 4) appeared in front of peak 1 and peak 2 (Fig. 5). Since the addition of oxygen in the proteins resulted in more polar compounds, the oxidized peaks appeared prior to the non-oxidized peak. As the concentration of H_2O_2 was increased, the area of peaks 3 and 4 increased, and the area of peaks 1 and 2 decreased. This supports the view that peaks 3 and 4 are due to oxidized lysozyme.

Fig. 6 shows the graphs between $\ln(C_t/C_0)$ vs. time. The degradation kinetics at 4 °C (Fig. 6a) and 25 °C (Fig. 6b) showed good linearity ($R^2 > 0.99$), indicating first-order kinetics. This result was consistent with the previous studies [31,32]. Conversely, the degradation kinetics at 40 °C did not show good linearity ($R^2 < 0.99$; Fig. 6c), perhaps due to physical degradation of the protein along with chemical degradation at high temperature. However, the rate constants were calculated assuming that no physical degradation occurred and the order of all the reactions was considered as first-order. The rate constant was obtained using the following rate equation for the first-order reaction:

$$\ln[C_t] = -kt + \ln[C_0]$$

where C_0 and C_t stand for the initial concentration and concentration of the protein at time 't', respectively, 'k' is the rate constant, and 't' is the time of reaction.

The rate constant increased with the increasing concentration of H_2O_2 and temperature. Initially, the rate constant of

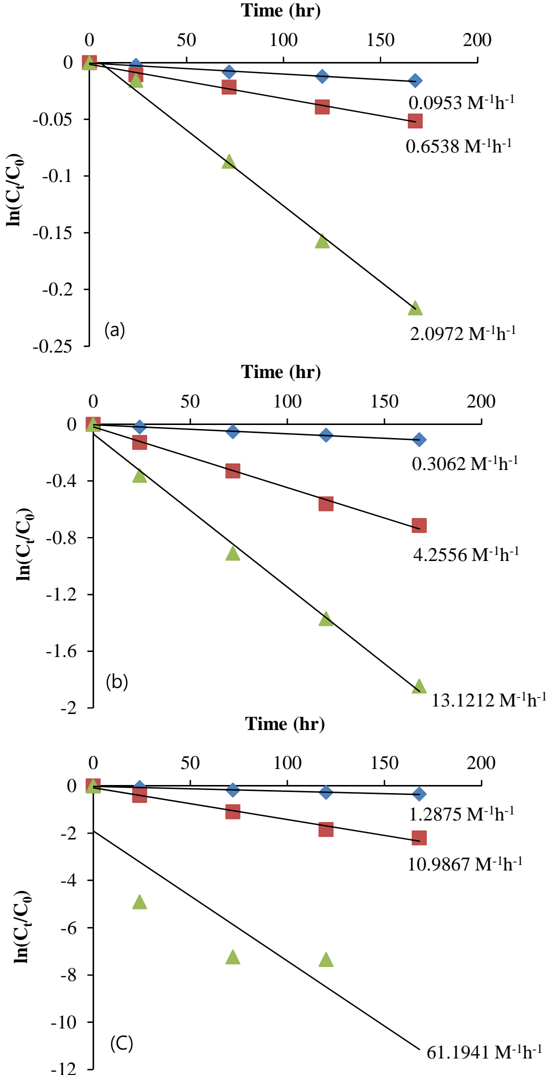

Fig. 6. Plots of $\ln(C_t/C_0)$ vs. time, where C_0 and C_t denote the concentration of non-oxidized lysozyme at time zero and time '*t*', respectively, representing the degradation kinetics at (a) 4 °C, (b) 25 °C, and (c) 40 °C. (◆), (■) and (▲) represent samples containing 0.15%, 1.0%, and 3.0% H_2O_2, respectively. The rate constants were calculated according to the first order rate equation.

reaction involving 0.15% of H_2O_2 was 0.0953 M^{-1} h^{-1} at 4 °C, which increased to 0.3062 M^{-1} h^{-1} and 1.2875 M^{-1} h^{-1} at 25 °C and 40 °C, respectively. The rate constant of reactions in the presence of 3.0% H_2O_2 was 2.0972 M^{-1} h^{-1}, 13.1212 M^{-1} h^{-1}, and 61.194 M^{-1} h^{-1} at 4 °C, 25 °C, and 40 °C, respectively. This clearly showed that oxidation kinetics were significantly impacted by the high thermal environment.

Fig. 7 shows the effects of various antioxidants on the oxidative degradation of the protein in different temperatures. Similar to the results of DLS, DSC, and CD, methionine was significantly protective in the oxidation of the protein with its increasing concentration. When stored at 4 °C, samples with 100 mM and 10 mM methionine retained 91.49% and 82.02% non-oxidized lysozyme on day 14, respectively. On the other hand, 73.76% non-oxidized lysozyme was retained in the sample without any antioxidants. However, degradation at 40 °C was so pronounced that all the samples were degraded by day 10 of incubation (Fig. 6c). N-acetyl-L-cysteine and ascorbic acid exhibited relatively less protecting role than methionine.

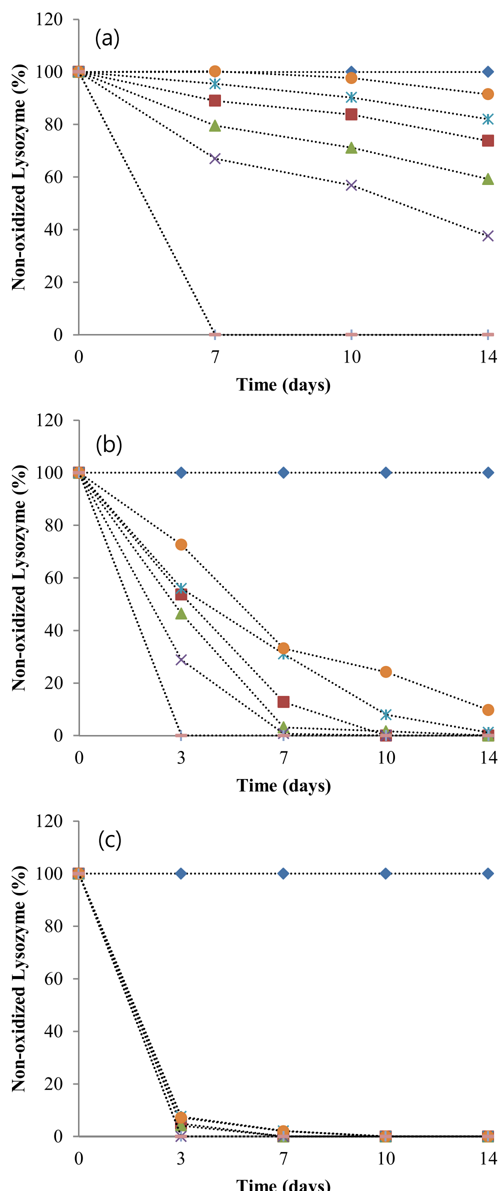

Fig. 7. Plots of percentage of non-oxidized lysozyme vs. days, showing the protecting role of methionine in presence of H_2O_2. The samples were incubated at (a) 4 °C, (b) 25 °C, and (c) 40 °C. The representation of symbols are: (◆) Control, (■) lysozyme + H_2O_2, (✳) 10 mM methionine, (●) 100 mM methionine, (▲) 10 mM N-acetyl-L-cysteine, (✕) 100 mM N-acetyl-L-cysteine, (|) 10 mM ascorbic acid and (▬) 100 mM ascorbic acid.

4. Discussion

Biophysical methods and chromatography were utilized together to evaluate protein stability as exposed to oxidative stress depending on temperature. DLS, DSC, CD, and RP-HPLC were used to investigate the protein stability with or without antioxidants. DLS is based on the magnitude of random or Brownian movement of particles as a result of small fluctuations in the bulk solution and here it was used to evaluate the oxidation on the protein hydrodynamic size [33]. Increased hydrodynamic size was evident as the concentration of H_2O_2 and storage temperature increased (Table 1). The result might indicate physical change along with the chemical change in the protein molecules. This outcome may also suggest that the most likely site of oxidation is methionine. Methionine residues in proteins can be readily oxidized by a wide range of oxidants including H_2O_2 to produce methionine

sulfoxide [7,32,34,35]. If partially or fully buried methionine is oxidized then the hydrophobic interactions may be disrupted by a local increase in hydrophilicity. This may cause the hydrophobic portions to unfold or loosen [36,37], which could increase hydrodynamic size, as seen in the results, indicating the most probable site of oxidation as methionine residues. The hydrodynamic size of samples with N-acetyl-L-cysteine and ascorbic acid was also increased as the both accelerated the rate of oxidation. On the other hand, the size of samples with 100 mM methionine remained nearly constant even on day 14. As methionine acted as an effective antioxidant through conversion to methionine sulfoxide, it decreased the concentration of H_2O_2 for the oxidization of methionine residues in protein. Consequently, oxidation of the methionine residue was suppressed and the hydrodynamic size of the monomer was retained in samples with methionine.

DSC was used to investigate the effects of oxidative stress on the conformational stability of protein. Native lysozyme showed two domains after the deconvolution, which separated significantly with thermal stress. This may suggest the presence of the two domains each having a different sensitivity to the thermal stress. The oxidative stress shifted both of the domains to a lower unfolding transition temperature. In the presence of oxidative stress, methionine residues are oxidized to methionine sulfoxide which increases its polarity and susceptibility toward thermal degradation [38]. This may support the thermal destabilization in the presence of an oxidant. Three different antioxidants were evaluated with DSC to see if they influenced the T_m. Samples containing methionine showed a significant increase in T_m with increasing concentration as compared to the samples containing N-acetyl-L-cysteine and ascorbic acid. As the T_m increased, the stability against the thermal unfolding increased, indicating a substantial protective behavior of methionine against oxidation.

CD revealed significant changes, particularly in the percentage of α-helix and antiparallel β-sheets. The percentage of α-helix decreased and β-sheet increased with the increasing oxidative stress. Along with the DLS and DSC findings, this change in secondary structure also indicates methionine oxidation. As methionine residues oxidize to form more hydrophilic methionine sulfoxide, α-helix is destabilized because of its higher preference for β-sheets [39], decreasing the content of α-helix. On the other hand, methionine addition minimized the decrease in α-helix and increase in β-sheet.

DSC, DLS, and CD results were consistent with RP-HPLC findings. The oxidation reaction of the protein followed the first-order reaction at 4 °C and 25 °C. However, due to the involvement of high thermal degradation along with the chemical degradation at 40 °C, the reaction seemed to deviate from first-order kinetics. Nonetheless, supposing that there was no physical degradation, the kinetic rate constant was calculated based on the first-order rate law. The rate constant increased with increasing concentration of H_2O_2 and temperature. This also supports the DSC results that the oxidation degradation kinetic also depends on the temperature at which the reaction takes place.

Methionine aided in retaining more percentage of non-oxidized lysozyme in comparison to samples without antioxidants. Samples containing N-acetyl-L-cysteine and ascorbic acid displayed accelerated oxidation. Methionine as an antioxidant reacts with molecular oxygen that is produced from the H_2O_2 and forms methionine sulfoxide. This prevents the oxidant from reacting with methionine or other amino acid residues in the protein molecule. In addition, ascorbic acid and N-acetyl-L-cysteine have a tendency to act both as an oxidant and a pro-oxidant [20,40,41], which may be a reason behind their decreased effectiveness in oxidation. Along with these outcomes, a change in color from colorless to yellow was seen in the solution with ascorbic acid and N-acetyl-L-cysteine. However, in the samples containing methionine, any change in color was not observed.

5. Conclusions

Oxidative degradation is of greater concern in the formulation development of protein therapeutics. In this study a degradation pattern was deduced including the conformational and structural stability during the oxidation of the protein through the analysis from different biophysical methods. Increase in hydrodynamic size and decrease in α-helix in the secondary structure were evident in the oxidized protein molecules. These changes implicate methionine as the most probable site of oxidation in lysozyme. Among the three antioxidants used, methionine was the most efficient in suppressing oxidation, suggesting amino acids as a promising component in the protein formulation. These results provide a basis for further examination of activities of other amino acids such as histidine and arginine for their application as an antioxidant. Antioxidants need to be carefully evaluated to achieve the maximum stability of therapeutic proteins in the development of protein formulations.

Acknowledgement

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ESTATUTO SOCIAL DA ASSOCIAÇÃO BRASILEIRA INTERDISCIPLINAR DE AIDS - ABIA

CAPÍTULO I - DENOMINAÇÃO, SEDE, DURAÇÃO E FINS

Artigo 1º - A Associação Brasileira Interdisciplinar de AIDS - ABIA, doravante designada simplesmente ABIA, é uma associação, de direito privado, sem fins lucrativos e de fins não econômicos, de natureza social e filantrópica, fundada em 12 de março de 1987, cujas atividades reger-se-ão pelo presente Estatuto Social, devidamente aprovado por Assembleia Geral, e pela legislação em vigor.

Parágrafo Único – Para a sua identificação, o ABIA poderá adotar logomarca.

Artigo 2º - A ABIA tem sua sede, foro e administração no município do Rio de Janeiro, Estado do Rio de Janeiro, na Av. Presidente Vargas n. 446, 13º andar, Centro, CEP: 20.071-907.

Parágrafo Primeiro – Por decisão da Assembleia Geral, a sede poderá ser transferida para outro local.

Parágrafo Segundo - A ABIA poderá atuar em todo território nacional, abrindo filiais, escritórios ou credenciando representantes regionais, no Brasil ou no exterior, respeitada a legislação aplicável.

Artigo 3º - A ABIA terá prazo de duração indeterminado.

Artigo 4º - A ABIA desenvolve ações voltadas à prevenção ao vírus da imunodeficiência humana (HIV), à Síndrome da Imunodeficiência Adquirida (AIDS/SIDA) e doenças associadas, a garantia dos direitos, à assistência à saúde de pessoas atingidas pelo HIV, promovendo o respeito aos direitos humanos e, em particular, pelo respeito aos direitos das pessoas vivendo com HIV/AIDS e dos grupos mais vulneráveis ao HIV/AIDS, cabendo-lhe:

I. Promover a assistência social;

II. Promover a educação e a informação visando prevenir e controlar a epidemia de AIDS/SIDA baseando suas ações no princípio da solidariedade;

III. Elaborar e implementar campanhas de prevenção adequadas à realidade brasileira.

IV. Acompanhar a formulação e a implementação de políticas públicas.

V. Coletar, armazenar e interpretar dados oriundos de pesquisas desenvolvidas.

VI. Reunir, sistematizar e divulgar informações, atualizadas e cientificamente fundamentadas sobre a epidemia, através de estudos, relatórios, e publicações por conta própria ou de terceiros.

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VII. Fornecer assessoria a diferentes grupos da sociedade tais como: empresas, escolas, universidades, sindicatos, associações comunitárias, igrejas, entidades de comunicação, prefeituras e outras instituições governamentais ou não governamentais.

VIII. Planejar, promover, coordenar e exercer atividades de promoção cultural e humana em suas áreas de atuação.

IX. Promover e/ou realizar projetos culturais, inclusive através das leis federais, estaduais e municipais de incentivo à cultura.
Parágrafo Primeiro - A ABIA não distribui entre os seus sócios, associados, conselheiros, diretores, empregados ou doadores eventuais excedentes operacionais, brutos ou líquidos, lucros, dividendos, bonificações, participações, resultados ou parcelas do seu patrimônio, auferidos mediante o exercício de suas atividades, e os aplica integralmente no território nacional, na manutenção e desenvolvimento de seus objetivos institucionais.

Parágrafo Segundo - À ABIA é vedada qualquer atividade político-partidária, eleitoral ou religiosa.

Parágrafo Terceiro – É vedado o uso da ABIA para qualquer espécie de promoção pessoal, política-partidária ou religiosa.

Artigo 5º - No desenvolvimento de suas atividades, a ABIA:

I. Não fará qualquer distinção de raça, cor, sexo, idade, condição física ou social, credo político ou religioso.

II. Prestará serviços permanentes e sem qualquer discriminação de clientela.

III. Poderá firmar termos de colaboração, termos de fomento, convênios, contratos, termos de cooperação, e outros instrumentos jurídico contratuais com pessoas jurídicas, públicas ou privadas, nacionais ou estrangeiras.

IV. Estimulará a atuação voluntária de pessoas interessadas em colaborar com suas finalidades.

CAPÍTULO II - DOS ASSOCIADOS

Seção I - Admissão, Exclusão e Penalidades.

Artigo 6º - A ABIA se constitui de número ilimitado de associados, pessoas naturais ou jurídicas, idôneas e interessadas, desde que:

I. Estejam na plenitude de sua capacidade civil.

II. Comunguem com suas finalidades sociais.

III. Concordem com o presente Estatuto Social e obriguem-se a cumpri-lo.

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IV. Sejam admitidos como associados pelo Conselho de Administração.

Parágrafo Primeiro - Os associados, membros ou não dos órgãos administrativos e consultivos, não respondem solidária nem subsidiariamente pelas obrigações sociais da ABIA.

Parágrafo Segundo – Os associados serão distribuídos nas seguintes categorias:

I. Associados Fundadores: as pessoas naturais que participaram da Assembleia Geral de Fundação da ABIA.

II. Associados Efetivos: as pessoas naturais, admitidas nesta qualidade, por deliberação da Assembleia Geral.

III. Associados Beneméritos: as pessoas naturais ou jurídicas, de caráter público ou privado, que tenham realizado doação, em bens ou espécie, ou tenham prestado relevantes serviços à ABIA, devendo ser recomendado por outros associados, sendo seus nomes aprovados pela Assembleia Geral.

Parágrafo Terceiro – Os associados, independentemente da sua qualificação, comprometem-se a envidar esforços para a consecução dos objetivos sociais da ABIA.

Parágrafo Quarto – Os associados beneméritos não terão direito a voto na Assembleia Geral.

Parágrafo Quinto – A condição de associado prevista neste Estatuto é intransferível a terceiros, a que título for.

Parágrafo Sexto – A ABIA poderá contar com mantenedores, pessoas naturais ou jurídicas, que não serão associados, mas que auxiliem com recursos financeiros ou com dedicação de atuação voluntária às atividades e projetos da ABIA.

Artigo 7º - O interessado em se associar deverá formular pedido por escrito ao Conselho de Administração da ABIA.

Parágrafo Único – O Conselho de Administração apreciará o pedido de filiação e, deferindo-o, o remeterá à aprovação da Assembleia Geral.

Artigo 8º - A exclusão de qualquer associado se dará apenas por justa causa, a critério do Conselho de Administração, sendo-lhe garantido:

I. Prévia notificação para que possa exercer plenamente seu direito de defesa; e

II. Recurso à Assembleia Geral, com efeito suspensivo.

Parágrafo Primeiro - Para fins do disposto nesse Estatuto, será considerado como justa causa:

I. A ausência não justificada em três Assembleias Gerais consecutivas;

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II. O não cumprimento do disposto no presente Estatuto Social ou em qualquer outro documento a que a ABIA seja submetida.

Parágrafo Segundo – Alternativamente à exclusão prevista no *caput* deste artigo, o Conselho de Administração poderá deliberar pela advertência do associado ou suspensão deste por até 90 (noventa) dias, contados da decisão.

Parágrafo Terceiro - O associado poderá se desligar a qualquer tempo se assim expressar formalmente e por escrito a sua intenção ao Conselho de Administração.

Seção II - Direitos e Deveres dos Associados

Artigo 9º - São direitos de todos os associados:

I. Frequentar a sede da ABIA.

II. Obter informações que desejarem sobre os objetivos sociais e funcionamento dos diversos órgãos da ABIA.

III. Participar das Assembleias Gerais e todos os eventos sociais, culturais e esportivos e demais atividades promovidos pela ABIA.
IV. Receber exemplares de todas as publicações da ABIA.

V. Propor a admissão de novos associados.

Parágrafo Único - Somente os associados fundadores e efetivos terão direito a voto.

Artigo 10 - São deveres dos associados, independente da categoria:

I. Colaborar com os órgãos da administração da ABIA, na realização dos atos necessários para a consecução de suas finalidades sociais.

II. Cumprir e fazer cumprir as disposições do presente Estatuto Social.

III. Pagar a contribuição financeira que venha a ser fixada pelo Conselho de Administração.

IV. Zelar pelos interesses morais, éticos e materiais da ABIA, cooperando com o seu desenvolvimento e maior prestígio.

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CAPÍTULO III – ADMINISTRAÇÃO

Artigo 11 - A ABIA será administrada por:

I. Assembleia Geral

II. Conselho de Administração

III. Conselho Fiscal

IV. Conselho Consultivo e de Sustentabilidade

Parágrafo Primeiro - Cada um desses órgãos será regido pelos artigos dispostos nas seções subsequentes e nos termos da legislação vigente.

Parágrafo Segundo – A ABIA poderá remunerar seus dirigentes e as pessoas naturais e jurídicas que lhe prestem serviços específicos, respeitados, em ambos os casos, as disposições legais aplicáveis.

Seção I - Assembleia Geral

Artigo 12 - A Assembleia Geral é o órgão soberano da ABIA, sendo constituída por todos os associados em pleno gozo de seus direitos estatutários.

Parágrafo Único - As decisões tomadas pela Assembleia Geral obrigam a todos os associados, ainda que ausentes ou discordantes.

Artigo 13 – Compete privativamente à Assembleia Geral:

I. Deliberar sobre todo e qualquer assunto de interesse da ABIA para o qual for convocada.

II. Eleger os membros do Conselho de Administração e do Conselho Fiscal.

III. Destituir os membros do Conselho de Administração e do Conselho Fiscal.

IV. Alterar o presente estatuto social.

V. Deliberar sobre a extinção da ABIA.

VI. Aprovar a Prestação de Contas, incluindo o Relatório de Atividades e Demonstrações Financeiras, formulados pelo Conselho de Administração, que deverão ser previamente aprovadas pelo Conselho Fiscal.

VII. Aprovar a admissão e exclusão de associados, após manifestação do Conselho de Administração.

VIII. Aprovar a Programação e o Orçamento anuais, formulados pelo Conselho de Administração.

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IX. Autorizar a aquisição, alienação e oneração de bens imóveis.

Parágrafo Primeiro – Todas as deliberações, salvo a prevista no parágrafo seguinte, da Assembleia Geral, inclusive as definidas nos incisos III e IV, deverão ser aprovadas pela maioria simples dos votos dos associados presentes.

Parágrafo Segundo – A deliberação quanto à extinção da ABIA e destinação do patrimônio remanescente, prevista no inciso V deste artigo, deverá ser aprovada por 2/3 (dois terços) dos associados presentes à Assembleia Geral especialmente convocada para esse fim.

Artigo 14 – A Assembleia Geral reunir-se-á, ordinariamente, por convocação do Presidente:

I. Anualmente, em até cento e vinte dias após o encerramento do exercício social da ABIA, para, dentre outros assuntos, examinar e aprovar o Relatório de Atividades, o Balanço Patrimonial e as demais demonstrações financeiras e contábeis.

II. A cada três anos, para a eleição dos membros do Conselho de Administração e do Conselho Fiscal.

Artigo 15 – A Assembleia Geral reunir-se-á, extraordinariamente, sempre que se faça necessário, quando convocada:

I. Pelo Presidente;

II. A qualquer tempo, por 1/5 (um quinto) dos associados.

Parágrafo Primeiro – Dentre os assuntos a ser objeto de Assembleia Geral Extraordinária estão:

I. Reforma estatutária.

II. Destituição de membros do Conselho de Administração e do Conselho Fiscal.

III. Dissolução ou liquidação da ABIA.

IV. Julgamento de recurso de exclusão de associado.

Parágrafo Segundo – As deliberações previstas neste artigo, inclusive as que dispuserem sobre os incisos I e II do parágrafo primeiro, deverão ser aprovadas pela maioria simples dos votos dos associados presentes à Assembleia Geral, especialmente convocada para esses fins.

Parágrafo Terceiro – A deliberação quanto à extinção da ABIA e destinação do patrimônio remanescente, prevista no inciso III do parágrafo primeiro, deverá ser aprovada por 2/3 (dois terços) dos associados presentes à Assembleia Geral especialmente convocada para esse fim.

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Artigo 16 – A Assembleia Geral será convocada para fins determinados, mediante prévio e geral anúncio, através de edital afixado na sede da ABIA, por carta enviada aos associados ou qualquer outro meio eficiente, inclusive eletrônicos, com antecedência mínima de cinco dias.

Parágrafo Primeiro – Qualquer Assembleia Geral instalar-se-á, em primeira convocação, com, no mínimo, 2/3 (dois terços) dos associados, e, em segunda convocação, decorridos trinta minutos, com qualquer número.

Parágrafo Segundo – Para melhor gestão operacional, as Assembleias Gerais poderão ser realizadas virtualmente.

Parágrafo Terceiro - Os atos relativos à reforma do Estatuto, para valerem contra terceiros, ficam sujeitos às formalidades de registro e arquivamento nos órgãos competentes.

Seção II – Conselho de Administração

Artigo 17 – O Conselho de Administração é o órgão de gestão estratégica e administração da ABIA, sendo composto por até oito membros, sendo um Presidente, um Vice-Presidente e um Tesoureiro.

Artigo 18 – O Conselho de Administração é eleito em Assembleia Geral, por maioria simples de votos, para um mandato de três anos, sendo permitida a reeleição.

Artigo 19 - Compete ao Conselho de Administração:

I. Definir as diretrizes estratégicas da ABIA, cumprindo suas prioridades.

II. Cumprir e fazer cumprir rigorosamente o Estatuto e as decisões da Assembleia Geral.

III. Deliberar sobre a convocação de Assembleias Gerais.

IV. Nomear e destituir os membros do Conselho Consultivo e de Sustentabilidade.

V. Estabelecer e fiscalizar as normas básicas de funcionamento.

VI. Elaborar o Orçamento Anual da ABIA e autorizar receitas e despesas extraordinárias.

VII. Autorizar investimentos e outros atos jurídicos, que representem ônus ou diminuição patrimonial para a ABIA.

VIII. Fixar a periodicidade e o valor da contribuição mínima a ser paga pelos associados.

IX. Elaborar o Relatório Anual de Atividades e as demonstrações financeiras, submetendo-os, em seguida, à aprovação do Conselho Fiscal e da Assembleia Geral.

X. Nomear os membros do Conselho Supervisor do Fundo Patrimonial e deliberar sobre as demais matérias relativas ao referido Fundo, nos termos deste Estatuto.

XI. Decidir sobre quaisquer matérias que não sejam da competência de outros órgãos ou instâncias da ABIA, inclusive as omissões e interpretações ao presente Estatuto.

Artigo 20 – O Conselho de Administração se reúne ordinariamente uma vez por semestre e, extraordinariamente, sempre que necessário, quando convocado por qualquer um de seus membros ou pelo Conselho Fiscal, sendo suas reuniões presididas pelo seu Presidente.

Parágrafo Único – O Conselho de Administração delibera, validamente, com a presença da maioria simples dos seus membros, sendo vedada a representação, reservado o voto de desempate ao Presidente.

Artigo 21 - Compete ao Presidente:

I. Representar institucionalmente a ABIA, ativa ou passivamente, judicial e extrajudicialmente.

II. Auxiliar nas atividades de mobilização de recursos.

III. Convocar e presidir as reuniões do Conselho de Administração e da Assembleia Geral.

IV. Autorizar pagamentos e movimentação bancária, observadas as diretrizes definidas neste Estatuto.

V. Coordenar, supervisionar e acompanhar as atividades, programas e projetos em realização.

VI. Admitir e demitir os empregados, colaboradores, estagiários e prestadores de serviços a qualquer título e definir as respectivas atribuições.

Parágrafo Único – Compete ao Vice-Presidente substituir o Presidente em suas ausências e impedimentos.

Artigo 22 – Compete ao Tesoureiro:

I. Se responsabilizar pela escrituração patrimonial da ABIA em livros próprios, tendo sob sua guarda e conservação todos os papéis, documentos, títulos e valores de qualquer interesse.

II. Fornecer ao Conselho de Administração balancetes periódicos extraídos da escrituração, bem como informes minuciosos sobre a vida financeira da ABIA.

Artigo 23 – Todos os documentos oficiais da ABIA, incluindo cheques e demais documentos bancários e financeiros, assim como todos os instrumentos contratuais, para serem válidos, deverão ter duas assinaturas, em conjunto, podendo ser:

I. A do Presidente em conjunto com a do Vice-Presidente.

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II. A do Presidente em conjunto com a do Tesoureiro.

III. A do Vice-Presidente em conjunto a do Tesoureiro.

III. A do Vice-Presidente em conjunto com a de um Procurador nomeado pelo Presidente.

IV. A do Tesoureiro em conjunto com a de um Procurador nomeado pelo Presidente.

Parágrafo Único – As procurações deverão ser firmadas, por instrumento público ou particular, pelo Presidente, com firma reconhecida.

Seção III - Conselho Fiscal

Artigo 24 - O Conselho Fiscal é um órgão colegiado, de avaliação, acompanhamento e controle, constituído por três membros, eleitos em Assembleia Geral para um mandato de três anos, permitida a reeleição.

Artigo 25 - Compete ao Conselho Fiscal:

I. Examinar os livros de escrituração da ABIA.

II. Fiscalizar a administração econômica, financeira e contábil, sugerindo ações e diretrizes ao Conselho de Administração, bem como à Assembleia Geral.

III. Emitir parecer sobre o Relatório Anual e as Demonstrações Financeiras apresentadas pelo Conselho de Administração.

IV. Contratar, quando necessário ou conveniente, auditoria externa independente, à custa da ABIA, devendo pronunciar-se sobre o relatório emitido pelos auditores.

V. Requisitar, para análise, a qualquer tempo, documentação comprobatória das operações econômico-financeiras realizadas.

Artigo 26 - O Conselho Fiscal se reunirá ordinariamente uma vez ao ano e, extraordinariamente, sempre que necessário.

Parágrafo Único - As reuniões do Conselho Fiscal deverão ser convocadas com antecedência mínima de cinco dias.

Seção V - Conselho Consultivo e de Sustentabilidade

Artigo 27 - O Conselho Consultivo e de Sustentabilidade, órgão auxiliar do Conselho de Administração, será constituído por número ilimitado de membros, escolhidos entre os associados, ou composto por pessoas

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de notório saber e reconhecimento em suas áreas de atuação, que possam contribuir tecnicamente com o desenvolvimento das finalidades da ABIA.

Parágrafo Único – Os membros do Conselho Consultivo e de Sustentabilidade serão nomeados pelo Conselho de Administração, que poderá destituí-los.

Artigo 28 - Compete ao Conselho Consultivo e de Sustentabilidade:

I. Orientar trabalhos de pesquisas.

II. Opinar em projetos, programas e orçamentos.

III. Colaborar com o aperfeiçoamento das atividades da ABIA.

IV. Auxiliar o Conselho de Administração no planejamento e implementação de ações que objetivem assegurar a sustentabilidade da ABIA.

V. Opinar sobre outras matérias que lhe sejam encaminhadas.

Artigo 29 - O Conselho Consultivo e de Sustentabilidade reunir-se-á anualmente ou sempre que convocado pelo Conselho de Administração.

CAPÍTULO IV – DAS FONTES DE RECURSOS E DO PATRIMÔNIO

Artigo 30 – Constituem fontes de recursos da ABIA:

I. As doações, dotações, legados, heranças, subsídios e quaisquer auxílios que lhe forem concedidos por pessoas físicas ou jurídicas, de direito privado ou de direito público, nacionais ou estrangeiras, bem como os rendimentos produzidos por esses bens e seu patrimônio.

II. As receitas provenientes dos serviços prestados atinentes às suas finalidades.

III. As receitas patrimoniais.

IV. A receita proveniente de contratos administrativos, termos de fomento, termos de colaboração, convênios e termos de cooperação, celebrados com o Poder Público.

V. A receita proveniente de contratos, convênios, parcerias ou acordos celebrados com pessoas jurídicas de direito público ou privado, nacionais ou estrangeiras.

VI. A receita proveniente das contribuições feitas pelos associados.

VII. Verbas provenientes de promoções organizadas pelos associados.

VIII. Recursos provenientes de projetos culturais enquadrados nas leis federais, estaduais e/ou municipais de incentivo à cultura.

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IX. Recursos advindos do recebimento de direitos autorais, conexos e de propriedade intelectual.

X. As receitas advindas da comercialização de produtos afins às atividades institucionais.

XI. Rendimentos financeiros e outras rendas eventuais.

Parágrafo Primeiro – As rendas, recursos, bens, direitos e eventuais resultados operacionais da ABIA serão aplicados integralmente no território nacional, na manutenção e desenvolvimento de seus objetivos institucionais.

Parágrafo Segundo - A ABIA se compromete a manter escrituração de suas receitas e despesas em livros revestidos de formalidades regulamentares capazes de comprovar sua exatidão.

Artigo 31 – O patrimônio da ABIA poderá ser constituído por bens móveis, imóveis, veículos, semoventes, ações e títulos da dívida pública ou privada.

Artigo 32 – No caso de dissolução da ABIA, o respectivo patrimônio líquido será transferido a outra entidade sem fins lucrativos e econômicos, com o mesmo objetivo social, congênere, registrada no Conselho Nacional de Assistência Social (CNAS), ou a entidade pública.

Artigo 33 - O exercício financeiro e fiscal da ABIA coincide com o ano civil.

Artigo 34 - O Fundo Patrimonial da ABIA, composto pelas aplicações financeiras da ABIA, tem por objetivo fortalecer, através de uma política de longo prazo, a sustentabilidade patrimonial da ABIA e a rentabilidade de suas reservas.

Parágrafo Primeiro - O Fundo Patrimonial, inclusive sua política de investimento, rege-se pelo disposto na legislação e regulamentação aplicáveis.

Parágrafo Segundo – Poderá ser constituído um Conselho Supervisor do Fundo Patrimonial com a função específica de acompanhar e supervisionar a administração, gestão e performance do Fundo Patrimonial.

Parágrafo Terceiro - O Conselho Supervisor do Fundo Patrimonial, quando constituído, será composto por três membros, todos com experiência em gestão de recursos de terceiros e indicados pelo Conselho de Administração.

Parágrafo Quarto - Observadas as disposições legais aplicáveis, o Fundo Patrimonial poderá ser usado também como instrumento de captação de recursos para a ABIA, inclusive mediante contribuições a ele destinadas, sendo certo que a sua existência não visa substituir ou diminuir outras fontes de receita da ABIA.

Artigo 35 - A prestação de contas da ABIA observará, no mínimo:

I. Os princípios fundamentais de contabilidade e as Normas Brasileiras de Contabilidade.

II. A publicidade, por qualquer meio eficaz, no encerramento do exercício fiscal, do relatório de atividades e das demonstrações financeiras da entidade, incluindo as certidões negativas de débitos junto ao INSS e FGTS, colocando-os à disposição para o exame de qualquer pessoa jurídica ou cidadão.

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III. A realização de auditoria, inclusive por auditores externos independentes se for o caso, da aplicação de eventuais recursos objeto de Termo de Parceria, observada a legislação aplicável.

IV. A prestação de contas de todos os recursos e bens recebidos de origem pública será feita conforme determina o parágrafo único do artigo 70 da Constituição Federal.

CAPITULO V – DISPOSIÇÕES GERAIS

Artigo 36 - A ABIA será dissolvida por deliberação da Assembleia Geral Extraordinária, especialmente convocada para esse fim, pelo voto concorde de 2/3 (dois terços) dos presentes, quando se tornar impossível a continuação de suas atividades, ou nos casos previstos em Lei.

Parágrafo Único – Em qualquer caso serão observados os dispositivos legais aplicáveis e o fixado no presente Estatuto.

Artigo 37 - Os membros da ABIA e seus empregados difundirão as finalidades e a filosofia da entidade, motivando a participação de outros membros da sociedade civil.

Artigo 38 - Os casos omissos serão resolvidos pelo Conselho de Administração, de acordo com a lei.

Rio de Janeiro, 06 de dezembro de 2016.

Richard Guy Parker
Presidente da Assembleia

Regina Maria Barbosa
Secretária da Assembleia

CARTÓRIO VANELE FALCÃO
21º OFÍCIO DE NOTAS - Vanele Falcão - Tabeliã de Notas
Av. Erasmo Braga nº 255 - RJ - Tel.: (21)2532-2121 11 de Abril de 2017
Reconheço por SEMELHANÇA as firmas de
REGINA MARIA BARBOSA..........
Em testemunho da verdade.
Mat. Jorge José da Silva Filho Escrevente
Emolumentos R$ 5,28 TJ-Fundos R$ 1,88 Total R$ 7,14
ECAR74853-RQE
Consulte em https://www3.tjrj.jus.br/sitepublico

Pedro Carpenter Genescá (OAB/RJ 121.340)

REGISTRO CIVIL DAS PESSOAS JURÍDICAS
DA CIDADE DO RIO DE JANEIRO

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C E R T I D Ã O

O Oficial do Registro Civil das Pessoas Jurídicas do Rio de Janeiro, conforme o art. 19, § 1º, da lei 6015/73 e do art.217 da Lei Civil, CERTIFICA que esta é a cópia fiel da ata da AGO datada de 11/11/2020 e arquivada em 14/12/2020 da ASSOCIAÇÃO BRASILEIRA INTERDISCIPLINAR DE AIDS, constituída neste ofício na matricula nº 92.514, num total de 09 páginas, numeradas e chanceladas digitalmente.

Rodolfo Pinheiro de Moraes
Mat. 90-00.00.00.00.02

Poder Judiciario - TJERJ
Corregedoria Geral de Justiça
Selo de Fiscalização Eletrônico
EDNC 38371 DQY
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Rio de Janeiro, **22 de janeiro de 2021**

Emol.: 153,16 Trib.: 58,79

REPÚBLICA FEDERATIVA DO BRASIL

ESTADO DO RIO DE JANEIRO

AAA 19710179

TERMO DE RESPONSABILIDADE E REQUERIMENTO DE REGISTRO

Requeiro ao Registro Civil de Pessoas Jurídicas o registro da presente documentação da

Pessoa Jurídica: **ASSOCIAÇÃO BRASILEIRA INTERDISCIPLINAR DE AIDS**

Matrícula da PJ: **92514** CNPJ: **29.263.068/0001-45**

Reconheço como verdadeiras todas as informações constantes neste documento, inclusive a autenticidade das assinaturas, sob pena de nulidade do ato, assumindo responsabilidade pessoal nos termos do **art. 14 da Lei 13874/19 e art. 6° §4° do Provimento 62/2018 CGJ publicado no DOJERJ de 20/12/18 pag. 42**.

[x] **Envio a documentação digitalmente com a minha assinatura ICP-BRASIL.**

Requeiro ainda vias impressas na seguinte forma:

OBS: Caso seja optado pelo envio de vias adicionais será cobrado os emolumentos referentes a quantidade de vias para este servico em decorrência do processo.

[ ] **Quantidade de** [ ] **Envio de via por** [ ] **Vou retirar no RCPJ**

Informar o(s) endereço(s) de entrega para o SEDEX ou o(s) e-mails para envio:

Rio de Janeiro, 17 de novembro de 2020

Pedro Carpenter Genesca
OAB/RJ 121.340
Advogado

ICP BRASIL do Advogado, Contador ou Participante do ato (Sócio, Administrador, Presidente, Diretor, Presidente da Assembleia e Testemunhas)

(*) OBS: 1) Em caso de registro de livro PDF as assinaturas digitais caberão aos: Representantes Legais e o Contador.
2) O Registro do documento será feito digitalmente, vias em papel deverão ser solicitadas acima.

REPÚBLICA FEDERATIVA DO BRASIL
AAA 19710180
CERTIDÃO
22/01/2021 EDNC 38371 DQY

ASSOCIAÇÃO BRASILEIRA INTERDISCIPLINAR DE AIDS – ABIA

CNPJ 29.263.068.0001/45

Assembleia Geral Ordinária

Convocação

São convocados os associados da ABIA, a se reunirem em Assembleia Geral Ordinária no dia 11 de novembro de 2020, em primeira convocação às 10:00 e, em segunda convocação, às 10:30 horas, a ser realizada de forma virtual, pela plataforma Zoom, a fim de deliberarem sobre a seguinte pauta:

1 – Apresentação das Demonstrações e relatório de Auditoria do Exercíco de 2019;

2- A situação da ABIA em 2020;

3 - Caminhos e perspectivas em 2021;

4 – Eleição do Conselho de Administração;

5 – Solicitação para compartilhamento de salas;

6 – Solicitação para baixa do imobilizado, doação e descarte.

Rio de Janeiro, 20 de outubro de 2020.

Richard Guy Parker
Diretor Presidente

REPÚBLICA FEDERATIVA DO BRASIL
ESTADO DO RIO DE JANEIRO
AAA 19710181
CERTIDÃO
22/01/2021 EDNC 38371 DQY

Lista de Presença da Assembleia Geral Ordinária da Associação Brasileira Interdisciplinar de AIDS – ABIA

1. Veriano de Souza Terto Júnior
2. Richard Guy Parker
3. Simone Souza Monteiro
4. Regina Maria Barbosa
5. Luis Felipe Rios de Nascimento
6. Fátima Maria Gomes da Rocha
7. Alexandre Domingues Granjeiro
8. Carlos Alberto Ebeling Duarte
9. Kenneth Rochel Camargo Júnior
10. Fernando Seffener
11. Simone Lima

Rio de Janeiro, 11 de novembro de 2020.

Richard Guy Parker
Presidente da Assembleia

Veriano de Souza Terto Júnior
Secretário da Assembleia

Av. Presidente Vargas, 446/13º andar - Centro - CEP- 20071-907 - Rio de Janeiro/RJ - Brasil
Tel.: +55 (21) 2223-1040 - (21) 2223-1185 - (21) 2223-1391
E-mail: abia@abiaids.org.br - Site: www.abiaids.org.br

REPÚBLICA FEDERATIVA DO BRASIL
ESTADO DO RIO DE JANEIRO
AAA 19710182
CERTIDÃO
22/01/2021 EDNC 36371 DQY

RCPJ-RJ 14/12/2020-62
EDNC32286VAC
fl.: 4/9

REPÚBLICA FEDERATIVA DO BRASIL

ATA DA ASSEMBLEIA GERAL ORDINÁRIA DA ASSOCIAÇÃO BRASILEIRA INTERDISCIPLINAR DE AIDS – ABIA - CNPJ 29.263.068.0001/45

Em segunda convocação, às 10:30 horas, do dia 11 de novembro de 2020, por convocação do Diretor Presidente, Sr. Richard Guy Parker, reuniram-se de forma virtual, pela plataforma Zoom, os senhores e senhoras associados da Associação Brasileira Interdisciplinar de Aids - ABIA, devidamente convocados por meio de correio eletrônico, atingindo o quórum estatutário, com a presença de 11 membros, sendo a Assembleia presidida pelo Sr. Richard Guy Parker e secretariada pelo Sr. Veriano de Souza Terto Júnior.

O Sr Richard Guy Parker deu inicio à reunião cumprimentando os presentes e abrindo a discussão da pauta, acerca dos seguintes itens:

1 - Apresentação das Demonstrações e relatório de Auditoria do Exercíco de 2019: A Sra. Simone Lima apresentou para o relatório da auditoria referente ao exercício de 2019 e parecer dos auditores independentes. Após votação, foi aprovada por unanimidade as Demonstrações Financeiras de 2019.

2- A situação da ABIA em 2020: Foi feito informe da situação da ABIA em 2020, com projetos iniciados e concluídos e situação financeira.

3 - Caminhos e perspectivas em 2021: Foi conduzida discussão sobre caminhos e perspectivas para 2021, com iniciativas para a sustentabilidade, possíveis mudanças e adaptações, perspectiva financeira.

4 – Eleição do Conselho de Administração: Foram eleitos, por unanimidade, os seguintes membros para o Conselho de Administração, para o mandato que se iniciará em 16/12/2020 e terminará em 15/12/2023: como Diretor-Presidente, **RICHARD GUY PARKER**, americano, solteiro, antropólogo, residente à Avenida Nossa Senhora de Copacabana, 13/1101, Leme. Rio de Janeiro RJ, CEP: 22.010-120, portador do CPF 017.881.517-98 e da identidade VO 62673-P CGP/DIREX/DPF; como Diretor Vice-Presidente, **VERIANO DE SOUZA TERTO JÚNIOR**, brasileiro, solteiro, psicólogo, residente à Rua Visconde de Figueiredo, nº 72 aptº 301- Tijuca – Rio de Janeiro CEP:20.550-050, portador do CPF 667.972.337-04 e da identidade 06.677.516-4, emitida pelo IFP/RJ; e como Tesoureira, **SIMONE SOUZA MONTEIRO**, brasileira, casada, psicóloga, residente à Rua Visconde de Pirajá, 592/503 – Ipanema, RJ, CEP: 22410-002. Rio de Janeiro, RJ, portadora do CPF 986.589.997-34 e da identidade n. 06210329-6, IFP/RJ. Os demais cargos do Conselho de Administração permanecem vacantes.

Declaração de Desimpedimento: Para fins de cumprimento de exigências legais, o administrador, abaixo assinado, declara, sob as penas da lei, de que não está impedido de exercer a administração da associação, por lei especial, ou em virtude de condenação criminal, ou por se encontrar sob os efeitos dela, a pena que vede, ainda que temporariamente, o acesso a cargos públicos; ou por crime falimentar, de prevaricação, peita ou suborno, concussão, peculato; ou contra a economia popular, contra o sistema financeiro nacional, contra normas de defesa da concorrência, contra as relações de consumo, fé pública ou a propriedade.

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AAA 19710183

5 – Solicitação para compartilhamento de salas: Foi aprovada por unanimidade autorização para o compartilhamento de salas da sede da ABIA com o IBASE, outra associação sem fins lucrativos.

6 – Solicitação para baixa do imobilizado, doação e descarte: Foi aprovada por unanimidade a baixa do ativo imobilizado da ABIA, dos bens móveis abaixo listados, podendo ser descartados ou doados a terceiros:

Nº DO PATRIMÔNIO	DESCRIÇÃO DO BEM	DATA DE AQUISIÇÃO	FINANCIADOR
MU 006	Cadeira tipo secretária s/braços com rodízio estofado preto.	25.04.03	HIVCENTER
MU 014	Mesa medindo 120x60, mobicom com 3 gavetas cinza/preto.	25.04.03	HIV CENTER
MU 026	Mesa medindo 106x60, mobicom, cor cinza.	25.04.03	HIV
MU 034	Cadeira tipo secret. s/braço giratória tecido preto.	05.04.04	EED
MU 049	Mesa de madeira c/03 gavts. c/tampo de 1,20x0,60m	30.06.92	FORD
MU 057	Estante de aço c/06 prateleiras	28.08.92	FORD
MU 058	Estante de aço c/06 prateleiras	28.08.92	FORD
MU 059	Estante de aço c/06 prateleiras	28.08.92	FORD
MU 135	Cadeira estofada, cor preta c/rodízio	23.11.92	MACARTHUR
MU 175	Estante de aço c/06 prateleiras	30.09.92	FORD
MU 220	Mesa em madeira c/03 gavts. medindo 1,30x0,40x0,70m	06.05.93	MACARTHUR
MU 271	Estante de aço c/04 prateleiras	02.02.96	EZE
MU 273	Estante de aço c/06 prateleiras.	02.02.96	EZE
MU 294	Mesa com 3 gavetas 1.20 x 60, cor ovo/preto.	23.09.02	FORD
MU 358	Banqueta alta com 80cm de alt. Cor preta.	22.03.07	ABIA
MU 362	Cadeira giratória tipo secretária fixa preta.	05.06.07	PRISMA
MU 363	Cadeira giratória tipo secretária fixa preta.	05.06.07	PRISMA

CERTIDÃO
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MU 365	Cadeira giratória tipo secretária fixa preta.	05.06.07	PRISMA
MU 366	Cadeira giratória tipo secretária fixa preta.	05.06.07	PRISMA
MU 367	Cadeira giratória tipo secretária fixa preta.	05.06.07	PRISMA
MU 368	Cadeira giratória tipo secretária fixa preta.	05.06.07	PRISMA
MU 369	Cadeira giratória tipo secretária fixa preta.	05.06.07	PRISMA
MU 370	Cadeira giratória tipo secretária fixa preta.	05.06.07	PRISMA
MU 371	Cadeira giratória tipo secretária fixa preta.	05.06.07	PRISMA
MU 372	Cadeira giratória tipo secretária fixa preta.	05.06.07	PRISMA
MU 373	Cadeira giratória tipo secretária fixa preta.	05.06.07	PRISMA
MU 374	Cadeira giratória tipo secretária fixa preta.	05.06.07	PRISMA
MU 375	Cadeira giratória tipo secretária fixa preta.	05.06.07	PRISMA
MU 376	Cadeira giratória tipo secretária fixa preta.	05.06.07	PRISMA
MU 377	Cadeira giratória tipo secretária fixa preta.	05.06.07	PRISMA
MU 378	Cadeira giratória tipo secretária fixa preta.	05.06.07	PRISMA
MU 379	Cadeira giratória tipo secretária fixa preta.	05.06.07	PRISMA
MU 380	Cadeira giratória tipo secretária fixa preta.	05.06.07	PRISMA
MU 381	Cadeira giratória tipo secretária fixa preta.	05.06.07	PRISMA
MU 382	Cadeira giratória tipo secretária fixa preta.	05.06.07	PRISMA
MU 383	Cadeira giratória tipo secretária fixa preta.	05.06.07	PRISMA
MU 384	Cadeira giratória tipo secretária fixa preta.	05.06.07	PRISMA
MU 385	Cadeira giratória tipo secretária fixa preta.	05.06.07	PRISMA
MU 387	Cadeira giratória tipo secretária fixa preta.	05.06.07	PRISMA
MU 388	Cadeira giratória tipo secretária fixa preta.	05.06.07	PRISMA
MU 389	Cadeira giratória tipo secretária fixa preta.	05.06.07	PRISMA

REPÚBLICA FEDERATIVA DO BRASIL
AA 19710185

MU 390	Cadeira giratória tipo secretária fixa preta.	05.06.07	PRISMA
M U 391	Cadeira giratória tipo secretária fixa preta.	05.06.07	PRISMA
MU 392	Cadeira secretária giratória cor preta.	05.06.07	PRISMA
MU 393	Cadeira secretária giratória cor preta.	05.06.07	PRISMA
MU 394	Mesa diretor cor cinza méd. 75x150, c/gaveteiro, pé.	05.06.07	PRISMA
MU 395	Mesa diretor cor cinza méd. 75x150, c/gaveteiro, pé.	05.06.07	PRISMA
MU 397	Armário multi uso cinza méd. 170x75x35.	05.06.07	PRISMA
MU 398	Armário multi uso cinza méd. 170x75x35.	05.06.07	PRISMA
MU 399	Armário multi uso cinza méd. 170x75x35.	05.06.07	PRISMA
MU 400	Arquivo 4 gavetas cinza arte 24.	05.06.07	PRISMA
MU 401	Arquivo móvel	26.11.07	PRISMA
MU 402	Arquivo móvel 2 gavetões.	16.08.07	PRISMA
MU 403	Armário ferramenta 1 porta	16.08.07	PRISMA
MU 404	Mesa tampo quadrado	16.08.07	PRISMA
MU 405	Armário Multi PA25 CZ	02.04.08	PRISMA
MU 406	Armário Multi PA 25 CZ.	02.04.08	PRISMA
MU 409	Armário Multi uso CE N. fiscal 67693	02.06.08	PRISMA
MU 410	Armário Multi uso CE N. fiscal nº 67693	02.06.08	PRISMA
MU 411	Arquivo 4 gavetas CZ. N. fiscal 63855	13.05.08	PRISMA
MU 412	Arquivo 4 gavetas CZ. N. fiscal 63855	13.05.08	PRISMA
MU 413	Armário 25 CZ N. Fiscal 55951	01.04.08	PRISMA
MU 414	Armário 25 CZ N. Fiscal 55951	01.04.08	PRISMA
MU 415	Arquivo 25 CZ N. Fiscal 55951	01.04.08	PRISMA
MU 416	Arquivo 25 CZ N. Fiscal 55951	01.04.08	PRISMA
MU 442	Arquivo deslizante c/28 faces mod. 1.6 ½	21.11.06	EED

REPÚBLICA FEDERATIVA DO BRASIL
AAA 19710186

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MU 444	POLTRONA PRESIDENTE MILANO LUXO EM CREPE	23.05.14	PRISMA
MU 445	CADEIRA EMPILHAVÉL 120 KG 1003 MS SYSTEM	23.05.14	PRISMA
MU 446	CADEIRA GIRATÓRIA 658 MS SYSTEM VENEZA	23.05.14	PRISMA
MU 447	ARQUIVO DE AÇO 4 GAVETAS PARA PASTA SUSPENSA EM CHAPA	23.05.14	PRISMA
MU 448	VENTILADOR DE COLUNA OSCILANTE PREMIUM 60 CM	23.05.14	PRISMA
MU 449	MESA AUXILIAR SEM GAVEYEIRO A 74 CM X L 1,06 CM X 60 CM ALFAMOB GAMA	23.05.14	PRISMA
MU 450	GAVETEIRO DE MESA ALFAMOB LIGTH 15 MM	23.05.14	PRISMA
MU 451	BEBEDOURO ELETRÔNICO NEO MASTERFRIO 110 BRANSCO GRANDE	23.05.14	PRISMA
MU 463	Mesa reta 40 MM 1200x600x740 castanho	08.12.16	UNITAID
MU 464	GAVETEIRO VOLANTE 03 GAVETAS 15MM VINHO	08.12.16	UNITAID
MU 466	Mesa de reunião méd. 2,00 x 1,00m.	08.12.16	UNITAID

Nada mais havendo a tratar, foi encerrada a reunião.

Rio de Janeiro, 11 de novembro de 2020.

Richard Guy Parker
Presidente da Assembleia

Veriano de Souza Terto Júnior
Secretário da Assembleia

REPÚBLICA FEDERATIVA DO BRASIL

AAA 19710187

CERTIDÃO
22/01/2021 EDNC 38371 DQY

RCPJ-RJ 14/12/2020-62
EDNC32286VAC
fl.: 9/9

REPÚBLICA FEDERATIVA DO BRASIL

Registro Civil de Pessoas Jurídicas
Comarca da Capital do Rio de Janeiro
Rua México, 148, 3º andar, Centro
CERTIFICO A AVERBAÇÃO NA MATRÍCULA, PROTOCOLO E DATA ABAIXO
Matr. 92514
202011231132121 14/12/2020

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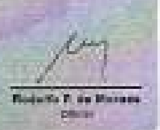

RIO DE JANEIRO

AAA 19710188

CERTIDÃO
22/01/2021 EDNC 38371 DQY

9

PROCURAÇÃO *ad judicia*

ASSOCIAÇÃO BRASILEIRA INTERDISCIPLINAR DE AIDS, pessoa jurídica de direito privado, sem fins lucrativos, constituída na forma da lei, registrada no CNPJ sob o n° 29.263.068/0001-45, com sede na Avenida Presidente Vargas, n° 446, 13° andar, Centro, Rio de Janeiro - RJ, CEP: 20071-907, na pessoa de seu representante nos termos de seu Estatuto Social, por seu Diretor Vice-presidente VERIANO DE SOUZA TERTO JÚNIOR, brasileiro, solteiro, psicólogo, RG n° 06.677.516-4 emitido pelo IFP/RJ, e inscrito no CPF n° 667.972.337-04, e por seu Coordenador de projetos JUAN CARLOS DE LA CONCEPCION RAXACH, cubano, naturalizado brasileiro, solteiro, médico, RG n° 32.847.134-7 emitido pelo DETRAN/RJ, e inscrito no CPF n° 052.549.417-07, vem pelo presente instrumento outorgar procuração ad judicia à advogada SUSANA RODRIGUES CAVALCANTI VAN DER PLOEG, inscrita no CPF 013.497.254-63 e na OAB/MG 181.599, com escritório na Avenida Presidente Vargas, 446, 13º andar, Centro - Rio de Janeiro - RJ, CEP 20071-907, concedendo-lhe poderes da cláusula ad judicia et extra, inclusive substabelecer com reserva de poderes, especificamente para apresentação de subsídio ao exame técnico e/ou processo administrativo de nulidade perante o INPI - Instituto Nacional da Propriedade Industrial relacionado à patente de invenção BR112019022972-8.

Rio de Janeiro, 19 de maio de 2025.

Veriano de Souza Terto Júnior	Juan Carlos de La Concepcion Raxach
Diretor Vice-presidente da Abia	Coordenador de projetos

Av. Presidente Vargas, n° 446 – 13º andar - 20.071 -001 -Rio de Janeiro /RJ . Brasil
Tel.:+55 21 2223 1040 - Fax: +55 21 2253 8495
E-mail: abia@abiaids.org.br - Site: www.abiaids.org.br

4º Oficial de Registro de Títulos e Documentos
e Civil de Pessoa Jurídica da Capital - SP
Robson de Alvarenga

CDT

CERTIDÃO DE INTEIRO TEOR DE ATO ESPECÍFICO
REGISTRO CIVIL DE PESSOA JURÍDICA

CERTIFICO e dou fé que a presente **certidão de inteiro teor de ato específico** foi materializada em folhas devidamente autenticadas e numeradas de 412666 a 412698 com os números de segurança deste Oficial de Registro, e reproduz integralmente apenas o documento original com **32** páginas objeto do **REGISTRO Nº 675220/18.**

Fica expressamente ressalvado que o ato específico reproduzido na presente certidão está vinculado ao **registro primitivo nº 237636/92**, que até o dia anterior à expedição desta certidão sofreu **alteração** em razão da averbação, sendo a última **registrada sob nº 675223/18**, do que dou fé.

A presente certidão tem o mesmo valor probante que o documento original, conforme disposto no art. 217 do Código Civil e no art. 161 da Lei nº 6.015/1973.

São Paulo, 3 de setembro de 2018.

Aroldo Virginio dos Santos - Escrevente

Fabio da Silva Avelar - Substituto do Oficial

Emolumentos	Estado	Ipesp	Registro Civil	Tribunal de Justiça
R$ 123,62	R$ 35,49	R$ 24,12	R$ 6,37	R$ 8,38
Ministério Público	**ISS**	**Condução**	**Outras Despesas**	**Total**
R$ 6,03	R$ 2,59	R$ 0,00	R$ 0,00	R$ 206,60

Para verificar o conteúdo integral do

servicos.cdtsp.com.br/validarcertidao

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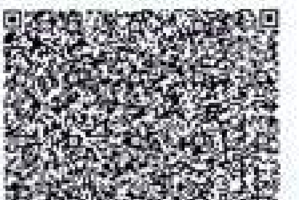

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https://selodigital.tjsp.jus.br

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4º Oficial de Registro de Títulos e Documentos e Civil de Pessoa Jurídica da Comarca de São Paulo

Oficial de Registro: Robson de Alvarenga

Rua Quinze de Novembro, 251 - 5º Andar - Centro
Tel.: (11) 37774040 - Email: contato@4rtd.com.br - Site: www.4rtd.com.br

REGISTRO PARA FINS DE PUBLICIDADE E EFICÁCIA CONTRA TERCEIROS

Nº 675.220 de 28/08/2018

Certifico e dou fé que o documento em papel, foi apresentado em 13/08/2018, o qual foi protocolado sob nº 375.068, tendo sido registrado sob nº **675.220** e averbado à margem do registro nº 237636/92 no Livro de Registro A deste 4º Oficial de Registro Civil de Pessoas Jurídicas da Comarca de São Paulo, na presente data.

Natureza:
ALTERAÇÃO DE ESTATUTO

São Paulo, 28 de agosto de 2018

Marco Aurélio Ribeiro
Substituto do Oficial

Este certificado é parte **integrante e inseparável** do registro do documento acima descrito.

Emolumentos	Estado	Ipesp	Registro Civil	Tribunal de Justiça
R$ 215,06	R$ 61,06	R$ 41,92	R$ 11,28	R$ 14,73
Ministério Público	ISS	Condução	Outras Despesas	Total
R$ 10,37	R$ 4,50	R$ 0,00	R$ 0,00	R$ 358,92

Para verificar o conteúdo integral do documento, acesse o site: servicos.cdtsp.com.br/validarregistro e informe a chave abaixo ou utilize um leitor de qrcode.

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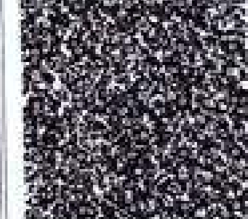

Para verificar a autenticidade do documento, acesse o site da Corregedoria Geral da Justiça: https://selodigital.tjsp.jus.br

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2

ILMO SR. OFICIAL DE REGISTRO CIVIL DE PESSOAS JURÍDICAS DA CAPITAL - SP

Nome do Representante Legal
Ronald Ferreira dos Santos

E-mail	Telefone
info@fenafar.org.br	(11) 32591-191

nacionalidade	estado civil	profissão	portador do RG.
brasileira	casado	farmacêutico	2039575
inscrito no CPF.MF.	**residente à**	**número**	**complemento**
834.631.789-15	rua joão de barro	124	
bairro	**cidade**	**CEP**	**UF**
canasvieiras	florianopolis	88054-620	Santa Catarina

representante legal da pessoa jurídica denominada:
federação nacional dos farmacêuticos

CNPJ*	com sede à	número	complemento
00.679.357/0001-48	rua br de itapetining	255	cj 1105
Bairro	**Cidade**	**CEP**	**UF**
centro	são paulo	01042-001	São Paulo

vem requerer, nos termos do art. 121 da Lei 6.015/73 e da Lei 10.406/02, o registro/averbação do instrumento em anexo, juntando 01 vias de igual teor e forma.

REGISTRO PESSOAS JURÍDICAS 20 AGO 2018 PROTOCOLO - MICROFILME 675220

nestes termos
pede deferimento

São Paulo, 13 de agosto de 2018

assinatura do representante legal

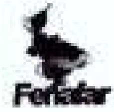
OBS.: (Para preenchimento do requerimento)

1- Não é necessário reconhecer firma no requerimento, se a assinatura estiver compatível;

2- Deve ser assinado pelo representante legal, conforme contrato ou estatuto;

* Campo não obrigatório em caso de constituição.

PRENOTADO
4º RCPJ/SP



ATA DA ASSEMBLEIA CONGRESSUAL EXTRAORDINÁRIA DA FEDERAÇÃO NACIONAL DOS FARMACÊUTICOS - CNPJ 00.679.357/0001-48

Ao 1° dia do mês de agosto de dois mil e dezoito, nas dependências do SESC Praia Formosa, localizado à Rodovia ES-010, Km 35 Norte, s/n, Santa Cruz, município de Aracruz/ES, CEP: 29199-548, às 19:00 horas, teve início o Congresso Extraordinário da FENAFAR, conforme previsto no Edital de Convocação publicado no Diário Oficial da União, em 15 de junho de 2018, Seção 3, fls. 205. Estiveram presentes 34 (trinta e quatro) delegados dos Sindicatos filiados à FENAFAR dos Estados do Ceará, Piauí, Maranhão, Acre, São Paulo, Goiás, Mato Grosso, Amapá, Rio Grande do Sul, Santa Catarina, Paraná, Minas Gerais, Sergipe, Espírito Santo, Roraima, Rio de Janeiro e Paraíba, conforme lista de presença anexa. Assumiu a presidência da Assembleia o presidente da FENAFAR, o farmacêutico Ronald Ferreira dos Santos, que convidou a mim, farmacêutica Maria Maruza Carlesso, Secretária Geral da Entidade, para secretariar os trabalhos. Lido o Edital de Convocação, são os seguintes os pontos de pauta: I - ALTERAÇÕES ESTATUTÁRIAS a seguir especificadas: **1**. Inserção do endereço da sede social no atual art. 2º. **2**. Adequação da redação do atual Artigo 12, Parágrafo primeiro, IV, a fim de excluir o termo "e posse". **3**. Inclusão do inciso "VII" ao atual Artigo 26, com a seguinte redação: "VII - Dar posse à diretoria e conselho fiscal eleitos para o mandato consecutivo"; **4**. Dar nova redação ao atual Artigo 26, I, para constar: "representar a FENAFAR em Juízo ou fora dele podendo delegar poderes a outro diretor, ou ainda, nomear mandatário por instrumento de procuração, se necessário for, para o desempenho de funções técnicas, burocráticas e ou administrativas da entidade"; **5**. Dar nova redação ao atual Artigo 49, para constar: "A posse dos membros da Diretoria e do Conselho Fiscal ocorrerá na data do início do mandato para o qual foram eleitos". **6**. Excluir do Estatuto o atual texto do parágrafo único do Artigo 49; **7**. Criação de novos cargos de Diretoria com sua inserção no atual art. 23 e respectivas atribuições, e também, o modo de constituição e ocupação desses cargos e exercício do primeiro mandato. **8**. Adequação dos atuais Arts. 12 a 15 do Estatuto ao Art. 54, V, da Lei 10.406/02 (Código Civil), para descrever o modo de constituição e funcionamento do Congresso. **9**. Adequação textual de todos os dispositivos necessários e renumeração de artigos em conformidade com as alterações deliberadas consoante os objetos acima especificados. II – Outros assuntos de interesse geral.

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Feitas essas colocações, o presidente passou a expor o fato constante do item "I" da pauta, relembrando que a necessidade de alterações já foi detectada em anteriores reuniões de diretoria da Entidade, tendo sido inclusive criada uma Comissão para estudo do estatuto. Assim, passou a expor a proposta de Reforma do Estatuto, conforme o texto do Edital acima reproduzido. Esclareceu também a necessidade de se ampliar a Diretoria da entidade em razão da ampliação de sua base e que haverá inclusão e previsão nas disposições transitórias.

Foram prestados os esclarecimentos e encaminhado para votação em plenário os seguintes termos para alteração do estatuto, inclusive a revogação do parágrafo único do Art. 49 e a ampliação da diretoria com a criação dos cargos de Primeiro Diretor de Relações Institucionais, Primeiro Diretor de Relações Internacionais, Primeiro Diretor de Organização Sindical e Primeiro Diretor de Assuntos Jurídicos, com as suas respectivas atribuições, o modo de constituição e ocupação desses cargos e exercício do primeiro mandato:

"Art. 2° - A Federação Nacional dos Farmacêuticos tem sua sede jurídica e administrativa em São Paulo/SP, à Rua Barão de Itapetininga, 255, 11° andar, Cj. 1105, Centro, CEP: 01055-900, e sua jurisdição em todo o território nacional, ficando a critério do Conselho de Representantes, criar subsedes regionais e ou transferir a sede para a Capital Federal".

"Art. 12 - O Congresso do FENAFAR é o órgão máximo de deliberação da categoria farmacêutica, sendo composto por delegados eleitos na sua base e observadores convidados:

I - Cada entidade sindical filiada poderá se fazer representar por delegados e observadores, com igual direito a voz, cabendo somente aos delegados eleitos o direito a voto.

II – O número de delegados de cada entidade sindical filiada deverá ser proporcional ao número de farmacêuticos inscritos no Conselho Regional de Farmácia de sua base até 31 de dezembro do ano anterior ao CONGRESSO da FENAFAR, sendo garantido 5 (cinco) delegados para cada 2000 (dois mil) farmacêuticos inscritos no Conselho Regional de Farmácia respectivo, e, mais 1 (um) delegado para cada fração superior a 2000 (dois mil) farmacêuticos inscritos no Conselho Regional de Farmácia da sua base;

III - Cada entidade sindical filiada poderá eleger para o CONGRESSO da FENAFAR delegados suplentes na proporção de 20% (vinte por cento) do número máximo de delegados titulares;

IV - Não haverá restrição à quantidade de observadores por entidade sindical filiada.

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V – Os delegados deverão ser credenciados perante a secretaria do Congresso, ficando garantido ao delegado suplente sua inscrição no caso de não comparecimento ou impedimento do delegado titular;

Parágrafo primeiro (...):

(...)

IV- Eleição da Diretoria da FENAFAR e Conselho Fiscal, conforme normas estabelecidas no Regulamento Eleitoral".

"Art. 13 – (...)

§ 1º- O Congresso, que será dirigido pelo Presidente da FENAFAR, sendo substituído em seus impedimentos pelo Primeiro Vice-presidente e assim sucessivamente, observará as competências privativas definidas no Art. 12 deste Estatuto, não necessariamente na ordem que estão elencadas através de seus incisos, podendo ser dispostas de modo diverso, mediante aprovação na plenária de início do mesmo, salvo a eleição da Diretoria Executiva, Conselho Fiscal e Suplentes, que terão que obedecer a data e hora estipulados em edital designado para esse fim.

§ 2º - Durante os trabalhos eleitorais, o Congresso será presidido pelo Presidente da Comissão Eleitoral, sendo substituído em seus impedimentos pelo primeiro secretário da comissão eleitoral, e, no impedimento deste, pelo segundo secretário da comissão eleitoral.

§ 3º - Outras atividades pertinentes aos objetivos sociais da FENAFAR, tais como eventos sociais e culturais, palestras e debates, também poderão ocorrer no Congresso, conforme deliberado na plenária de início do mesmo".

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"Art. 15 – (...).

§ 1º – A publicização do Edital que convoca o Congresso da FENAFAR será subscrita pelo Presidente da Federação e deverá conter a pauta, o local, o período e horário de sua realização, o qual será publicado no Diário Oficial da União com antecedência de 6 (seis) meses de sua realização;

§ 2º - Em caráter extraordinário a antecedência mínima será de 30 (trinta) dias;

§ 3º - O Congresso só poderá se instalar com a presença de delegações de no mínimo 50% (cinquenta por cento) das entidades filiadas em primeira convocação, ou, com o

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número de presentes em segunda convocação, devendo as deliberações do CONGRESSO da FENAFAR ser tomadas por maioria simples dos presentes.

"Art. 23 - A Diretoria é composta por membros efetivos e suplentes assim distribuídos:

(...)

XI - Primeiro Diretor de Relações Institucionais;

(...)

XIII – Primeiro Diretor de Relações Internacionais;

(...)

XV - Primeiro Diretor de Organização Sindical;

(...)

XIX - Primeiro Diretor de Assuntos Jurídicos;

(...)

"Art. 26 - Compete ao Presidente:

I- representar a FENAFAR em Juízo ou fora dele podendo delegar poderes a outro diretor, ou ainda, nomear mandatário por instrumento de procuração, se necessário for, para o desempenho de funções técnicas, burocráticas e ou administrativas da entidade;

(...)

VII – Dar posse à diretoria e conselho fiscal eleitos para o mandato consecutivo;

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"Art. 34 A – Compete ao Primeiro Diretor de Relações Institucionais:

I – Auxiliar o Diretor Relações Institucionais em todas as suas atividades e para as quais for designado e substituí-lo em suas ausências";

"Art. 35 A – Compete ao Primeiro Diretor de Relações Internacionais:

I – Auxiliar o Diretor Relações Internacionais em todas as suas atividades e para as quais for designado e substituí-lo em suas ausências";

"Art. 36 A – Compete ao Primeiro Diretor de Organização Sindical:

I – Auxiliar o Diretor de Organização Sindical em todas as suas atividades e para as quais for designado e substituí-lo em suas ausências";

"Art. 38 B Caberá ao Primeiro Diretor de Assuntos Jurídicos:

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I – Auxiliar o Diretor de Assuntos Jurídicos em todas as suas atividades e para as quais for designado e substituí-lo em suas ausências";

"Art. 49 - A posse dos membros da Diretoria e do Conselho Fiscal ocorrerá na data do início do mandato para o qual foram eleitos";

"DISPOSIÇÕES TRANSITÓRIAS

Art. 1º - Excepcionalmente o primeiro mandato para os cargos de Primeiro Diretor de Relações Institucionais, Primeiro Diretor de Relações Internacionais, Primeiro Diretor de Organização Sindical e Primeiro Diretor de Assuntos Jurídicos será exercido por Diretores indicados pela Plenária do 9º Congresso da FENAFAR, convocado para os dias 02, 03 e 04 de agosto de 2018, em Aracruz/ES, e aprovados pela mesma, vindo a ser empossados na primeira reunião do Conselho de Representantes que ocorrer após o registro deste estatuto no órgão competente, com término do mandato coincidente com o da Diretoria eleita durante o referido Congresso".

Submetida em plenário para votação a proposta de alteração do Estatuto, restou aprovada por unanimidade dos presentes, não havendo voto contrário nem abstenções. A plenária também autoriza que se proceda a consolidação e renumeração do estatuto, bem como a dar redação às disposições transitórias para incluir o que restou aprovado no item "I" da pauta. Passado para segundo da pauta, "II - Outros assuntos de interesse geral", o Presidente esclarece que o Conselho de Representantes foi procurado em carater de urgência pela Comissão organizadora do 9º Congresso da Fenafar e pela Presidente da Comissão Eleitoral a farmacêutica Fabiola Cristiane de Macêdo Mota que informaram a ausência dos seguintes membros da Comissão Eleitoral: Jose Vilmore Junior; Albano do Valle Verona; Gilmario Cesar Sousa de Carvalho que não conseguirão se fazer presentes no 9º Congresso da Fenafar e a renúncia de Andre Nunes Cavalcante, e que para o prosseguimento dos trabalhos eleitorais existe a necessidade de recomposição de ao menos dois nomes para integrarem a referida Comissão Eleitoral. Informou também que o Conselho de Representantes aprovou a indicação das farmacêuticas Ratsa Ferraz Aguiar e Ernestina Rocha de Sousa e Silva para essa recomposição e assim integrarem a Comissão Eleitoral juntamente com as farmacêuticas Fernanda Mazzini e Fabiola Cristiane de Macêdo Mota, membros da Comissão Eleitoral ora presentes, com a finalidade de dar sequência nos trabalhos eleitorais. Após alguns esclarecimentos, restou deliberada a ratificação dos citados atos do Conselho de Representantes, o que restou aprovado por unanimidade dos presentes.

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E, não havendo mais nada a tratar, a reunião foi encerrada, cabendo a mim Maria Maruza Carlesso, Secretaria Geral lavrar esta ata que também contará com a assinatura do Presidente Ronald Ferreira dos Santos.

Aracruz, 1° de agosto de 2018.

Maria Maruza Carlesso
Secretaria Geral

Ronald Ferreira dos Santos
Presidente

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4º Oficial de Registro de Títulos e Documentos

ESTATUTO DA FEDERAÇÃO NACIONAL DOS FARMACÊUTICOS

CAPÍTULO I

DA ENTIDADE, SEUS FINS, SEDE E DURAÇÃO

Art. 1º - A Federação Nacional dos Farmacêuticos, entidade Sindical de 2º grau, também denominada FENAFAR, fundada em 25 de outubro de 1974 e reconhecida pela Carta Sindical outorgada pelo Ministério do Trabalho em 1 de outubro de 1981 (Mtb - 11.448/75, Mtb - 318-408/80), é uma entidade autônoma, de natureza civil, sem fins lucrativos, desvinculada do Estado e de duração indeterminada, que representa o conjunto dos trabalhadores da categoria dos Farmacêuticos, independentemente das suas convicções políticas, partidárias e religiosas.

Art. 2º - A Federação Nacional dos Farmacêuticos tem sua sede jurídica e administrativa em São Paulo/SP, à Rua Barão de Itapetininga, 255, 11º andar, Cj. 1105, Centro, CEP: 01055-900, e sua jurisdição em todo o território nacional, ficando a critério do Conselho de Representantes criar subsedes regionais e ou transferir a sede para a Capital Federal.

Ar. 3º - A Federação Nacional dos Farmacêuticos tem por finalidades precípuas a união, a defesa de direitos e interesses da categoria e a assistência a seus Sindicatos filiados,

Art. 4º - A Federação Nacional dos Farmacêuticos tem por objetivos precípuos:

I - Congregar e representar os sindicatos farmacêuticos e seus filiados em todo o País;

II - Expressar as reivindicações e lutas dos Farmacêuticos nos planos educacional, econômico, social, cultural e político;

III - Defender condições adequadas para o bom desempenho do trabalho do profissional Farmacêutico, condizentes com sua formação superior;

IV - Incentivar a participação dos filiados nas reuniões, congressos e demais atividades inerentes à Entidade;

V - Fortalecer e estimular a organização da categoria, respeitando sua autonomia, nos limites deste Estatuto;

VI - Coordenar e unificar o movimento dos Farmacêuticos nas iniciativas de alcance nacional, respeitando as dinâmicas regionais e setoriais;

VII - Buscar a Integração com movimentos e entidades nacionais e internacionais dos Farmacêuticos, dos trabalhadores em geral e de outros setores, na luta pela democracia e pelos interesses do povo brasileiro;

VIII - Defender a saúde enquanto bem público, conceituada nos termos da Constituição Federal e garantida mediante políticas sociais de alimentação, habitação, moradia, acesso e posse da terra, visando assegurar através de sua promoção e proteção o exercício pleno da cidadania;

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IX - Defender o direito à assistência farmacêutica entendida como o conjunto de atividades e pesquisa, produção, distribuição, armazenamento e dispensação, que garanta o acesso qualitativo e quantitativo da população ao medicamento, assim como a sua farmacovigilancia e farmacoepidemiologia;

X - Celebrar convênios com entidades sindicais, órgãos públicos ou privados, com vistas à integração de recursos de esforços, visando a execução dos objetivos expressos neste estatuto;

XI - Prestar assistência jurídica, celebrar dissídios e assinar acordos coletivos de trabalho nas unidades federativas onde não houver Sindicato;

XII - Promover ações de defesa e proteção ao meio ambiente, ao consumidor, à ordem econômica, ao patrimônio artístico, estético, histórico, turístico e paisagístico;

XIII - Promover e organizar estudos, pesquisas, seminários, congressos, palestras, cursos profissionalizantes de qualificação e requalificação profissional, isoladamente ou em convênio com outras entidades da categoria, órgãos governamentais e não governamentais, universidades e demais segmentos da sociedade a fim de:

a - colaborar com a atualização dos conhecimentos dos profissionais sobre temas inerentes às ciências farmacêuticas e demais áreas da saúde;

b - colaborar com o estudo e solução dos problemas que se relacionem direta ou indiretamente com a categoria e o profissional farmacêutico;

c - colaborar com a conscientização e conhecimento dos profissionais sobre o conjunto dos projetos políticos, econômicos, sociais e culturais existentes;

d - colaborar com estudos sobre as relações de trabalho, especialmente as questões sindicais e trabalhistas;

Art. 5° - Constituem prerrogativas e deveres da Federação Nacional dos Farmacêuticos de acordo com este Estatuto:

I - Representar perante as autoridades administrativas e judiciárias os interesses gerais da categoria e os interesses de seus filiados.

II - Estabelecer contribuições financeiras para todos os filiados de acordo com as decisões tomadas no CONGRESSO DA FEDERAÇÃO NACIONAL DOS FARMACEUTICOS, denominado CONGRESSO DA FENAFAR e ou CONSELHO DE REPRESENTANTES.

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CAPÍTULO II

DOS FILIADOS, SEUS DIREITOS E DEVERES

Art. 6° - A todo Sindicato de Farmacêutico no Território Nacional, satisfazendo as exigências da legislação em vigor e do presente Estatuto, assiste o direito de filiar-se à FENAFAR.

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Art. 7° - Para filiar-se à FENAFAR. o sindicato encaminhará a solicitação à diretoria da Federação, acompanhada de ata de assembleia, carta sindical ou registro do Estatuto da entidade em cartório, número de associados inscritos, nominata da direção mencionando o

respectivo período de mandato.

Art. 8° - Até 30 (trinta) dias após o recebimento do pedido de filiação, a diretoria da FENAFAR aprovará sua filiação de acordo com os requisitos do art. 7°.

Art. 9 - A desfiliação de um sindicato da FENAFAR se dará por deliberação de sua assembleia geral, lavrada em ata própria, na forma que dispuser o respectivo estatuto, não cabendo à Diretoria da FENAFAR o julgamento de mérito da petição e vigorará a partir da entrada da comunicação na secretaria da Federação, contra recibo.

Art. 10 - São direitos dos sindicatos filiados:

I - gozar dos direitos oferecidos pela FENAFAR;

II - solicitar e receber da diretoria da FENAFAR, do Conselho de Representantes e do Congresso da FENAFAR as medidas que julgar necessárias para defender seus interesses e de seus associados. Incluem-se aqui prestação de ajuda material, financeira, jurídica e social, quando for o caso, e o apoio as suas iniciativas e reinvindicações inclusive promovendo a solidariedade nacional e internacional da categoria, desde que não contrarie deliberação adotada pelo Congresso da FENAFAR e pelo Conselho de Representantes, ou este estatuto;

III - desfilar-se da FENAFAR, obedecida as exigências do Art.9°;

IV – participar de todas as atividades e instancias decisórias da FENAFAR nos termos deste estatuto;

V - ser informado regularmente das decisões adotadas pela entidade, assim como das atividades desenvolvidas e programadas;

VI - recorrer de decisões à instância superior, na forma deste Estatuto;

VII - votar e ser votado através de seus representantes e delegados nos organismos da entidade, na forma desde estatuto;

Art. 11 - São deveres dos Sindicatos filiados:

I- prestigiar a FENAFAR por todos os meios ao seu alcance, cumprindo e fazendo cumprir este estatuto, acatando as deliberações dos Congressos da FENAFAR e de seu Conselho de Representantes, zelando pela unificação do movimento sindical dos farmacêuticos e dos trabalhadores em geral;

II - participar do Conselho de Representantes da FENAFAR, por Intermédio de seus delegados, através de intercâmbio constante e permanente, comparecendo às plenárias e reuniões para as quais for convocado;

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III - executor em sua base territorial, os planos de trabalho conjunto propostos e aprovados pelo plenário do Conselho de Representantes da FENAFAR ou Congresso de caráter nacional da categoria, procurando também entrosar-se com a Federação e os demais sindicatos filiados, nos planos de âmbito regional;

IV - recolher à FENAFAR a anuidade de filiado, conforme deliberação, valor e prazo definido pelo Congresso da FENAFAR e/ou Conselho de Representantes:

V - informar anualmente à FENAFAR os nomes e endereços profissionais e residenciais de seus delegados representantes, coordenadores e presidentes;

VI - manter atualizado o cadastro dos farmacêuticos em sua base e repassá-lo à FENAFAR;

VII - informar a federação sobre suas atividades, eleições e deliberações de suas instâncias;

CAPÍTULO III

DO CONGRESSO DA FEDERAÇÃO NACIONAL DOS FARMACÊUTICOS

Art. 12 - O Congresso do FENAFAR é o órgão máximo de deliberação da categoria farmacêutica, sendo composto por delegados eleitos na sua base e observadores convidados:

I - Cada entidade sindical filiada poderá se fazer representar por delegados e observadores, com igual direito a voz, cabendo somente aos delegados eleitos o direito a voto.

II – O número de delegados de cada entidade sindical filiada deverá ser proporcional ao número de farmacêuticos inscritos no Conselho Regional de Farmácia de sua base até 31 de dezembro do ano anterior ao CONGRESSO da FENAFAR, sendo garantido 5 (cinco) delegados para cada 2000 (dois mil) farmacêuticos inscritos no Conselho Regional de Farmácia respectivo, e, mais 1 (um) delegado para cada fração superior à 2000 (dois mil) farmacêuticos inscritos no Conselho Regional de Farmácia da sua base;

III - Cada entidade sindical filiada poderá eleger para o CONGRESSO da FENAFAR delegados suplentes na proporção de 20% (vinte por cento) do número máximo de delegados titulares;

IV - Não haverá restrição à quantidade de observadores por entidade sindical filiada.

V – Os delegados deverão ser credenciados perante a secretaria do Congresso, ficando garantido ao delegado suplente sua inscrição no caso de não comparecimento ou impedimento do delegado titular;

Parágrafo primeiro - o Congresso da FENAFAR delibera sobre:

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I- Alterações deste Estatuto;

II - A linha política e organizativa da Entidade;

III - Os planos de ação sindical e de luta para os trabalhadores representados considerando a situação político, social e econômico do país;

IV- Eleição da Diretoria da FENAFAR e Conselho Fiscal, conforme normas estabelecidas no Regulamento Eleitoral.

Parágrafo segundo: é dever do Congresso da FENAFAR promover a solidariedade nacional e internacional da classe trabalhadora;

Art. 13 - O regimento interno do Congresso deverá ser elaborado por uma comissão designada pelo Conselho de Representantes e aprovado na plenária de início do mesmo.

§ 1º- O Congresso, que será dirigido pelo Presidente da FENAFAR, sendo substituído em seus impedimentos pelo Primeiro Vice-presidente e assim sucessivamente, observará as competências privativas definidas no Art. 12 deste Estatuto, não necessariamente na ordem que estão elencadas através de seus incisos, podendo ser dispostas de modo diverso, mediante aprovação na plenária de início do mesmo, salvo a eleição da Diretoria Executiva, Conselho Fiscal e Suplentes, que terão que obedecer a data e hora estipulados em edital designado para esse fim.

§ 2º - Durante os trabalhos eleitorais, o Congresso será presidido pelo Presidente da Comissão Eleitoral, sendo substituído em seus impedimentos pelo primeiro secretário da comissão eleitoral, e, no impedimento deste, pelo segundo secretário da comissão eleitoral.

§ 3º - Outras atividades pertinentes aos objetivos sociais da FENAFAR, tais como eventos sociais e culturais, palestras e debates, também poderão ocorrer no Congresso, conforme deliberado na plenária de início do mesmo.

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Art. 14 - A pauta e data do Congresso serão definidas pelo Conselho de Representantes da FENAFAR que designará uma comissão organizadora para auxiliar a diretoria nos encaminhamentos necessários,

Art. 15 - O Congresso reunir-se-á ordinariamente trienalmente, ou em caráter extraordinário quando convocado por dois terços dos membros do Conselho de Representantes.

§ 1º – A publicização do Edital que convoca o Congresso da FENAFAR será subscrita pelo Presidente da Federação e deverá conter a pauta, o local, o período e horário de sua realização, o qual será publicado no Diário Oficial da União com antecedência de 6 (seis) meses de sua realização;

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§ 2º - Em caráter extraordinário a antecedência mínima será de 30 (trinta) dias;

§ 3º - O Congresso só poderá se instalar com a presença de delegações de no mínimo 50% (cinquenta por cento) das entidades filiadas em primeira convocação, ou, com o número de presentes em segunda convocação, devendo as deliberações do CONGRESSO da FENAFAR ser tomadas por maioria simples dos presentes.

CAPITULO IV

DO CONSELHO DE REPRESENTANTES DA FEDERAÇÃO NACIONAL DOS FARMACÊUTICOS

Art. 16 - O Conselho de Representantes da Federação Nacional dos Farmacêuticos é a segunda instância deliberativa.

Parágrafo único - O Conselho de Representantes (CR) é composto por um representante de cada sindicato filiado conforme seu estatuto.

Art. 17· São atribuições do Conselho de Representantes:

I - Implantar e cumprir as deliberações do Congresso;

II - Regulamentar, quando necessário, as deliberações do Congresso;

III - Examinar e apresentar parecer ao Congresso dos relatórios financeiros, prestações de contas e previsões orçamentárias apresentadas pela Diretoria.

IV - Decidir sobre os recursos interpostos às decisões da Diretoria;

V - Convocar o Congresso, com antecedência mínima de 6 meses de sua realização, definindo a data e temário do mesmo;

VI - Aplicar penalidades de suspensão aos filiadas da FENAFAR;

VII- As penalidades serão aplicadas quando o filiado se contrapor às normas e objetivos deste estatuto, bem como às deliberações do Conselho de Representantes e de seu Congresso;

VIII - Propor a criação de comissões ou grupos de trabalho permanentes ou temporários, sobre questões de interesse da categoria e da população em geral, indicando seus componentes;

IX - Alterar o valor e forma da cobrança da anuidade de seus filiados, definida na última reunião anual do Conselho de Representantes para o próximo exercício;

X - Elaborar o Regulamento Eleitoral da Federação Nacional dos Farmacêuticos, eleger uma Comissão eleitoral, que é responsável pelo processo eleitoral de acordo com o previsto neste estatuto.

XI - Aprovar alterações aditivas e complementares ao Estatuto Social, desde que não contrariem as deliberações do Congresso ou qualquer dispositivo deste estatuto.

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Parágrafo único: A deliberação da proposta depende do apoio de maioria absoluta de seus membros, devendo ser aprovado por 2/3 dos presentes, em reunião convocada especialmente para este fim.

CAPITULO V

DO CONSELHO FISCAL DA FEDERAÇÃO NACIONAL DOS FARMACÊUTICOS

Art. 18 - O Conselho Fiscal da FENAFAR será composto por três farmacêuticos efetivos e três suplentes eleitos na forma prevista no Art. 12, com mandato coincidente ao da diretoria.

Art. 19 - Compete ao Conselho Fiscal:

I - dar parecer sobre o orçamento do FENAFAR para o exercício financeiro.

II - opinar sobre as despesas extraordinárias, sobre os balancetes mensais e sobre o balanço anual.

III - reunir-se semestralmente coincidindo com a realização da reunião do Conselho de Representantes;

IV - dar parecer sobre o balanço do exercício financeiro.

V - examinar e apresentar parecer sobre os relatórios financeiros, prestação de contas e previsões orçamentárias apresentadas pela diretoria.

CAPITULO VI

DA DIRETORIA DA FEDERAÇÃO NACIONAL DOS FARMACÊUTICOS

Art. 20 - A Diretoria é o órgão executivo da FENAFAR.

Art. 21 - À Diretoria, coletivamente compete:

I - Representar a Entidade e defender os interesses da categoria perante os poderes públicos, empresas estatais e privadas, fundações e autarquias podendo a diretória nomear mandatários por procuração, de acordo com o inciso I do artigo 5°;

II - Cumprir e fazer cumprir este estatuto, os regimentos e as normas administrativas da FENAFAR, bem como as decisões do Congresso da FENAFAR e do Conselho de Representantes;

III - Gerir o patrimônio, garantindo sua utilização para cumprimento deste estatuto e das deliberações do Congresso e do Conselho de Representantes:

IV - Organizar os serviços administrativos internos da FENAFAR;

V - Elaborar relatórios financeiros, prestações de contas e previsão orçamentária anuais da FENAFAR, remetendo ao Conselho Fiscal;

VI - Convocar as reuniões ordinárias e extraordinárias do Conselho de Representantes;

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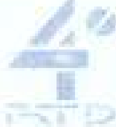


VII - Constituir comissões, coordenações e grupos de trabalho permanente e temporários sobre quaisquer assuntos, indicando seus componentes;

VIII - Remeter à reunião do Conselho de Representantes, parecer do pedido de filiação do Sindicato, como disposto no Artigo 7º;

Art. 22 - O mandato da Diretoria será de 3 (três) anos, eleita pelo Congresso da FENAFAR.

Parágrafo único: serão permitidas reeleições de qualquer membro da diretoria a qualquer cargo.

Art. 23 - A Diretoria é composta por membros efetivos e suplentes assim distribuídos:

I - Presidente:

II - Primeiro Vice-Presidente;

III - Segundo Vice-Presidente:

IV - Secretario Geral;

V - Primeiro Secretário;

VI - Tesoureiro Geral;

VII - Primeiro Tesoureiro;

VIII - Diretoria de Comunicação;

IX - Diretoria de Formação Sindical;

X - Diretoria de Relações Institucionais;

XI - Primeiro Diretor de Relações Institucionais;

XII - Diretoria de Relações Internacionais;

XIII – Primeiro Diretor de Relações Internacionais;

XIV - Diretoria de Organização Sindical;

XV - Primeiro Diretor de Organização Sindical;

XVI - Diretoria da Mulher;

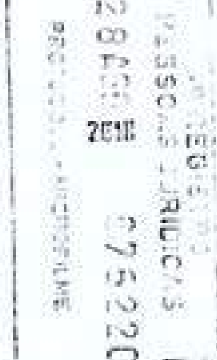
XVII - Diretoria de Educação;

XVIII – Diretoria de Assuntos Jurídicos;

XIX - Primeiro Diretor de Assuntos Jurídicos;

XX – Diretoria de Saúde e Segurança do Trabalho;

XXI – Diretoria de Relações Trabalhistas;



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XXII – Diretoria de Juventude e Direitos Humanos;

XXIII – Diretoria da Região Norte;

XXIV – Diretoria da Região Nordeste;

XXV – Diretoria da Região Centro-Oeste;

XXVI – Diretoria da Região Sudeste;

XXVII – Diretoria da Região Sul;

Parágrafo primeiro - os suplentes para a Diretoria serão em número de 7 (sete);

Parágrafo segundo - é vedada a acumulação de cargos na Diretoria;

Parágrafo terceiro - os suplentes poderão assumir outras funções pela diretoria e/ou Conselho da FENAFAR.

Parágrafo quarto - Os diretores devem participar e representar a entidade nas atividades que tenham sido convidados, sempre se orientando nos princípios da unidade dos trabalhadores e do fortalecimento das entidades sindicais.

Art. 24 - A Diretoria se reúne:

I- Ordinariamente, duas vezes ao ano;

II – Extraordinariamente, quando convocada pelo presidente ou por 1/3 (um terço) de seus membros, em data e local fixados.

Art. 25 - As deliberações da Diretoria são adotadas por maioria simples de votos.

Art. 26 - Compete ao Presidente:

I- representar a FENAFAR em Juízo ou fora dele podendo delegar poderes a outro diretor, ou ainda, nomear mandatário por instrumento de procuração, se necessário for, para o desempenho de funções técnicas, burocráticas e ou administrativas da entidade;

II - Abrir, instalar e presidir o Congresso, o Conselho de Representantes e as reuniões da Diretoria:

III - Convocar as eleições para a nova Diretoria de acordo com o previsto no Artigo 41;

IV Abrir, rubricar e encerrar os livros do FENAFAR;

V - Assinar a correspondência oficial da FENAFAR e, juntamente com o Secretário Geral, toda correspondência que estabeleça quaisquer obrigações para a FENAFAR;

VI - Movimentar com o tesoureiro em exercício as contas da FENAFAR;

VII – Dar posse à diretoria e conselho fiscal eleitos para o mandato consecutivo;

Art. 27 - Compete aos Vice-Presidentes:

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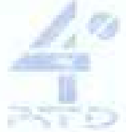

I - Auxiliar o Presidente em todas as suas atividades e para os quais forem designados;

II - Executar todas as atribuições que lhes forem outorgadas pela Diretoria;

Parágrafo primeiro: além das funções atribuídas pelo artigo 27, incisos I e II, será de responsabilidade do segundo Vice-presidente assumir os assuntos de ordem parlamentar;

Parágrafo segundo: no caso de afastamento definitivo, vacância e impedimento do Presidente, assume o 1° Vice-presidente a presidência, o 2° Vice-presidente assume a 1° Vice-presidência, e o 1° suplente assume a Vice-presidência.

Art. 28 - Compete ao Secretário Geral:

I - Ter sob sua guarda e responsabilidade o arquivo da Secretaria;

II - Secretariar as reuniões da Diretoria e do Conselho de Representantes;

III - Encarregar-se do expediente e da correspondência da FENAFAR;

Art. 29 - Compete ao Primeiro Secretário, além de assumir a Secretaria Geral, no caso de falta e/ou impedimento do Secretário Geral, cooperar em todas as atividades inerentes ao cargo;

Parágrafo único: no caso de vacância, impedimento ou afastamento definitivo do Secretário geral, compete do Primeiro Secretário assumir a Secretaria Geral e ao suplente assumir a Primeira Secretaria.

Art. 30 Compete ao Tesoureiro Geral:

I - Ter sob sua responsabilidade e guarda os bens e valores da FENAFAR.

II - Ser responsável pelos recebimentos e pagamentos das despesas.

III - Assinar, com o presidente, os cheques para pagamentos em geral.

IV - Movimentar, com o Presidente, as contas bancárias da FENAFAR.

V - Organizar o balancete semestral e o balanço anual.

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Parágrafo único – caso o Tesoureiro se afaste definitivamente, deverá apresentar o balanço ao Presidente dentro de 30 (trinta) dias após seu afastamento;

Art. 31 - Compete ao Primeiro Tesoureiro assumir a Tesouraria geral, no caso de falta e/ou impedimento do Tesoureiro geral e cooperar em todas as atividades inerentes ao cargo.

Parágrafo único: No caso de vacância, impedimento ou afastamento definitivo do Tesoureiro Geral, compete ao Primeiro Tesoureiro assumir a Tesouraria Geral e o suplente assumir a Primeira Tesouraria;

Art. 32 - Compete ao Diretor de Comunicação:

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I - Promover a divulgação das ações junto aos sindicatos, a categoria e a sociedade;

II- Desenvolver e acompanhar as campanhas publicitárias definidas pelos órgãos de deliberação da FENAFAR;

III - Ter sob sua responsabilidade os setores de imprensa, comunicação, publicidade, biblioteca e centro de memória;

IV - Manter a publicação e divulgação do "Boletim da FENAFAR".

Parágrafo único - No caso de afastamento do diretor de comunicação, compete ao suplente assumir e cumprir o restante do mandato.

Art. 33 - Compete ao Diretor de Formação Sindical:

I - Implementar o departamento de formação sindical;

II – Propor, organizar e coordenar, em conjunto com o Diretor Intersindical, a realização de seminários, cursos, palestras, encontros de área, dentro dos interesses da categoria e dos trabalhadores em geral, com base nos princípios fixados por este estatuto;

III - Formar dirigentes, representantes sindicais, organizando cursos e seminários de formação sindical e capacitação política:

IV - Elaborar e submeter à apreciação e deliberação do Conselho de Representantes, na última reunião anual o plano de formação sindical a ser executado no exercício seguinte;

Parágrafo único - No caso de afastamento do diretor de formação sindical, compete ao suplente assumir e cumprir o resto do mandato.

Art. 34 - Compete à Diretoria de Relações Institucionais:

I- Planejar, propor e executar as políticas e projetos de relações institucionais da FENAFAR visando o estreitamento das relações institucionais com entidades de representação sindical e de trabalhadores, de associações, fundações, organismos não governamentais, órgãos e poderes governamentais do Brasil e organizações da sociedade civil em geral, especialmente com as entidades farmacêuticas;

Art. 34 A – Compete ao Primeiro Diretor de Relações Institucionais:

I – Auxiliar o Diretor Relações Institucionais em todas as suas atividades e para as quais for designado e substituí-lo em suas ausências;

Art. 35 - Compete à Diretoria de Relações Internacionais:

I - Planejar e propor atividades que promovam e incrementem as relações internacionais da FENAFAR com as demais entidades sindicais e de trabalhadores das nações estrangeiras, associações internacionais de trabalhadores, entidades congêneres da sociedade civil dos diversos povos do mundo, especialmente com as entidades farmacêuticas bem como com governos e representações diplomáticas.

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II- Promover a interação sindical, cultural, política e social com as entidades acima nominadas;

III- Executar as políticas e projetos de Relações Internacionais da FENAFAR;

Art. 35 A – Compete ao Primeiro Diretor de Relações Internacionais:

I – Auxiliar o Diretor Relações Internacionais em todas as suas atividades e para as quais for designado e substituí-lo em suas ausências;

Art. 36 - Compete à Diretoria de Organização Sindical

I - Planejar as ações e promover o debate permanente e pontual dos diversos aspectos que envolvem a organização sindical dos trabalhadores no Brasil;

II- Acompanhar todos os debates e discussões legislativas sobre a Organização Sindical:

III - Ter e dispor à Diretoria e à categoria todas as informações referentes ao tema Organização Sindical;

IV - Fomentar, no âmbito da categoria o debate de questões específicas da organização sindical farmacêutica.

Art. 36 A – Compete ao Primeiro Diretor de Organização Sindical:

I – Auxiliar o Diretor de Organização Sindical em todas as suas atividades e para as quais for designado e substituí-lo em suas ausências;

Art. 37- Compete à Diretoria da Mulher:

I - Planejar e executar as ações e atividades que promovam o debate, a conscientização e o avanço das questões de gênero no meio sindical e da categoria;

II - Propor a realização de estudos, pesquisas e projetos que tenham por finalidade promover a emancipação social, política, econômica e profissional da mulher:

III - Promover a luta permanente pelas conquistas e pela melhoria das condições de vida, trabalho e saúde da mulher;

Art. 38 - Compete à Diretoria de Educação:

I – Planejar, propor e coordenar a execução das ações da FENAFAR relacionadas à Educação, em especial à educação farmacêutica:

II - Estabelecer e coordenar as relações e interfaces da Fenafar com as demais instituições e organismos, nacionais e internacionais, no campo da educação farmacêutica, participando das atividades relacionadas;

III - Contribuir para a construção e implementação da política pública de educação, como representação formal da categoria farmacêutica;

Art. 38 A – Caberá ao Diretor de Assuntos Jurídicos:

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I – Coordenar a elaboração de Assuntos Jurídicos e ter sob seu comando a responsabilidade das tarefas desenvolvidas pelo setor jurídico da Federação e outros correlatos;

Art. 38 B - Caberá ao Primeiro Diretor de Assuntos Jurídicos:

I – Auxiliar o Diretor de Assuntos Jurídicos em todas as suas atividades e para as quais for designado e substituí-lo em suas ausências;

Art. 38 C– Caberá ao Diretor de Saúde e Segurança do Trabalho:

I – Promover estudos, pesquisas e seminários visando a melhoria das condições de vida, de trabalho e relacionadas ao ambiente do trabalho;

Art. 38 D– Caberá ao Diretor de Relações Trabalhistas:

I – Promover estudos e pesquisas relacionadas a legislação trabalhista aplicáveis à categoria;

II – Promover estudos, pesquisas e seminários relacionados às reivindicações da categoria em negociações coletivas;

Art. 38 E – Caberá ao Diretor de Juventude e Direitos Humanos:

I – Promover atividades e eventos culturais como seminários, palestras, programações artísticas, etc, de interesse dos jovens farmacêuticos;

II – Coletar, sistematizar e processar dados de interesse da categoria, elaborando análises sobre as relações de trabalho envolvendo os jovens farmacêuticos;

Art. 38 F - Caberá às Diretorias Regionais:

I - exercer juntamente com os demais membros da Diretoria a representação política e institucional da Fenafar dentro da respectiva área regional;

II - apresentar e desenvolver um planejamento regional no sentido de promover a capilaridade e efetivar maior presença federativa nos respectivos estados;

III - apresentar aos demais Diretores planos e propostas de ampliação e fortalecimento da base e da representatividade da Federação;

Art. 39 - Qualquer membro da Diretoria poderá ser destituído em Congresso, convocado para este fim, sendo que o mesmo se aplica a Diretoria coletivamente;

Parágrafo primeiro: Fica garantido o direito de ampla defesa às partes;

Parágrafo segundo: No caso de destituição de metade mais um dos membros da Diretoria, efetivos e suplentes, o Congresso deverá eleger uma diretoria provisória que promoverá novas eleições congressuais no prazo de até 180 dias.

Parágrafo terceiro: O diretor que for destituído ficará impedido de se candidatar pelo período correspondente a dois mandatos seguintes ao da sua destituição, não considerado o tempo da diretoria provisória.

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CAPÍTULO VII

DO CONSELHO CONSULTIVO

Artigo 40 - O Conselho Consultivo é um órgão permanente e de auxílio aos órgãos deliberativo e executivo da Federação Nacional dos Farmacêuticos, composto por profissionais farmacêuticos, cabendo a ele, quando solicitado, se pronunciar emitindo pareceres, estudos e reflexões, com a finalidade de aconselhamento, consultar orientação à Diretoria da Fenafar sobre os temas de relevância nacional e que possam representar grande interesse ao exercício profissional e aos rumos que serão adotados pela entidade, respeitadas as atribuições de seus órgãos deliberativos;

Parágrafo primeiro - O Presidente do Conselho Consultivo será eleito na Plenária da Assembleia estatutária do Congresso da Fenafar que deliberar a sua criação, sendo que os demais membros serão indicados Executiva após consulta aos sindicatos de base e aprovados pelo Conselho de Representantes;

Parágrafo segundo - O Conselho de Representantes deverá regulamentar a composição, critérios de participação, o funcionamento e atribuições do Conselho Consultivo;

Parágrafo terceiro - O Conselho Consultivo, quando solicitado pela Diretoria, se reunirá junto com uma das Reuniões do Conselho de Representantes da Fenafar;

Parágrafo quarto - Em caso de desistência, saída ou afastamento de quaisquer membros do Conselho Consultivo, a vacância será recomposta na primeira reunião posterior do Conselho de Representantes, por proposta da Diretoria Executiva.

CAPÍTULO VIII

DAS ELEIÇÕES

Art. 41 - A eleição da Diretoria é convocada para o mês de agosto, pelo Presidente em exercício com pelo menos 90 (noventa) dias de antecedência ressalvando o disposto no parágrafo segundo do Artigo 39;

Parágrafo primeiro: A diretoria da FENAFAR, bem como seus suplentes serão eleitos pelo Congresso da FENAFAR;

Parágrafo segundo: A eleição dar-se-á pelo voto direto dos Delegados, conforme o regulamento eleitoral e regimento interno do Congresso.

Parágrafo terceiro: Não sendo convocada a eleição nos prazos previstos neste artigo, cabe ao Conselho de Representantes convocá-la no máximo 30 (trinta) dias após este prazo ter se esgotado;

Art. 42 - O Conselho de Representantes elaborará o Regulamento Eleitoral, elegerá uma Comissão Eleitoral que será responsável pelo processo eleitoral, de acordo com o previsto neste estatuto;

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CAPITULO IX

DO PATRIMÔNIO E FINANÇAS, DA AQUISIÇÃO E ALIENAÇÃO DE BENS DO ATIVO PERMANENTE

Art. 43 - O patrimônio da FENAFAR é constituído por:

I - O valor do rateio que lhe couber, na forma da legislação vigente, do produto das arrecadações das contribuições confederativa e sindical;

II - O valor da contribuição (anuidade) dos sindicatos para custeio das suas despesas, como disposto no Artigo 43;

III - Bens e imóveis que a FENAFAR venha a adquirir;

IV - Móveis e utensílios;

V - Doações e legados recebidos com especificações para o patrimônio;

Art. 44 – Aquisição, alienação ou aceitação de doações de bens imóveis e títulos de valores mobiliários, classificados como investimentos de caráter permanente da FENAFAR, deverão ter a aprovação do Conselho de Representantes;

Parágrafo único: excetuam-se do disposto neste artigo as aquisições de móveis e utensílios caracterizados como investimentos transitórios, que podem ser efetuados por deliberação da Diretoria;

CAPÍTULO X

DA RECEITA E DESPESA

Art. 45 - A receita da FENAFAR é classificada em ordinária e extraordinária:

I - Constituem receita ordinária:

a) produto das contribuições dos Filiados, como disposto no artigo 43;

b) juros provenientes de depósitos bancários realizados pela FENAFAR, bem como de títulos incorporados ao patrimônio;

c) repasses das contribuições sindical e confederativa;

d) renda dos imóveis, dos bens e valores de propriedade da FENAFAR, quando os possuir;

II - Constituem receita extraordinária:

a) subvenções de qualquer natureza;

b) multas e rendas eventuais;

c) renda de doações feitas à FENAFAR:

Art. 46 - Os sindicatos filiados deverão pagar anuidade a FENAFAR de acordo com o Artigo 5º, inciso II;

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CAPÍTULO XI

DAS DISPOSIÇÕES GERAIS

Art. 47 - Nenhuma entidade filiada, individual ou coletivamente, responderá subsidiariamente pelos encargos que seus representantes contraírem.

Art. 48 - Os membros da Diretoria não receberão remuneração pelas atividades que desempenharem na FENAFAR, ressalvando o ressarcimento de despesas feitas para o desempenho de atividades sindicais, bem como eventual ônus de liberação de diretor (es) pela categoria, aprovado em Conselho de Representantes;

Art. 49 - A posse dos membros da Diretoria e do Conselho Fiscal ocorrerá na data do início do mandato para o qual foram eleitos;

Art. 50 - A FENAFAR poderá ser voluntariamente dissolvida em Congresso convocado especificamente para este fim;

Parágrafo único - No caso de dissolução, o destino dos bens da FENAFAR será definido pelo congresso que a dissolver;

Art. 51 - O presente Estatuto poderá ser reformado pelo Congresso da Fenafar e observando-se o disposto no inciso XI e parágrafo único do artigo 17, pelo Conselho de Representantes, em reunião convocada para este fim;

Art. 52 - Os casos omissos neste Estatuto serão resolvidos pelo Congresso da FENAFAR.

Art. 53 - Este estatuto entra em vigor na data de seu registro no órgão competente.

Art. 54 - Revogam-se as disposições em contrário.

DISPOSIÇÕES TRANSITÓRIAS

Art. 1º - Excepcionalmente o primeiro mandato para os cargos de Primeiro Diretor de Relações Institucionais, Primeiro Diretor de Relações Internacionais, Primeiro Diretor de Organização Sindical e Primeiro Diretor de Assuntos Jurídicos será exercido por Diretores indicados pela Plenária do 9º Congresso da FENAFAR, convocado para os dias 02, 03 e 04 de agosto de 2018, em Aracruz/ES, e aprovados pela mesma, vindo a ser empossados na primeira reunião do Conselho de Representantes que ocorrer após o registro deste estatuto no órgão competente, com término do mandato coincidente com o da Diretoria eleita durante o referido Congresso.

São Paulo, 1º de agosto de 2018.

Ronald Ferreira dos Santos

Presidente

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EÓLICA HERMENEGILDO II S.A.

AVISO DE LICITAÇÃO
PREGÃO PRESENCIAL Nº 3/2018

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FEDERAÇÃO NACIONAL DOS EMPREGADOS NAS EMPRESAS CONCESSIONÁRIAS DO RAMO DE RODOVIAS PÚBLICAS, ESTRADAS EM GERAL E PEDÁGIOS

EDITAL DE CONVOCAÇÃO
ASSEMBLEIA GERAL ORDINÁRIA

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FEDERAÇÃO NACIONAL DOS FARMACÊUTICOS

EDITAL DE CONVOCAÇÃO
ASSEMBLEIA CONGRESSUAL EXTRAORDINÁRIA

[illegible]

RONALD FERREIRA DOS SANTOS
Presidente da Federação

FEDERAÇÃO NACIONAL DOS FARMACÊUTICOS

EDITAL

[illegible]

RONALD FERREIRA DOS SANTOS
Presidente da Federação

FSB COMUNICAÇÕES LTDA

EDITAL DE CONVOCAÇÃO
ASSEMBLEIA GERAL

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OTAVIO DOS SANTOS VENTURA DA SILVA
Administrador

FUNDAÇÃO DE APOIO À EDUCAÇÃO, PESQUISA E DESENVOLVIMENTO CIENTÍFICO E TECNOLÓGICO DA UNIVERSIDADE TECNOLÓGICA FEDERAL DO PARANÁ

AVISO DE LICITAÇÃO FRACASSADA
PREGÃO ELETRÔNICO Nº 1/2018

[illegible]

FUNDAÇÃO DE APOIO À PESQUISA, DESENVOLVIMENTO E INOVAÇÃO - EXÉRCITO BRASILEIRO

EXTRATO DE DOAÇÃO

[illegible]

FUNDAÇÃO DE APOIO À TECNOLOGIA E CIÊNCIA

EXTRATO DE INEXIGIBILIDADE DE LICITAÇÃO

[illegible]

EXTRATO DE INEXIGIBILIDADE DE LICITAÇÃO

[illegible]

AVISO DE LICITAÇÃO

[illegible]

AVISO DE LICITAÇÃO
TOMADA DE PREÇOS

[illegible]

FUNDAÇÃO APOLÔNIO SALLES DE DESENVOLVIMENTO EDUCACIONAL

AVISO DE COTAÇÃO DE PREÇOS

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FUNDAÇÃO DE ASSISTÊNCIA SOCIAL DE PONTA GROSSA

AVISO DE LICITAÇÃO
PREGÃO ELETRÔNICO

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AVISO DE LICITAÇÃO
PREGÃO PRESENCIAL

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PRENOTADO
4º RCPJ-SP

REGISTRO PESSOAS JURÍDICAS 28 AGO 2018 675230 MICROFILME

PRENOTADO 4º RCPJ/SP

Lista de Presença da Assembleia Congressual Extraordinária da Federação Nacional dos Farmacêuticos – FENAFAR, realizada em 1º/08/2018, nas dependências do SESC Praia Formosa, localizado à Rodovia ES-010, Km 35 Norte, s/n, Santa Cruz, município de Aracruz/ES.

NOME COMPLETO	CPF/MF	SINDICATO	Assinatura
Lavinia Salete de Melo Maia Magalhães	928.413.173-15	Ceará Sinfarce	[signature]
Malena Cadelha Cavalcante	641.091.603-59	Ceará Sinfarce	[signature]
Josefa Nilza Fernandes Nata	566 228 303-63	Sinfapi	[signature]
Luciano Manoel de Freitas Junior	741.891.173-34	SINFARMA	[signature]
Luiz Ricardo de Moraes Rangford	011.304.343-03	SINFARMA	[signature]
Carlos Augusto Barbosa Toledo	601.187.203 68	SINFARMA	Carlos Augusto B. Toledo

4º Oficial de Registro de Títulos e Documentos

PESSOAS JURÍDICAS
28 AGO 2018 075220
PROTOCOLO / MICROFILME

PRENOTADO
4º RCPJ/SP

Lista de Presença da Assembleia Congressual Extraordinária da Federação Nacional dos Farmacêuticos – FENAFAR, realizada em 1º/08/2018, nas dependências do SESC Praia Formosa, localizado à Rodovia ES-010, Km 35 Norte, s/n, ~~Santa Cruz,~~ município de Aracruz/ES.

NOME COMPLETO	CPF/MF	SINDICATO	Assinatura
Flávia Gonçalves Ferreira	770.210.962-91	SINDIFAC ACRE	
Kathlen Cristina da Silva [illegible]	602.256.812-24	Sindifac Acre	
Renata [illegible] Gonzaga Pereira	159 444 598 18	SINFAR-SP	
Isabela de O. Sobrinho	741.128.332-34	SINDIFAC-ACRE	
Maria Cristina Ramirez	015 503 871-20	Sinfar GO	
Willi Marcio Colazans	395361571-87	Sinfar-MT	

4º Oficial de Registro de Títulos e Documentos

4º REGISTRO PESSOAS JURÍDICAS 28 AGO 2018 075220

Lista de Presença da Assembleia Congressual Extraordinária da Federação Nacional dos Farmacêuticos – FENAFAR, realizada em 1º/08/2018, nas dependências do SESC Praia Formosa, localizado à Rodovia ES-010, Km 35 Norte, s/n, Santa Cruz, município de Aracruz/ES.

NOME COMPLETO	CPF/MF	SINDICATO	Assinatura
Otavio Eutiquio Vasconcelos Pinheiro da Silva	657454692-34	SINFAR-AP	[assinatura]
Celia Machado Gervasio Chaves	262770000-63	SINDIFARS	[assinatura]
Helena Cavalcanti Ransolin	382102190-00	SINDIFARS	[assinatura]
Fernanda Manzini	28995028-29	SindFar SC	[assinatura]
Mônica Cristina Nunes da Trindade	058.338.349-00	Sindfar SC	[assinatura]
Lia Mello de Almeida	405.058.449-49	SINDIFAR-PR	[assinatura]

PRENOTADO 4º RCPJ/SP

PRENOTADO
4º RCPJ/SP

REGISTRO PESSOAS JURÍDICAS
28 AGO 2018 ... 75220

Lista de Presença da Assembleia Congressual Extraordinária da Federação Nacional dos Farmacêuticos – FENAFAR, realizada em 1°/08/2018, nas dependências do SESC Praia Formosa, localizado à Rodovia ES-010, Km 35 Norte, s/n, Santa Cruz, município de Aracruz/ES.

NOME COMPLETO	CPF/MF	SINDICATO	Assinatura
Rillke Novato Públio	545828796-68	Sinfarmig	[signature]
Cristiane O. Costa	652000715-34	Sindifarma/SE	[signature]
Josias Pinz	002760891-15	Sinfar/MT	[signature]
Silvana Leite	93983042953	Sindfar SC	[signature]
DEBORA R Melecchi	63275643053	SINDIFARS	[signature]
MARCOS VINICIUS GHENO	005189150-69	SINDIFARS	Marcos Vinicius Gheno

4º Oficial de Registro de Títulos e Documentos

PESSOAS JURIDICAS
28 AGO 2018 075220
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PRENOTADO
4º RCPJ/SP

Lista de Presença da Assembleia Congressual Extraordinária da Federação Nacional dos Farmacêuticos – FENAFAR, realizada em 1º/08/2018, nas dependências do SESC Praia Formosa, localizado à Rodovia ES-010, Km 35 Norte, s/n, Santa Cruz, município de Aracruz/ES.

NOME COMPLETO	CPF/MF	SINDICATO	Assinatura
Priscila Vautier	15603465824	SINFAR-SP São Paulo	[assinatura]
Maria José Sartório	813.195.107-91	SINFES	[assinatura]
Neudo Magnago Heleodoro	890.897.237-49	SINFES	[assinatura]
Gilda Almeida Souza	912544008-00	SINFAR-SP	[assinatura]
Fabio José Basilio	830864801-06	SINFAR-GO	[assinatura]
RONALD F. SANTOS	834631189-15	SINDFAR-SC	[assinatura]

PESSOAS JURIDICAS
28 AGO 2018 075220
PROTOCOLO MICROFILME

Lista de Presença da Assembleia Congressual Extraordinária da Federação Nacional dos Farmacêuticos – FENAFAR, realizada em 1º/08/2018, nas dependências do SESC Praia Formosa, localizado à Rodovia ES-010, Km 35 Norte, s/n, Santa Cruz, município de Aracruz/ES.

NOME COMPLETO	CPF/MF	SINDICATO	Assinatura
Mª Maruza Carlesso	252.227.327-20	Sind. Farmcos no Estado do Esp. Santo	[assinatura]
Humberto Alves Nogueira	015.330.801-00	Sindicato dos Farmacêuticos de Roraima	[assinatura]
Catarine Bezerra Cavalcanti	081.918.297-46	cavalcanti.catery@gmail.com SINFAERJ.	[assinatura]
Sergio Luis Gomes da Silva	715.004-644-49	Sind. dos Farmacêutico da PB	[assinatura]

PRENOTADO
4º RCPJ/SP

4º Oficial de Registro de Títulos e Documentos

4º Oficial de Registro de Títulos e Documentos e Civil de Pessoa Jurídica da Comarca de São Paulo

Oficial de Registro: Robson de Alvarenga

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REGISTRO CIVIL DE PESSOA JURÍDICA

Nº 705.961 de 26/10/2022

Certifico e dou fé que o documento em papel, contendo **12 (doze) páginas**, foi apresentado em 03/10/2022, protocolado sob nº 417.903, tendo sido registrado eletronicamente sob nº **705.961** e averbado no registro nº 237636/92 no Livro de Registro A deste 4º Oficial de Registro Civil de Pessoas Jurídicas da Comarca de São Paulo, na presente data.

Denominação
FEDERACAO NACIONAL DOS FARMACEUTICOS FENAFAR
CNPJ nº 00.679.357/0001-48

Natureza:
ATA

São Paulo, 26 de outubro de 2022

(ASSINADO ELETRONICAMENTE)

Carlos Augusto Peppe
Escrevente

Este certificado é parte **integrante e inseparável** do registro do documento acima descrito.

Emolumentos	Estado	Secretaria da Fazenda	Registro Civil	Tribunal de Justiça
R$ 100,03	R$ 28,49	R$ 19,53	R$ 5,30	R$ 6,84
Ministério Público	**ISS**	**Condução**	**Outras Despesas**	**Total**
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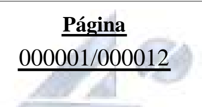

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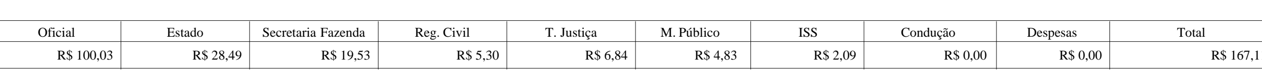

Oficial	Estado	Secretaria Fazenda	Reg. Civil	T. Justiça	M. Público	ISS	Condução	Despesas	Total
R$ 100,03	R$ 28,49	R$ 19,53	R$ 5,30	R$ 6,84	R$ 4,83	R$ 2,09	R$ 0,00	R$ 0,00	R$ 167,11

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Oficial	Estado	Secretaria Fazenda	Reg. Civil	T. Justiça	M. Público	ISS	Condução	Despesas	Total
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ILUSTRÍSSIMO SENHOR OFICIAL DO 4º REGISTRO DE TÍTULOS E DOCUMENTOS E CIVIL DE PESSOA JURÍDICA DA CAPITAL – SP

A Federação Nacional dos Farmacêuticos, inscrita no CNPJ sob o nº 00.679.357/0001-48, com sede à Rua Barão de Itapetininga, 255, Sala 1105, Centro, São Paulo/SP, CEP 01042-001, representada neste ato por seu representante legal Ronald Ferreira dos Santos, brasileiro, casado, farmacêutico, portador do RG nº 2039575/SSP-SC, CPF nº 834.631.789-15, CRF/SC nº 2592, residente e domiciliado à Rua João De Barro, 124, Casa - Florianópolis-SC CEP: 88.054-620 vem, respeitosamente, à presença de Vossa Senhoria requerer o registro da Ata de Eleição da Diretoria, do Conselho Fiscal e seus respectivos suplentes para o mandato 2022/2025 anexos, declarando que foram cumpridos todos os requisitos estatutários vigentes.

São Paulo, 18 de outubro de 2022

FEDERACAO NACIONAL DOS FARMACEUTICOS: 00679357000148

Ronald Ferreira dos Santos

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Registro Nº
705.961
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Protocolo nº 417.903 de 03/10/2022 às 11:17:39h: Documento **registrado eletronicamente para fins de publicidade e/ou eficácia contra terceiros** sob nº **705.961** em **26/10/2022** e averbado no registro nº 237636/92 neste **4º Oficial de Registro Civil de Pessoas Jurídicas da Comarca de São Paulo**. Assinado digitalmente por Carlos Augusto Peppe - Escrevente.

Oficial	Estado	Secretaria Fazenda	Reg. Civil	T. Justiça	M. Público	ISS	Condução	Despesas	Total
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ATA GERAL DA ELEIÇÃO DA DIRETORIA, CONSELHO FISCAL E SUPLENTES DA FEDERAÇÃO NACIONAL DOS FARMACÊUTICOS FENAFAR, CNPJ 00.679.357/0001-48, TRIÊNIO 2022/2025

Aos 06 dias do mês de agosto de 2022, na sede do 10º CONGRESSO DA FEDERAÇÃO NACIONAL DOS FARMACÊUTICOS - FENAFAR, situada à rua Fonte do Boi, 215 - Rio Vermelho, Salvador - BA, 41940-360, a partir das 12 horas reuniu-se a Comissão Eleitoral que presidiu o pleito eleitoral que elegeu a nova Diretoria Executiva, Conselho Fiscal e Suplentes da FENAFAR para o triênio 2022/2025, em eleição congressual realizada no dia 06 de agosto de 2022, das 10 horas às 12 horas, nas dependências do Hotel Mercure, sito à Rua da Fonte do Boi, durante o 10º Congresso da FENAFAR convocado para os dias 04, 05 e 06 de agosto de 2022 na cidade de Salvador/BA. Participaram da presente reunião os farmacêuticos Masurquede Coimbra, Elister de Oliveira Rodriguês, Bruno Fernandes Baltazar de Oliveira, Eliana Cristina de Santana Fiais, José Jorge Silva Júnior e Bruna Barboza Murada. O processo eleitoral foi realizado através de sistema de votação eletrônica, utilizando uma plataforma previamente desenvolvida pela Empresa Pandgora Tecnologia Ltda, CNPJ 03.283.183/0001-98, que permite relatório de apuração e de votantes pela identificação do CPF e IP do equipamento utilizado para votação, garantindo o sistema, a inviolabilidade e sigilo do voto. A votação foi aberta às 10 horas permitindo que os delegados dos Sindicatos filiados à FENAFAR dos Estados do ACRE, ALAGOAS, AMAZONAS, AMAPÁ, BAHIA, CEARÁ, GOIÁS, MATO GROSSO, MINAS GERAIS, PARAÍBA, PARANÁ, PERNAMBUCO, PIAUÍ, RIO DE JANEIRO, RIO GRANDE DO SUL, SANTA CATARINA, SÃO PAULO e SERGIPE, sendo 114 (cento e quatorze) delegados aptos a votar cujos nomes constam de lista anexa a esta ata, presencialmente e de forma remota. A votação foi encerrada precisamente às 12 horas dando início imediato à apuração dos votos através do sistema de votação supracitado com a utilização de chave criptografada de segurança. O Presidente da Comissão Eleitoral, atendendo o disposto no Regulamento Eleitoral e no Estatuto Social da entidade, fez uso da palavra e proclamou o resultado, tendo 91 (noventa e um) votos registrados, na plataforma, sendo 90 votos para a chapa 1 e um voto nulo. Em seguida, a Comissão Eleitoral declarou eleita a nova Diretoria Executiva, Conselho Fiscal e Suplentes da FENAFAR para o mandato do triênio 2022/2025, iniciando-se o mandato em 01-09-2022 e término em 31 de agosto de 2025, conforme Ata do Congresso

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Oficial	Estado	Secretaria Fazenda	Reg. Civil	T. Justiça	M. Público	ISS	Condução	Despesas	Total
R$ 100,03	R$ 28,49	R$ 19,53	R$ 5,30	R$ 6,84	R$ 4,83	R$ 2,09	R$ 0,00	R$ 0,00	R$ 167,11

Extraordinário da Federação Nacional dos farmacêuticos que de 09 de junho de 2021, prorrogou o mandato até 31 de agosto de 2022. Segue qualificação dos eleitos:

PRESIDENTE: Fábio José Basílio, brasileiro, casado, farmacêutico, portador do RG nº 3207835/SSP-GO, CPF nº 830.864.801-06, CRF/GO nº 3104, residente e domiciliado à Rua RB, 16, Qd 41 lt 22 - Goiânia-GO CEP: 74.474-377;

PRIMEIRO VICE-PRESIDENTE: André Nunes Cavalcante, brasileiro, casado, farmacêutico, portador do RG nº 95002322087/SSP-CE, CPF nº 868.401.773-00, CRF/CE nº 3042, residente e domiciliado à Rua Barão De Aratanha, 444, casa - Fortaleza-CE CEP: 60.050-070;

SEGUNDO VICE-PRESIDENTE: Debora Raymundo Melecchi, brasileira, solteira, farmacêutica, portador do RG nº 4051802314/SSP-RS, CPF nº 632.756.430-53, CRF/RS nº 5911, residente e domiciliado à Rua Ramiro Barcelos, 1552, apto 11 - Porto Alegre-RS CEP: 90.035-002;

SECRETÁRIO GERAL: Maria Maruza Carlesso, brasileira, solteira, farmacêutica, portador do RG nº 167541/SSP-ES, CPF nº 252.227.327-20, CRF/ES nº 274, residente e domiciliado à Av. Luiz Manoel Vellozo, 121, apto 302 - Vitória-ES CEP: 29.060-040;

PRIMEIRO SECRETÁRIO: Dalmare Anderson Bezerra de Oliveira Falcão e Sá, brasileiro, casado, farmacêutico, portador do RG nº 11062560/SSP-PE, CPF nº 028.425.735-44, CRF/PE nº 07736, residente e domiciliado à Estrada De Belém, 1286, Casa - Recife-PE CEP: 52.031-000;

TESOUREIRO: Célia Machado Gervásio Chaves, brasileira, solteira, farmacêutica, portador do RG nº 1017839836/SSP-RS, CPF nº 262.770.000-63, CRF/RS nº 1894, residente e domiciliado à Rua Marcílio Dias, 589, apto. 1701 - Porto Alegre-RS CEP: 90.130-001;

PRIMEIRO TESOUREIRO: Alexandre Correia dos Santos, brasileiro, casado, farmacêutico, portador do RG nº 1027718/SSP-AL, CPF nº 563.359.544-00, CRF/AL nº 541, residente e domiciliado à Rua Rosalvo Ribeiro, casa - Pilar-AL CEP: 57.150-000;

DIRETOR DE COMUNICAÇÃO: Sérgio Luís Gomes da Silva, brasileiro, divorciado, farmacêutico, portador do RG nº 1326941/SSP-PB, CPF nº 715.004.644-49, CRF/PB nº 1813, residente e domiciliado à Rua Professor Cardoso, 105, - João Pessoa-PB CEP: 58.040-310;

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R$ 100,03	R$ 28,49	R$ 19,53	R$ 5,30	R$ 6,84	R$ 4,83	R$ 2,09	R$ 0,00	R$ 0,00	R$ 167,11

DIRETOR DE FORMAÇÃO SINDICAL: Júnia Dark Vieira Lelis Ligório, brasileira, casada, farmacêutica, portador do RG nº MG10657902/SSP-MG, CPF nº 036.133.366-85, CRF/MG nº 14135, residente e domiciliado à Rua Geraldo Magela De Almeida, 135, - Belo Horizonte-MG CEP: 30.840-640;

DIRETORA DE RELAÇÕES INSTITUCIONAIS: Renata Tereza Gonçalves Pereira, brasileiro, casado, farmacêutico, portador do RG nº 209558325/SSP-SP, CPF nº 159.144.598-18, CRF/SP nº 18176, residente e domiciliado à Avenida Manoel Da Nóbrega, 200, apto 106 - São Vicente-SP CEP: 11.320-200;

PRIMEIRA DIRETORIA DE RELAÇÕES INSTITUCIONAIS: Isabela de Oliveira Sobrinho, brasileira, solteira, farmacêutica, portador do RG nº 303082/SSP-AC, CPF nº 741.123.332-34, CRF/AC nº 145, residente e domiciliado à Rua Dos Engenheiros, 464, Casa - Rio Branco-AC CEP: 69.919-053;

DIRETOR DE REL. INTERNACIONAIS: Ronald Ferreira dos Santos, brasileiro, casado, farmacêutico, portador do RG nº 2039575/SSP-SC, CPF nº 834.631.789-15, CRF/SC nº 2592, residente e domiciliado à Rua João De Barro, 124, Casa - Florianópolis-SC CEP: 88.054-620;

PRIMEIRA DIRETORA DE RELAÇÕES INTERNACIONAIS: Gilda Almeida de Souza, brasileira, divorciada, farmacêutica, portador do RG nº 10153588-0/SSP-SP, CPF nº 912.544.008-00, CRF/SP nº 6430, residente e domiciliado à Rua Inácio Manuel Alvares, 360, 64 Bloco A - São Paulo-SP CEP: 05.372-110;

DIRETOR DE ORGANIZAÇÃO SINDICAL: Rilke Novato Públio, brasileiro, solteiro, farmacêutico, portador do RG nº MG3231185/PC-MG, CPF nº 545.826.796-68, CRF/MG nº 7851, residente e domiciliado à Rua Geraldo Magela Pereira, Apto 201 - Belo Horizonte-MG CEP: 30.840-380;

PRIMEIRA DIRETORIA DE ORGANIZAÇÃO SINDICAL: Otávio Eutíquio Vasconcelos Pinheiro da Silva, brasileiro, casado, farmacêutico, portador do RG nº 1804766, CPF nº 657.454.692-34, CRF/AP nº 404, residente e domiciliado à Av. Brunei, 466, Condomínio Parque Novo Mundo - Macapá-AP CEP: 68.906-802;

DIRETORIA DA MULHER: Maria Soraya Pinheiro de Amorim, brasileira, solteira, farmacêutica, portador do RG nº 142154890/SSP-BA, CPF nº 373.979.665-00, CRF/BA nº 2114, residente e domiciliado à Travessa Orlando Moscoso, 1, casa - Salvador-BA CEP: 41.706-665;

DIRETORIA DE EDUCAÇÃO: Silvana Nair Leite Contezini, brasileira, casada, farmacêutica, portador do RG nº 2864322/SSP-SC, CPF nº 939.830.429-53,

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Oficial	Estado	Secretaria Fazenda	Reg. Civil	T. Justiça	M. Público	ISS	Condução	Despesas	Total
R$ 100,03	R$ 28,49	R$ 19,53	R$ 5,30	R$ 6,84	R$ 4,83	R$ 2,09	R$ 0,00	R$ 0,00	R$ 167,11

CRF/SC nº 3268, residente e domiciliado à Rua Pedro Vieira Vidal, 280, 702 - Florianópolis-SC CEP: 88.040-010;

DIRETORIA DE ASSUNTOS JURÍDICOS: Clóvis de Santana Reis, brasileiro, casado, farmacêutico, portador do RG nº 480872201/SSP-BA, CPF nº 928.939.455-20, CRF/BA nº 3030, residente e domiciliado à Rua Nivaldo Domingos, 439, Cond.Residencial Boulevard Ipitanga, Casa 2 - Lauro De Freitas-BA CEP: 42.706-320;

PRIMEIRA DIRETORIA DE ASSUNTOS JURÍDICOS: Allan Kardec de Lima, brasileiro, solteiro, farmacêutico, portador do RG nº 163853757/SSP-SP, CPF nº 117.506.788-10, CRF/PR nº 30671, residente e domiciliado à Avenida Dos Estados, 577, Apto 5 - Curitiba-PR CEP: 80.610-040;

DIRETORIA DE SAÚDE E SEGURANÇA DO TRABALHO: Carlos Augusto Barboza Toledo, brasileiro, solteiro, farmacêutico, portador do RG nº 20825194-4/SSP-MA, CPF nº 801.187.203-68, CRF/MA nº 1506, residente e domiciliado à Avenida Dos Marinheiros,7, quadra 2, bairro Aracagy - São José De Ribamar-MA CEP: 65.110-000;

DIRETORIA DE RELAÇÕES TRABALHISTAS: Ulisses Nogueira de Aguiar, brasileiro, casado, farmacêutico, portador do RG nº 3103636/SSP-PB, CPF nº 514.451.403-06, CRF/PI nº 607, residente e domiciliado à Endereço Vila Da Fab Casa 5 Aeroporto, Casa - Teresina-PI CEP: 64.006-030;

DIRETORIA DE JUVENTUDE E DIREITOS HUMANOS: Daniela Santos Oliveira, brasileira, solteira, farmacêutica, portador do RG nº 1398669/SSP-SE, CPF nº 005.583.855-32, CRF/SE nº 610, residente e domiciliado à Rua Maria De Nazareth Barros Santos,114, Casa - Aracaju -SE CEP: 49.030-830;

DIRETOR REGIONAL NORTE: Lituânia Mustafá Paes de Almeida, brasileira, casada, farmacêutica, portador do RG nº 1026132-0/SSP-AM, CPF nº 417.095.782-20, CRF/AM nº 1996, residente e domiciliado à Rua Monte Moriá (Cj Galiléia Ll),06, - Manaus-AM CEP: 69.092-015;

DIRETOR REGIONAL NORDESTE: Holdack Velloso Gomes Pedroza, brasileiro, divorciado, farmacêutico, portador do RG nº 1316509/SSP-PE, CPF nº 231.312.094-53, CRF/PE nº 5397, residente e domiciliado à Rua Sebastião Ribeiro da Silva, 321, Casa - Olinda-PE CEP: 53.240-060;

DIRETOR REG. CENTRO-OESTE: Wille Marcio Nascimento Calazans, brasileiro, casado, farmacêutico, portador do RG nº 373031/SSP-MT, CPF nº 395.361.571-87,

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CRF/MT nº 931, residente e domiciliado à Rua Mestre Albertino, 95, Apto 1302 DUQUE DE CAXIAS - Cuiabá-MT CEP: 78.043-356;

DIRETOR REGIONAL SUDESTE: Catarine Bezerra Cavalcanti, brasileira, solteira, farmacêutica, portador do RG nº 11377721-3/DETRAN/RJ, CPF nº 081.918.297-46, CRF/RJ nº 23233, residente e domiciliado à Rua Nossa Senhora de Lourdes, 32, 11 - Rio de Janeiro-RJ CEP: 20.540-370;

DIRETOR REGIONAL SUL: Fábio Augusto do Carmo Santana, brasileiro, casado, farmacêutico, portador do RG nº 8.879.996-8/SSP/PR, CPF nº 006.086.049-92, CRF/PR nº 16985, residente e domiciliado à Rua Xavier Da Silva, 978, apto 403, bloco 05 - Paranaguá-PR CEP: 83.203-620;

PRIMEIRO SUPLENTE: Leonardo Légora de Abreu, brasileiro, casado, farmacêutico, portador do RG nº 12159023-6/SSP-RJ, CPF nº 054.409.627-43, CRF/RJ nº 13010, residente e domiciliado à Rua Doutor Sílvio e Silva, 162, Casa - Duque de Caxias-RJ CEP: 25.071-030;

SEGUNDO SUPLENTE: Deick Rodrigues Quaresma, brasileiro, divorciado, farmacêutico, portador do RG nº 2868019/IIPC-PA, CPF nº 628.511.102-25, CRF/PA nº 2235, residente e domiciliado à TV Monte Alegre, 397, Casa - Belém-PA CEP: 66.020-700;

TERCEIRO SUPLENTE: Francisco Ielano Vasconcelos Mesquita, brasileiro, casado, farmacêutico, portador do RG nº 95002523147/SSP-CE, CPF nº 771.922.653-49, CRF/CE nº 3392, residente e domiciliado à Rua Das Gaivotas, 1403, Casa - Fortaleza-CE CEP: 60.821-160;

QUARTO SUPLENTE Iohanna Emanuelle Martins, brasileira, casada, farmacêutica, portador do RG nº 2796573, CPF nº 023.448.711-95, CRF/DF nº 5652, residente e domiciliado à SHVP Rua 06, chácara 245, casa 27, Condomínio - Brasília DF CEP: 72.006-465;

QUINTO SUPLENTE: Ricardo Jorge Bouez Ribeiro, brasileiro, solteiro, farmacêutico, portador do RG nº MG-7.342.340/SSPMG, CPF nº 595.006.702-91, CRF/RO nº 1309, residente e domiciliado à Avenida Costa Marques, 988, Casa - Guajará-Mirim-RO CEP: 76.850-000;

SEXTO SUPLENTE: Renan de Figueiredo Ferraz, brasileiro, casado, farmacêutico, portador do RG nº 7604610/SDS-PE, CPF nº 068.349.874-60, CRF/PE nº 05286, residente e domiciliado à Rua Mário Bhering, 171, Apto 202 - Recife-PE CEP: 52.110-090;

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SÉTIMO SUPLENTE: Caetano Carloni Camargo, brasileiro, solteiro, farmacêutico, portador do RG nº 0333131/SSP-AC, CPF nº 777.141.992-53, CRF/AC nº 341, residente e domiciliado à Rua Bom Destino, 77, N tem - Rio Branco-AC CEP: 69.918-306;

CONSELHO FISCAL EFETIVO: Maria Cristina Ramirez, brasileira, viúva, farmacêutica, portador do RG nº 494834/SSP-GO, CPF nº 215.503.871-20, CRF/GO nº 11473, residente e domiciliado à Sres Qd 07 Bl A Casa, 8, fundos - Brasília-DF CEP: 70.640-018;

CONSELHO FISCAL EFETIVO: Josias Pina, brasileiro, casado, farmacêutico, portador do RG nº 1660918/SSP-RJ, CPF nº 002.160.891-15, CRF/MT nº 26R, residente e domiciliado à Rua Cel. Barros 150, casa - Cuiabá-MT CEP: 78.020-000;

CONSELHO FISCAL EFETIVO: Eline Cristina Souto Maior Baracho, brasileira, solteira, farmacêutica, portador do RG nº 98001246039/SSP-AL, CPF nº 009.432.504-98, CRF/AL nº 910, residente e domiciliado à Rua Estatistico Teixeira De Freitas, 86, Apto 304 Pinheiro - Maceió-AL CEP: 57.055-660;

SUPLENTE DO CONSELHO FISCAL: Rosilane Reis Rocha, brasileira, casada, farmacêutica, portador do RG nº 11670827/SSP-AM, CPF nº 475.966.782-20, CRF/RR nº 230, residente e domiciliado à Rua Libra, 725, Casa - Boa Vista-RR CEP: 69.317-520;

SUPLENTE DO CONSELHO FISCAL: Josenildo Segundo Chaves de Araújo, brasileiro, solteiro, farmacêutico, portador do RG nº 1545074SSDS-PB, CPF nº 014.013.244-95, CRF/PB nº 3797, residente e domiciliado à Rua Paulo Roberto De Souza Acioly, 1030, Casa - João Pessoa-PB CEP: 58.035-110;

SUPLENTE DO CONSELHO FISCAL: Maria do Socorro Cordeiro Ferreira, brasileira, viúva, farmacêutica, portador do RG nº 135900SSP-PI, CPF nº 022.726.363-49, CRF/PI nº 122, residente e domiciliado à Rua Desembargador Adalberto Correia Lima, 1882, 1882 - Teresina-PI CEP: 64.049-680.

Nada mais havendo para ser tratado, eu, Elister de Oliveira Rodriguês, lavrei a presente Ata que segue assinada por mim e pelos demais membros da Comissão Eleitoral.

Salvador-BA, 06 de agosto de 2022.

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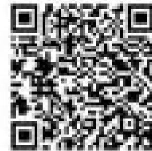

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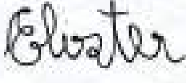

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Assinou

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Documento 9842c3a8-171c-4916-8a04-004a6db0f8da **criado** por ADELIR RODRIGUES DA VEIGA (573e50d1-ec16-4cb0-b79c-04191a1e4315). Email:efarmaceuticos@gmail.com. - DATE_ATOM: 2022-09-22T14:26:02-03:00

22 Sep 2022, 14:44:18
Assinaturas **iniciadas** por ADELIR RODRIGUES DA VEIGA (573e50d1-ec16-4cb0-b79c-04191a1e4315). Email: efarmaceuticos@gmail.com. - DATE_ATOM: 2022-09-22T14:44:18-03:00

22 Sep 2022, 15:30:43
BRUNO FERNANDES BALTAZAR DE OLIVEIRA **Assinou** - Email: farmasantos1@yahoo.com.br - IP: 177.51.63.203 (203.63.51.177.isp.timbrasil.com.br porta: 22124) - Documento de identificação informado: 219.609.298-36 - DATE_ATOM: 2022-09-22T15:30:43-03:00

22 Sep 2022, 15:33:30

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MASURQUEDE DE AZEVEDO COIMBRA **Assinou** - Email: masurr@gmail.com - IP: 200.198.136.144 (144.136.198.200.ippool.procergs.com.br porta: 64152) - Documento de identificação informado: 924.418.150-91 - DATE_ATOM: 2022-09-22T15:33:30-03:00

22 Sep 2022, 15:43:47
ELIANA CRISTINA DE SANTANA FIAIS **Assinou** - Email: elianacristinafiais@gmail.com - IP: 177.20.3.181 (177-20-3-181.salvador.ba.gov.br porta: 14874) - Documento de identificação informado: 538.346.315-15 - DATE_ATOM: 2022-09-22T15:43:47-03:00

22 Sep 2022, 17:43:14
ELISTER DE OLIVEIRA RODRIGUES **Assinou** - Email: elisteroliveira20@gmail.com - IP: 187.69.87.73 (187-69-87-73.3g.claro.net.br porta: 48114) - Geolocalização: -16.7146045 -49.2767058 - Documento de identificação informado: 950.556.731-68 - DATE_ATOM: 2022-09-22T17:43:14-03:00

22 Sep 2022, 18:29:28
BRUNA BARBOZA MURADA **Assinou** - Email: brunamurada@yahoo.com.br - IP: 187.27.149.73 (187-27-149-73.3g.claro.net.br porta: 22458) - Documento de identificação informado: 659.722.792-15 - DATE_ATOM: 2022-09-22T18:29:28-03:00

30 Sep 2022, 17:55:35
ADELIR RODRIGUES DA VEIGA (573e50d1-ec16-4cb0-b79c-04191a1e4315). Email: efarmaceuticos@gmail.com. **ALTEROU** o signatário **jjfarma@gmail.com** para **jj_farma@yahoo.com.br** - DATE_ATOM: 2022-09-30T17:55:35-03:00

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JOSÉ JORGE SILVA JUNIOR **Assinou** - Email: jj_farma@yahoo.com.br - IP: 187.44.210.222 (187-44-210-222.STATIC.itsweb.com.br porta: 58536) - Geolocalização: -12.4902937 -38.8610657 - Documento de identificação informado: 012.521.535-54 - DATE_ATOM: 2022-09-30T18:31:31-03:00

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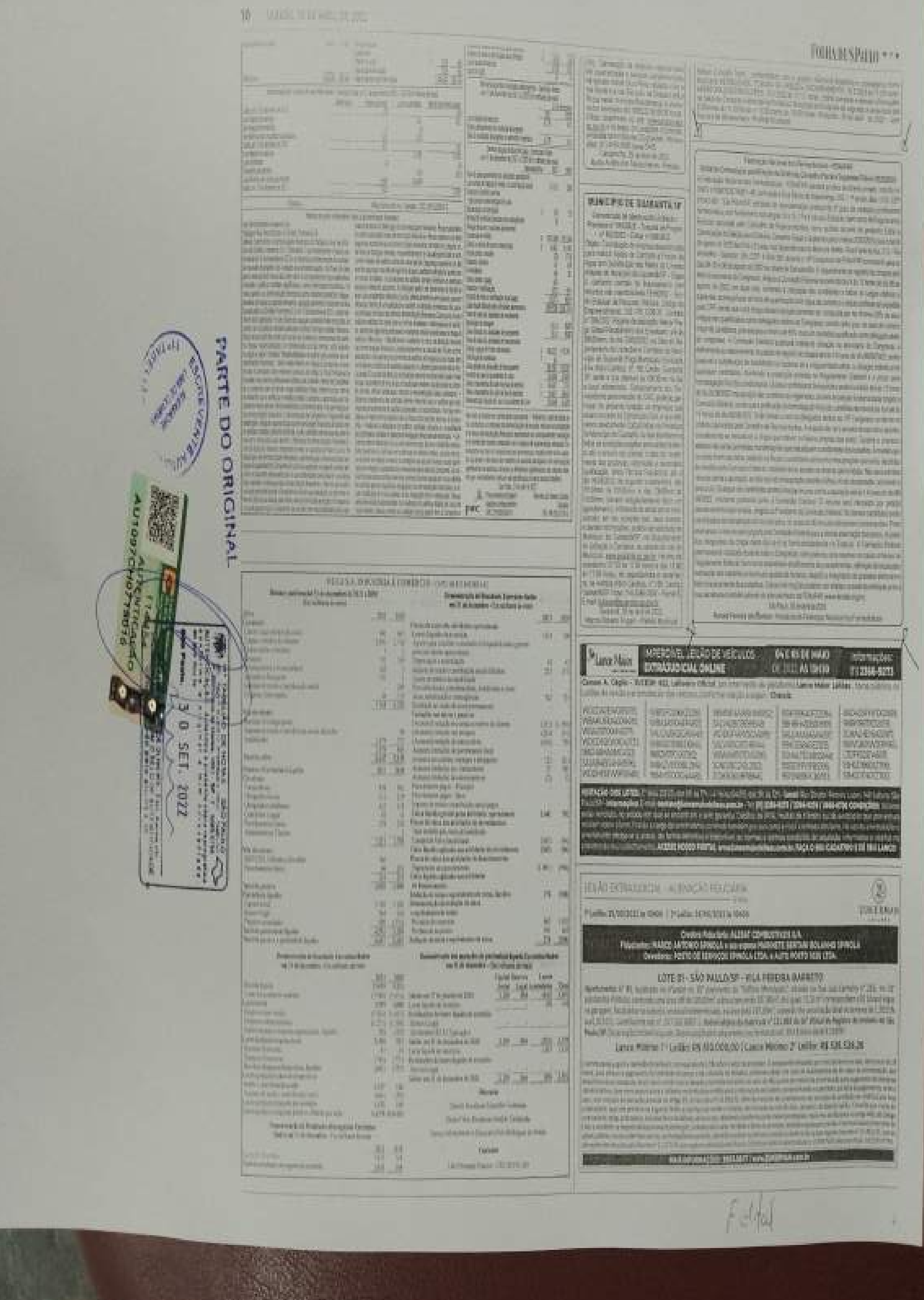

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FEDERAÇÃO NACIONAL DOS ATLETAS PROFISSIONAIS DE FUTEBOL

EDITAL
ABERTURA DE PRAZO

Conforme votado e aprovado pelos presentes, de forma unânime na assembleia realizada em 24 de janeiro de 2022, Felipe Augusto Leite foi afastado da presidência sob as considerações que obedecem rigorosamente os ditames estatutários, artigo 59 do Código Civil, legislação vigente e decisão assemblear, sendo assim, a partir da presente data fica ABERTO O PRAZO para, querendo, Felipe Augusto Leite, apresente sua defesa com suas razões e instruindo com os documentos comprobatórios quanto as irregularidades apontadas: 1.Falta de capacidade sindical por nunca ter sido jogador profissional; 2. Uso do PIS do Atleta Álvaro Felipe da Silva; 3. Utilização de CTPS de série 009 quando a do atleta profissional válida era a de número 600; 4. Apresentação de cópia de contrato de trabalho com registro na CBF com período igual ou superior a dois anos; que responda as afrontas estatutárias: artigo 7º, retirou o valor de 4.000,00 a título de solidariedade dos sindicatos que não tinham receita e manteve a liberação do valor mensal para o seu sindicato, RN; inciso XIV do mesmo artigo, promoveu a suspensão dos recursos de todos os sindicatos, menos do sindicato do RN, e remeteu, por busca de apoio político, R$ 5.000,00 de solidariedade para os sindicatos filiados que lhe davam a base de sustentação; mesmo artigo, inciso XVI, omissão ao não promover cobranças judiciais referente a 0,2% das transferências nacionais e internacionais conforme art. 57 da lei 9.615/98, cujo valor atual ultrapassa a 12 milhões de reais; artigo 10, o Sindicato dos Atletas Profissionais de Futebol do Município de São Paulo, admitido não poderia figurar como filiado na Fenapaf que é uma entidade que reúne sindicatos estaduais; também não tem Código Sindical fornecido pelo órgão competente, logo, encontra-se irregular e mesmo assim foi beneficiado com o valor de 5.000,00 mil reais mês, sindicato municipal que foi articulado pelo presidente da Fenapaf dentro da FPF, entidade patronal que somente defende interesse dos clubes; no artigo 11, na caracterização de busca de apoio político, acolhe o Sindicato do município de São Paulo que não possui os requisitos exigidos no estatuto; artigos 12 e 13: o Sindicato do Município de São Paulo além de irregular teve o seu representante aceito como se admitido fosse; e a irregularidade transcende a questão de simples participação porque teve voto nas assembleias, assinando todas as atas e reuniões que participou; artigo 14, irregularidade, a Fenapaf, conforme confessado em edital publicado por Felipe Augusto Leite no dia 17 de janeiro de 2022 (Diário Oficial da União nº 11 - Seção 3 - pag. 119), não tem composição de Conselho Fiscal, questão que deveria ser submetida a uma regular eleição; artigo 15, atuais Conselho Fiscal e Diretoria Executiva deveriam ser eleitos e recompostos no prazo estatutário de 90 dias; tentativa da supressão do Conselho Deliberativo; art. 21, Conselho Fiscal foi desfeito e teria que ser recomposto. Art.24, a Diretoria Executiva não deliberou, nem o Conselho Fiscal, as inúmeras doações a Academia Nacional de Direito Desportivo, instituição que vem trabalhando arduamente em termos políticos para suprimir direitos dos atletas profissionais; artigo 21: "o", "q" e "r", tais incisos deixam clara a obrigação de eleição para preenchimento das vacâncias e não indicação como fez o presidente; a atuação de Felipe Augusto Leite mostra que a série de irregularidades e improbidades constatadas e demonstradas por atitudes de descumprimentos ferem o estatuto, não cumpre com as determinações mandamentais; o artigo 25: como ordenador de despesas, explicita na alínea "c", não é possível isentá-lo das irregularidades financeiras/administrativas; item "d", condição de ordenador de despesas lhe impõe todas as responsabilidades que não foram atendidas; artigo Art.28, entre outras competências do diretor financeiro, a principal é de apresentar ao presidente os balanços do exercício e patrimonial, previsão de orçamento, dar ciência da situação econômica e financeira da FENAPAF, sendo obrigado a promover, inclusive, medidas cabíveis para resguardar a saúde financeira da entidade, se tornando conivente e principal responsável pelos desmandos financeiros; artigo 30, total desrespeito às ordens internas, não recompôs o Conselho Fiscal, além de tentar extinguir o Conselho Deliberativo ignorando completamente a determinação estatutária; artigo: Art. 47, deveria promover uma nova eleição para o preenchimento das vagas; artigo 67, a análise aponta como principais irregularidades neste quesito o fato de o presidente não ter sido atleta profissional, ou seja, não pertencer a categoria a, ainda, se utilizar de número de documento falso no caso do PIS, quando das assinaturas das atas de eleição e posse nas eleições de 2016 e 2020. A análise identifica que o número do PIS em questão pertence ao atleta Álvaro Felipe da Silva, natural do Rio Grande do Norte; o artigo 68: ações de mesma responsabilidade Felipe Augusto Leite, porém, não executadas. Outras irregularidades. Publicou edital no DOU no dia 17 de janeiro de 2022 (Diário Oficial da União nº 11 - Seção 3 - pag. 119), sem o prazo mínimo estatutário de 15 dias com o intuito de embaraçar a realização da assembleia que discutiria seu afastamento, sendo que foi devidamente convocado para tanto; nova tentativa de exclusão dos sindicatos filiados sem qualquer regular processo visando garantir a ampla defesa e o contraditório. Salientando que essa é uma prática de Felipe Augusto Leite, a das exclusões arbitrárias, fato já tratado e reparado, inclusive em segunda instância, no processo 0000313-13.2017.5.10.0017 da 17ª Vara do Trabalho de Brasília, sentença que ele vem resistindo e com isso causando prejuízos financeiros para a instituição, reconhece a decisão judicial reintegrando os membros e sindicatos em procedimento eleitoral para sua reeleição, mas não deu ciência a nenhum dos reclamantes, se utilizando desse artifício para tentar legitimar sua reeleição, ainda, contratou escritório de advocacia em Brasília, destituindo o patrono inicial, para seguir no processo contestando a decisão em forma de recurso, além do Recurso Ordinário, ajuizou outros dois, cujas decisões foram desfavoráveis a Fenapaf, causando mais prejuízos financeiros à Fenapaf; na Ação de Exigir Contas (1001573-16.2018.5.02.0086) a sentença confirma a retenção de R$ 4.350.000,00 (quatro milhões, trezentos e cinquenta mil reais) do Sindicato de São Paulo, valor ainda a ser atualizado, no qual ele resiste, aumentando os danos financeiros da Fenapaf; desmontou a Diretoria e o Conselho Fiscal e tentou governar nomeando membros irregulares sem indicação de sindicatos filiados, nomeou, embora não conseguisse o registro formal, uma diretoria e o Conselho Fiscal em total desrespeito às normas estatutárias; nomeou, inclusive, como diretor financeiro (Washington Luís Mascarenhas) que não era delegado de nenhum sindicato filiado e deu a ele acesso às finanças da entidade; pelas irregularidades provocou o bloqueio das contas correntes da Fenapaf, questão que causou um prejuízo enorme para os sindicatos filiados e a própria federação; por negligência de Felipe Augusto Leite em não responder às informações requeridas em dois processos que tramitam em Belo Horizonte a Fenapaf já teve bloqueado o valor de R$ 100.000,00 (cem mil reais) e conta com o risco e possibilidade de multa, pela reincidência, de R$ 1.000.000,00 (um milhão de reais), processos que tramitam na 19ª Vara do Trabalho sob números 00108272-52.2016.5.03.0019 e 0010827-57.2016.5.03.0113; promoveu perseguições aos sindicatos filiados causando verdadeiro estrago na representatividade; cedeu direitos à CBF em processo que a gestão anterior havia conseguido decisão favorável de 72 horas de intervalo de descanso reduzindo para 66 horas sem nenhuma contrapartida para a categoria; cedeu novamente direito da categoria reduzindo o intervalo ainda mais, para 44 horas, sem contrapartida novamente em prejuízo para a categoria; apoio a ANDD (Academia Nacional de Direito Desportivo) financeiramente, instituição formada somente por representantes patronais que trabalha arduamente contra os atletas profissionais no TST; se manifestou em processos judiciais contra os sindicatos e atletas; indicou para o STJD Paulo Feuz, advogado patronal da Federação Paulista de Futebol e Sindicato de Clubes de Futebol de SP; não realizou a prestação de contas e previsão orçamentária para os anos de 2019, 2020 e 2021; a tentativa de organização de assembleia virtual em 20 de janeiro de 2022, com publicação em edital no dia 17 de janeiro de 2022 somente para embaraçar a realização dessa sessão; como confirmado, questão de desconfiança anterior, não disponibilizou o link de acesso a nenhum dos sindicatos que não fazem parte do seu grupo de apoio político apesar de, ainda, terem solicitado; irregular toda a ordem do dia da assembleia convocada para o dia 20 de janeiro de 2022 por Felipe Augusto Leite; a retenção irregular de valores do direito de arena a serem repassados aos sindicatos; a retenção total de repasse aos sindicatos de Goiás e Paraíba e no caso de Goiás repassando direto aos atletas desrespeitando a legislação vigente; mudança da sede administrativa para Natal - RN, sem haver previsão estatutária para evitar a realização de assembleias que pudessem tratar de temas a ele desfavoráveis. Defesa que deverá ser encaminhada para o presidente em exercício, Alfredo Sampaio da Silva Junior, no endereço eletrônico alfredosampaiojunior@gmail.com e para o Conselheiro Deliberativo Rinaldo José Martorelli no endereço eletrônico martorelli@sapesp.com.br , ambos com solicitação de lido, até o dia 28 de fevereiro de 2022, tudo de acordo com os mandamentos estatutários. Após esse prazo o Conselho Deliberativo e Diretoria emitirão parecer que será analisado, votado, aprovado ou não na assembleia que será realizada em 07 de março de 2022. A presente notificação de abertura de prazo também é enviada para os endereços eletrônicos utilizados de forma oficial até a presente data por Felipe Augusto Leite: marcela@fenapafoficial.com.br ; fenapaf@fenapafoficial.com.br ; e para o seu endereço pessoal felipeaugustoleite@hotmail.com .

Brasília, 27 de janeiro de 2022.
ALFREDO SAMPAIO DA SILVA JUNIOR
Presidente em Exercício
RINALDO JOSÉ MARTORELLI
Conselheiro Deliberativo

FEDERAÇÃO NACIONAL DAS EMPRESAS DE SEGUROS PRIVADOS, DE CAPITALIZAÇÃO E DE PREVIDÊNCIA COMPLEMENTAR ABERTA FENASEG

CNPJ: 33.621.893/0001-80

AVISO DE REGISTRO DE CHAPA

Pelo presente edital de aviso de registro de chapas, faço saber que foi registrada a chapa única abaixo elencada, para concorrer as eleições da Federação Nacional das Empresas de Seguros Privados, de Capitalização e de Previdência Complementar Aberta - Fenaseg, para o mandato de 30/04/2022 a 29/04/2025, conforme edital publicado no Diário Oficial da União no dia seis de janeiro do corrente ano e enviado aos Sindicatos filiados. Nos termos do Estatuto Social da Entidade, o prazo para impugnação da chapa ou de candidato é de 3 (três) dias úteis, contados a partir da presente divulgação e poderá ser realizado através do encaminhamento ao e-mail secretariageral@cnseg.org.br, das 9h às 17h. Chapa única - Conselho Diretor - Membros: Antonio Eduardo Márquez de Figueiredo Trindade (Chubb Seguros Brasil S/A), Camila de Freitas Aichinger (Caixa Seguradora S/A), Edson Luís Franco (Zurich Minas Brasil Seguros S/A), Eduard Folch Rue (Allianz Seguros S/A), Eduardo Nogueira Domeque (Itaú Seguros S/A), Eduardo Stefanello Dal Ri (HDI Seguros S/A), Erika Médici Klaffke (Axa Seguros S/A), Felipe Costa da Silveira Nascimento (Mapfre Previdência S/A), Francisco Alves de Souza (COMPREV Vida e Previdência S/A), Helder Molina (Mongeral Aegon Seguros e Previdência S/A), Ivan Luiz Gontijo Junior (Bradesco Seguros S/A), José Adalberto Ferrara (Tokio Marine Seguradora S/A), Leonardo Deeke Boguszewski (Junto Seguros S/A), Luciano Snel Corrêa (Icatu Capitalização S/A), Manoel Antonio Peres (Bradesco Saúde S/A), Marcelo Gonçalves Farinha (Brasilcap Capitalização S/A), Marcelo Malanga (Zurich Santander Brasil Seguros e Previdência S/A), Patrícia Andrea Freitas Velloso dos Santos (Prudential do Brasil Seguros de Vida S/A), Patricia Chacon Jimenez (Liberty Seguros S/A), Pedro Cláudio de Medeiros Bocayuva Bulcão (Sinaf Previdencial Cia. de Seguros), Pedro Pereira de Freitas (American Life Companhia de Seguros), Ricardo Bottas Dourado dos Santos (Sul América Companhia de Seguro Saúde), Roberto de Souza Santos (Porto Seguro Companhia de Seguros Gerais) e Ulisses Christian Silva Assis (Brasilprev Seguros e Previdência S/A) - Conselho Fiscal - Efetivos: Haydewaldo Roberto Chamberlain da Costa (Bradesco Seguros S/A), Lúcio Pereira dos Santos (Sul América Companhia de Seguro Saúde) e Gleice Coelho Ferreira (Chubb Seguros Brasil S/A) e Suplentes: Celso Damadi (Porto Seguro Companhia de Seguros Gerais) e Maria Antonieta dos Reis Roto Scarlassari (Zurich Minas Brasil Seguros S/A).

Rio de Janeiro, 27 de janeiro de 2022.
MARCIO SERÔA DE ARAUJO CORIOLANO
Diretor Presidente

FEDERAÇÃO NACIONAL DOS FARMACÊUTICOS

EDITAL DE CONVOCAÇÃO
10º CONGRESSO DA FEDERAÇÃO NACIONAL DOS FARMACÊUTICOS

A Federação Nacional dos Farmacêuticos - FENAFAR, pessoa jurídica de direito privado, inscrita no CNPJ nº 00679357/0001- 48, com sede à rua Barão de Itapetininga, 255, 11º andar, sala 1105, CEP 01042-001, São Paulo/SP, entidade de representação sindical de 2º grau da categoria dos farmacêuticos, com fundamento em seu Estatuto Social, torna público que o Conselho de Representantes da Fenafar, reunido no dia 24 de janeiro de 2022, POR MEIO ELETRÔNICO, CONVOCOU o 10º Congresso da FENAFAR para realizar-se entre os dias 04, 05 e 06 de agosto de 2022 na cidade de Salvador/BA, se as condições sanitárias permitirem, ou de forma virtual ou híbrida e ainda que o mesmo Conselho de Representantes designou a Comissão Organizadora do 10º Congresso, a Comissão Eleitoral e aprovou o Regulamento Eleitoral. O Congresso delibera sobre as disposições contidas no Art. 12 do Estatuto da entidade, que dispõe: "Art. 12: O Congresso da FENAFAR é o órgão máximo de deliberação da categoria farmacêutica, sendo composto por delegados eleitos na sua base e observadores convidados. Parágrafo primeiro: o Congresso da FENAFAR delibera sobre: I- Alterações deste Estatuto; II- A linha política e organizativa da Entidade; III- Os planos de ação sindical e de luta para os trabalhadores representados considerando a situação política, social e econômica do país; IV- Eleição da Diretoria da FENAFAR e Conselho Fiscal, conforme normas estabelecidas no Regulamento Eleitoral. Parágrafo segundo: é dever do Congresso da FENAFAR promover a solidariedade nacional e internacional da classe trabalhadora". A Fenafar informa ainda que a Eleição deverá ser convocada pelo Presidente com pelo menos 90 dias de antecedência, da qual será publicado Edital específico, com as informações pertinentes a este processo eleitoral. Informa ainda que outras informações serão oportunamente fornecidas tais como o local, a programação etc.; e que quaisquer informações sobre o 10º Congresso e a Eleição poderão ser obtidas na sede da entidade junto à sua secretaria e através do sítio eletrônico da FENAFAR.

São Paulo, 26 de janeiro de 2022.
RONALD FERREIRA DOS SANTOS
Presidente da Federação Nacional dos Farmacêuticos

FEDERAÇÃO NACIONAL DOS SERVIDORES DOS MINISTÉRIOS PUBLICOS ESTADUAIS - FENAMP

EDITAL Nº 1/2022
CONVOCAÇÃO PARA II CONGRESSO ORDINÁRIO DA FENAMP

A Coordenação Executiva da Federação Nacional dos Servidores dos Ministérios Públicos Estaduais (FENAMP), com base no Art. 12, inciso I e § 1º, e Art.22, inciso I, do Estatuto, CONVOCA todos os Sindicatos Filiados à FENAMP para o II Congresso Ordinário, que ocorrerá no dia 27 de março de 2022, às 08h, em ambiente híbrido, sendo: Presencial no auditório do Cullinan Hplus Premium, situado no SHN (Setor Hoteleiro Norte), Quadra 04, Bloco E, Asa Norte, Brasília/DF, CEP 70.704-050, e Virtual, através da Plataforma Google Meet, para discutir e deliberar a seguinte pauta:

1. Análise de Conjuntura e Balanço Organizativo;
2. Definição da Pauta de Lutas 2022 e Aprovação do Plano de Ação 2022;
3. Prestação de Contas 2021 e Previsão Orçamentária 2022;
4. Proposta de Alteração da Mensalidade
5. Eleição do sistema diretivo da FENAMP;
6. Outros assuntos.

Brasília-DF, 26 de janeiro de 2022.
ERICA OLIVEIRA DE SOUZA
Coordenadora Executiva

<u>PROCURAÇÃO *ad judicia*</u>

Federação Nacional dos Farmacêuticos, pessoa jurídica de direito privado, sem fins lucrativos, constituída na forma da lei, registrada no CNPJ sob o n° 00.679.357/0001-48, com sede na Rua Barão de Itapetininga, 255, 3 andar sala 302, República, São Paulo, na pessoa de seu representante nos termos de seu Estatuto Social, por seu presidente, Fábio José Basílio, brasileiro, casado, Farmacêutico, RG n°3207835 emitido pelo SESP GO, e inscrito no CPF n° 830.864.801-06, vem pelo presente instrumento outorgar procuração *ad* judicia à advogada **SUSANA RODRIGUES CAVALCANTI VAN DER PLOEG**, inscrita no CPF 013.497.254-63 e na **OAB/MG 181.599**, com escritório na Avenida Presidente Vargas, 446, 13º andar, Centro - Rio de Janeiro - RJ, CEP 20071-907, concedendo-lhe poderes da cláusula *ad judicia et extra*, inclusive substabelecer com reserva de poderes, especificamente para apresentação de subsídio ao exame técnico e/ou processo administrativo de nulidade perante o INPI - Instituto Nacional da Propriedade Industrial relacionado à <u>patente de invenção **BR112019022972-8**</u>.

São Paulo, 12 de maio de 2025.

FEDERACAO NACIONAL DOS FARMACEUTICOS :00679357000148

Assinado de forma digital por FEDERACAO NACIONAL DOS FARMACEUTICOS:00679357000148
Dados: 2025.05.12 17:59:19 -03'00'

Fábio José Basílio

Presidente da Federação Nacional dos Farmacêuticos

ESTATUTO - FÓRUM ONG AIDS RS

CAPÍTULO I
DA DENOMINAÇÃO, SEDE E FINS

Art 1º O Fórum ong aids RS, fundado em 28 de agosto de 1999, com sede e foro em Porto Alegre, capital do Rio Grande do Sul, é uma Associação civil, privada, autônoma, sem vinculação político-partidária, social e / ou religiosa, sem fins econômicos e de duração indeterminada.
Parágrafo único: O Fórum não distribui a Dirigentes, Coordenadores, Associados, Instituidores, Credenciados, Conselheiros, Benfeitores ou Mantenedores, qualquer parcela de seu patrimônio ou de suas rendas, a título de lucro ou participação no seu resultado.

Art 2º O Fórum ong aids RS é uma articulação Estadual de organizações da sociedade civil que atuam no âmbito da síndrome da imunodeficiência adquirida (aids), e suas implicações.
Parágrafo único: A missão do Fórum ong aids RS é ampliar e articular políticas de prevenção e assistência às DST/HIV/aids e suas coinfecções, bem como colaborar no fortalecimento político das instituições que atuam no âmbito da aids no Rio Grande do Sul, incluindo o acesso aos direitos humanos e justiça social.

Art 3º São objetivos do Fórum ong aids RS:
I- Incentivar o intercâmbio e interajuda entre as associações e movimentos;
II- Analisar, incentivar e promover campanhas de prevenção, apoio e educação;
III- Orientar, acompanhar e denunciar qualquer tipo de violação das leis vigentes que prejudiquem os direitos e os deveres das associações que participam, ou não, do Fórum ong aids RS;
IV- Elaborar propostas conjuntas, visando fortalecer a ação das Instituições e movimentos sociais que atuam na luta contra a aids e suas coinfecções, no estado do Rio Grande do Sul, perante as autoridades públicas, civis e religiosas;
V- Influir na legislação pertinente no sentido de conquistar e assegurar novos direitos e/ou alterar dispositivos contrários ou prejudiciais à prevenção das DST/HIV/aids e suas coinfecções, bem como a assistência as pessoas infectadas pelo HIV/aids;
VI- Intervir e participar no processo de formulação de políticas públicas de saúde para que sejam definidas políticas de prevenção e controle da aids, bem como de assistência as pessoas que vivem com HIV/aids;
VII- Incentivar a participação das Instituições associadas nos Conselhos Municipais e Estaduais de Saúde, Assistência Social, Educação, Criança e Adolescente, Idosos, Direitos Humanos e Cidadania e outras instâncias deliberativas e nas comissões Municipais, Estadual e Nacional de DST/aids e outras instâncias consultivas, a fim de fortalecer o papel político-social das Instituições no desenvolvimento do controle social;
VIII- Denunciar todas as formas de omissão, transgressão e violação dos direitos humanos, civis, políticos e sociais, resultados de discriminação as pessoas que vivem com HIV/aids e buscar mecanismos para responsabilizar e punir os (as) infratores (as) de tais atos;
IX- Apoiar e repercutir as ações das Instituições associadas, sempre que estas coincidirem com os princípios do coletivo do Fórum, respeitando suas identidades, autonomia e dinâmicas próprias de funcionamento;
X- Divulgar informações e incentivar / promover ações (palestras, seminários, cursos, oficinas, assessorias e outros eventos) que visem a sustentabilidade das Instituições e seu desenvolvimento técnico e político.
XI- Promover campanhas e outros eventos com finalidade de levantar recursos que possibilitem a consecução da missão da Associação, bem como o fortalecimento político e técnico;
XII- Entrar em contato, desenvolver ações conjunta com entidades civis, públicas ou privadas, nacionais ou internacionais, para viabilizar o cumprimento dos objetivos;
XIII- Buscar financiamentos que propiciem a execução das atividades e ações, visando garantir o alcance da missão.
Parágrafo único – Para cumprir seu propósito, o Fórum ong aids RS atuará por meio da execução direta ou indireta de projetos, programas ou planos de ações, ou execução de ações e atividades de apoio a outras organizações e a órgãos do setor público que atuam em áreas afins.

CAPÍTULO II
DOS ASSOCIADOS, DIREITOS E DEVERES

Art 4º Poderão associar-se ao Fórum ong aids RS as Instituições, Redes e Movimentos que atuam no âmbito da aids, apresentando os seguintes documentos:
a) ata de fundação da Instituição;
b) ata de posse da atual Diretoria;
c) estatuto;
d) c n p j ;

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e) ofício solicitando adesão indicando um representante titular e um suplente, assinado pela coordenação da instituição
§ 1º: Em se tratando de Movimentos ou Redes não institucionalizados, esses poderão participar do Fórum com um representante do seu coletivo, e mediante apresentação de ata de eleição de reunião do seu coletivo. § 2º - Os interessados em associar-se ao Fórum ong aids RS terão o prazo máximo de 06 (seis) meses para regularização de sua situação jurídica dentro dos critérios supramencionados.

Art 5º O Fórum ong aids RS se comporá e funcionará com a participação de pelo menos um representante titular – ou seu suplente – de cada Instituição a ele associado.
I – somente terá direito a voto os representantes titulares ou, na sua ausência, os suplentes das Instituições associadas.
II – os representantes titulares e suplentes deverão defender posicionamentos de suas Instituições, responder por deliberações defendidas nos encontros do Fórum ong aids RS, bem como repassar as informações, propostas e encaminhamentos para os membros de suas organizações.
III – o Fórum ong aids RS está aberto à participação de qualquer pessoa física ou jurídica, interessada em colaborar para o crescimento do movimento de luta contra as DST/HIV/aids garantindo, no entanto, o direito a voto somente as Instituições associadas.
IV – em situações especiais, havendo interesse, o Fórum ong aids do RS poderá convidar profissionais ou representante de Órgãos, Instituições , Conselhos, Câmaras Éticas e Técnicas e outros que possam contribuir com os objetivos definidos neste estatuto, bem como auxiliar na execução da missão.
V – quando houver ações ou projetos em nome do Fórum ong aids RS, envolvendo recursos financeiros, cabe à Coordenação, o gerenciamento e a prestação de contas dos mesmos.

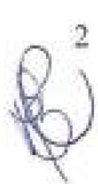
Art 6º São direitos dos associados, em dia com seus deveres:
a) participar das AssembleiaAssembleias
b) participar de todas as atividades a que esteja o Fórum ong aids RS direta ou indiretamente ligado;
c) ter acesso às atas de reuniões e Assembleias e aos livros contábeis
d) representar o Fórum ong aids RS, desde que autorizado pela Coordenação, em contatos com o público e com outras Instituições;
e) votar e ser votado em indicações para representações e participações.
Parágrafo Único: O direito a ser votado para os cargos que compõem a Coordenação do Fórum é privativo das Instituições associadas.

Art 7º São deveres dos associados:
a) respeitar, cumprir e fazer cumprir o estatuto e demais atos normativos do Fórum ong aids RS;
b) zelar pelo nome e imagem do Fórum ong aids RS, seu patrimônio e empenhar-se pela consecução de seus objetivos;
c) acatar os atos e decisões dos órgãos diretivos;
d) exercer o cargo para o qual for eleito, salvo se houver motivo de força maior, plenamente justificável;
e) manter-se informado e prestar informações ao público em geral sobre as formas de infecção e meios de prevenção das DST/HIV/aids e suas coinfecções, sempre que possível;
f) estimular atitudes que neutralizem o preconceito e a discriminação social às pessoas que vivem com HIV/aids e suas coinfecções;
g) denunciar à Coordenação qualquer atitude individual, coletiva ou Institucional que seja lesiva aos direitos humanos das pessoas que vivem com HIV/aids;
h) guardar sigilo ético sobre informações, nomes, dados pessoais que venha a receber sobre as pessoas que vivem com HIV/aids;

Art 8º Os associados não respondem, individual ou subsidiariamente pelas obrigações sociais do Fórum ong aids RS.
Parágrafo único: Não há entre as Instituições associadas, direitos e obrigações recíprocos.

CAPÍTULO III
DAS PENALIDADES

Art 9º Os associados que infringirem este Estatuto e as demais normas internas estarão sujeitos às seguintes penalidades:
I. advertência escrita;
II. Suspensão de 15 (quinze) dias a 12 (doze) meses;
III. Exclusão
Parágrafo único – No caso de aplicação das penalidades dos incisos "II" e "III" desse artigo, o associado poderá interpor recurso a Assembleia Ordinária no prazo de 15 dias, dirigido a Coordenação que deverá



convocar uma Assembleia nos termos e prazos determinados neste estatuto, contado da data em que teve ciência da penalidade, devendo, enquanto pendente a decisão, permanecer afastado da Instituição.

CAPÍTULO IV
DOS ÓRGÃOS CONSTITUITIVOS DO FÓRUM ONG AIDS RS

Art 10 São órgãos constitutivos do FÓRUM ONG aids RS:
I – Assembleia Ordinária
II – Coordenação
III - Conselho

SEÇÃO I
DA ASSEMBLEIA GERAL

Art 11 A Assembleia Ordinária é o órgão supremo do Fórum ong aids RS, dentro dos limites deste estatuto, para tomar toda e qualquer decisão, sendo para tanto soberana, reunindo-se ordinariamente uma vez por ano, em local determinado pela Coordenação, e convocada com antecedência mínima de 30 (trinta) dias. Poderá reunir-se extraordinariamente quando convocada pela Coordenação ou por 1/5 (um quinto) dos associados, obedecendo a antecedência mínima de 10 (dez) dias.
§ 1º Na realização da Assembleia Ordinária seguir-se-ão as seguintes normas:
a) instalar-se-á em primeira chamada com a presença mínima de 2/3 (dois terços) dos associados ou, em segunda chamada, com qualquer número de associados presentes, sendo as deliberações tomadas pela aprovação de maioria simples;
b) será dirigida por um membro da Coordenação;
c) a própria Assembleia decidirá sobre as normas específicas para o seu funcionamento.
§ 2º A Assembleia extraordinária será realizada sempre que algum assunto urgente de interesse social o exija, inclusive para eleger vacâncias de cargos da Coordenação e do Conselho.
§ 3º Na Assembleia ordinária será proibido o voto por representação
§ 4º A convocação das Assembleias gerais ordinárias e extraordinárias será feita através de correspondência para as Instituições associadas, podendo ser por meio eletrônico.

Art. 12 Em especial compete à Assembleia Ordinária:
I - Eleger a Coordenação e o Conselho;
II - Destituir a Coordenação e o Conselho;
III - Aprovar a escrituração contábil, balanços anuais e planos de trabalho;
IV -Realizar e aprovar alteração estatutária.
Parágrafo único: Para aprovar alteração no Estatuto e destituir a Coordenação é necessário o voto concorde de 2/3 dos associados presentes à Assembleia convocada especialmente para este fim, não podendo esta deliberar em 1ª convocação sem a maioria absoluta dos associados e nas convocações seguintes com menos de 1/3 dos associados.

SEÇÃO II
DA COORDENAÇÃO

Art 13 A Coordenação do Fórum ong aids RS é o órgão deliberativo e normativo no intervalo das Assembleias e com funções executivas através de seus membros; será eleita por voto direto, durante a Assembleia geral ordinária dos associados, e será constituída de três representantes titulares e três suplentes das Instituições associadas a saber :
I - **Coordenação Técnica**
II - Coordenação Executiva
III - Coordenação Financeira
§ 1º A duração do mandato dos membros da Coordenação é de 03 (três) anos renovado em 1/3 a cada ano, podendo cada um dos seus membros ser reeleito ou eleito mais de uma vez para outro cargo, em períodos consecutivos, desde que sujeitos a processo eleitoral. § 2º A posse da Coordenação dar-se-á após o término do ato eleitoral.
§ 3º Os membros da Coordenação são voluntários e não receberão, sob título algum remuneração por suas funções diretivas do Fórum ong aids RS.
§ 4º **Compete a Coordenação**:
a) Representar o Fórum ong aids RS – ativa, passiva, judicial e extrajudicialmente;
b) Reunir-se pelo menos uma vez ao mês;
c) Cumprir e fazer cumprir este Estatuto e Regimento Interno;
d) Interpretar e fiscalizar a observância do Estatuto e as decisões da Assembleia Geral;

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e) Redigir, submeter à aprovação da Assembleia Geral Ordinária e por em execução o plano de ação para cada exercício;
f) Aprovar balancetes anuais;
g) Autorizar a celebração de contratos e distratos;
h) Aceitar subvenções, doações, donativos e legados;
i) Aplicar os fundos sociais;
j) Autorizar operações de crédito e a compra de títulos, móveis e imóveis;
k) Gerir bens patrimoniais;
l) Apresentar à Assembleia geral ordinária o balanço do exercício anterior e o orçamento do exercício futuro para aprovação;
m) Convocar Assembleia Geral;
n) Autorizar despesas orçamentárias e extraordinárias;
o) Tomar conhecimento e apreciar os atos dos seus, praticados no desempenho de suas funções;
p) Apreciar e deliberar sobre os planos de trabalho do Fórum ong aids RS;
q) Apreciar e deliberar sobre os relatórios do Fórum ong aids RS.
§ 5º **Em especial compete a Coordenação Técnica**
a) Convocar as Assembleias Gerais;
b) Assinar e rubricar todos os livros do Fórum ong aids RS;
c) Representar o Fórum ong aids RS nas suas relações externas ou designar associados para representá-lo nesta função;
d) Defender, perante as autoridades, os interesses do Fórum ong/aids RS, assim como representá-lo em juízo;
e) Constituir advogado e acompanhar as causas judiciais do Fórum ong aids RS, ou designar associados para tanto;
f) Promover sindicância ou inquérito quando ocorrer irregularidade;
g) Decidir e tomar providências, em caso inadiável e imprevisto, submetendo o seu ato à Coordenação na primeira Assembleia ordinária que esta realizar;
h) Substituir o Coordenar Executivo ou Financeiro, em suas faltas ou impedimentos.
§ 6º **Em especial compete a Coordenação Executiva:**
a) Substituir o Coordenador Técnico ou Financeiro, nas suas faltas e impedimentos;
b) Lavrar e ler as atas das reuniões e Assembleias;
c) Organizar o arquivo, tendo sob sua responsabilidade papéis, livros e documentos;
d) Expedir e receber correspondências, designando associados para auxiliá-lo nesta tarefa;
e) Elaborar ao final de cada exercício, relatório geral das atividades do Fórum ong aids RS, que será submetido à Assembleia ordinária;
f) Assinar todos os livros, juntamente com o Coordenador Técnico; § 7º **Em especial compete a Coordenação Financeira**:
a) Ter sob sua guarda os valores e fundos pertencentes ao Fórum ong aids RS;
b) Controlar movimento financeiro;
c) Promover arrecadação de rendas e o recebimento de importâncias creditadas ao Fórum ong aids RS;
d) Realizar pagamentos;
e) Assinar, com a Coordenação Técnica ou com a Coordenação Executiva os livros contábeis;
f) Organizar e assinar toda a escrituração contábil, apresentando balanços anuais para apreciação da Coordenação e Conselho Fiscal em Assembleias Gerais.
g) Substituir o Coordenador Técnico ou Executivo, nas suas faltas ou impedimentos.

SEÇÃO III
DO CONSELHO

Art. 14 O Conselho será composto de três representantes titulares e três representantes suplentes dos associados, eleitos pelos associados votantes.
Parágrafo único: Compete ao Conselho:
a) Prestar assessoria e auxiliar a Coordenação em suas funções;
b) Reunir-se sempre que necessário ou por convocação da Coordenação;
c) Eleger, entre seus membros, um coordenador para dirigir os trabalhos do Conselho;
d) . Auxiliar a Coordenação nas representações externas.

Art 15 Cabe aos suplentes substituir qualquer membro do Conselho em caso de vacância do cargo.

Art 16 O mandato do Conselho é de 03 (três) anos, renovando 1/3 cada ano, podendo cada um dos seus membros ser reeleito, em períodos consecutivo, desde que sujeitos a processo eleitoral.

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CAPÍTULO IV
DO PATRIMÔNIO, ECONOMIA E FINANÇAS

Art 17 O patrimônio do Fórum ong aids RS será constituido pela totalidade de bens, direitos e obrigações que o mesmo venha a possuir ou contrair a qualquer titulo.
§ 1º: Em caso de extinção da Instituição, por nitida impossibilidade de funcionamento, a critério da Assembleia I Extraordinária especialmente convocada para este fim, e por decisão de 2/3 (dois terços) dos associados votantes presentes, seu patrimônio liquido será destinado à entidade de fins não econômicos, estadual ou federal, de fins idênticos.
I - Inexistindo esta, o remanescente do patrimônio será encaminhado para Instituições afins do Estado do Rio Grande do Sul, registrada no Conselho Estadual de Assistência Social.
II - Não existindo no Estado Instituições nas condições indicadas, o patrimônio será devolvido a Fazenda do Estado.
§ 2º - Para instalação da Assembleia Extraoridnária para este fim específico se requer a presença de 2/3 (dois terços) dos associados do Fórum ong aids RS.

Art 18 Constituem rendas as subvenções, legados, auxilios, remissões, doações, contribuições que forem feitas, os juros, aluguéis, dividendos provenientes de serviços e por outros recursos legalmente adquiridos.

Art 19 O Fórum ong aids RS poderá abrir contas e fazer operações bancárias de qualquer natureza.
Parágrafo único: Em todas as operações bancárias e outras do mercado financeiro, terá competência para assinar sempre o Coordenador Financeiro, podendo estar acompanhado de mais um Coordenador em conjunto.

Art 20 O Fórum ong aids RS poderá contratar serviços remunerados, tanto com vínculo empregatício, como por prestação de serviços, segundo a legislação em vigor, de acordo com as necessidades das ações e projetos a serem desenvolvidos.
Parágrafo único: Os membros dirigentes do Fórum ong aids RS, só responderão, individual ou subsidiariamente, pelas obrigações contraídas em nome da instituição, em caso de atos lesivos ao seu patrimônio e infringentes ao presente Estatuto.

CAPÍTULO V
DAS DISPOSIÇÕES GERAIS E TRANSITÓRIAS

Art 21 Como exercicio será considerado o ano civil.

Art 22 As eleições do Fórum ong aids RS serão regulamentadas pela Coordenação Geral resguardando o disposto neste estatuto.

Art 23 Os casos omissos neste Estatuto serão resolvidos pela Coordenação e ratificados pela Assembleia.
Parágrafo único: A organização, direção e forma de funcionamento da Instituição serão regidas pelo presente Estatuto, complementados, internamente pelo Regimento Interno.

Art. 24 O presente Estatuto, entrará em vigor, após o seu registro no Cartório competente da cidade de Porto Alegre.

Porto Alegre, 27 de setembro de 2012.

OAB RS 32459

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www.titulosedocumentos.com.br - titulosedocumentos@titulosedocumentos.com.br
Oficial: Bel. Pérsio Brinckmann Filho

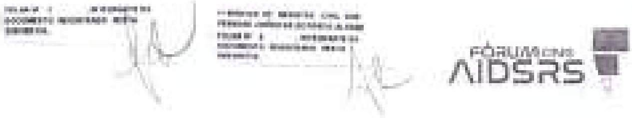
CERTIDÃO

Certifico que, nesta data, foi registrada a alteração estatutária da associação denominada "FORUM ONG AIDS RS", sob nº 81331, a folhas 046 F do Livro A nº 164 de Registro Civil das Pessoas Jurídicas. O referido é verdade e dou fé. Porto Alegre, RS, 5 de dezembro de 2012. Selos: (044801120001239978; 044801120001239979; 044901120001239980; 044801120001239981;0449020800007[illegible]6659;044903100000166518;044903100000166519; 044903100000166520; 0449[illegible]1200000200129)

Luciana Jardim dos Santos
Escrevente Autorizada

R$ 27,00

CRISTINA DE SIQUEIRA MÜLLER
Escrevente Autorizada



FÓRUM ONG AIDS RS

ATA DA ASSEMBLEIA ORDINÁRIA Nº 01/2022 PARA ELEIÇÃO DA COORDENAÇÃO E CONSELHO DO FÓRUM ONG AIDS RS

Ata de Assembleia do Fórum Ong aids RS para tratar da renovação de coordenação e do conselho. Aos trinta dias do mês de agosto de dois mil e vinte e dois, reuniram-se os representantes de instituições integrantes do Fórum Ong aids RS, inscrito no CNPJ nº 07.959.716/0001-60, às quatorze horas e quinze minutos em segunda chamada, na Rua Uruguai, 300/101, centro de Porto Alegre, com quórum suficiente conforme artigo 11 do Estatuto, para realizar processo de eleição de para a Coordenação e Conselho. Instalou-se a mesa eleitoral nomeando-se como secretaria Soila Mar Silveira da Silva, portadora do CPF nº 70610860097 e presidente Nicole de Aguiar Duarte, portadora do CPF nº 02785384016. Dando início aos trabalhos apresentaram-se os candidatos aos cargos da Coordenação, sendo eles: Coordenação Técnica – Rubens Raffo Pinto, Coordenação Executiva – Márcia de Avila Berni Leão, Coordenação Financeira – Horizontina Taborda Rovira; e para o Conselho as instituições: Coletivo Feminino Plural; GAPA RS; Fonte Colombo; APVHA; SOMOS e Construindo a Igualdade. Após informou-se sobre o procedimento para a eleição, com as necessárias explicações e em acordo com o Estatuto. Conduzido o processo eleitoral, a chapa única apesentada foi eleita por unanimidade, ficando o quadro diretivo da Associação, assim composto: a) **Coordenação Técnica – Rubens Raffo Pinto**, brasileiro, casado, funcionário público aposentado, portador do RG nº 7006437664 e CPF nº 289.941.910/20, residente e domiciliado à Rua Fernando de Andrade Prates, 247, CEP 94410-170, Viamão/RS, para desempenhar as funções constantes do art. 13, § 5º do citado dispositivo; b) **Coordenação Executiva – Márcia de Avila Berni Leão**, brasileira, solteira, maior, advogada, portadora do RG nº 1044601753 e CPF nº 549329630-68, residente e domiciliada a Rua Albion, 398/105, CEP 91530-010, Porto Alegre, para desempenhar as funções constantes do art. 13, § 6º do Estatuto da Instituição e, c) **Coordenação Financeira – Horizontina Taborda Rovira**, brasileira, casada, técnica em enfermagem, portadora do RG nº 2032624062 e CPF nº 178090100/34, residente e domiciliada nesta Capital, na Rua Upamaroti 400, bloco 4, apto 202, CEP 90820-140, Porto Alegre, para desempenhar as funções constantes do art. 13 § 7º do Estatuto do Fórum Ong aids RS; a composição do Conselho, para exercer o determinado no art. 14 e 15 do já citado dispositivo fica com: **Coletivo Feminino Plural; GAPA RS; Fonte Colombo; APVHA; SOMOS; e Construindo a Igualdade**, sendo os três primeiros titulares e os seguintes suplentes, todos com **mandato vigente até 30 de agosto 2025**. Nada mais havendo a tratar, eu secretária ad hoc encerro os trabalhos e lavro a presente ata que vai por mim e pela presidente da Assembleia, assinada.

Márcia Leão
OAB/RS 32459

FÓRUM ONG AIDS RS
forumongaidsrs@gmail.com
www.forumongaidsrs.org

RELATÓRIO

▾ [illegible] Arquivo de assinatura aprovado, em conformidade com MP 2.200-2/2001

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Tipo de assinatura	Destacada
Status da assinatura	Aprovado
Caminho de certificação	Aprovado
Estrutura da assinatura	Conformidade com o padrão (ISO 32000).
Cifra assimétrica	Aprovada
Resumo criptográfico	Correto
Data da assinatura	September 27, 2022 at 11:55:03 AM BRT
Status dos atributos	Aprovados

▸ Caminho de certificação

▸ Atributos

AVALIE ESTE SERVIÇO

EXPANDIR ELEMENTOS

1º Títulos e Documentos
Pessoas Jurídicas

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CERTIDÃO

Certifico que a presente cópia reprográfica, [illegible], é reprodução fiel do documento registrado e protocolado sob o nº 1779428, no Livro A-92 de protocolo [illegible], em 27 de setembro de 2022, e registrado em 3 de outubro de 2022, [illegible], sob o número de ordem 120000, no Livro A número 303 de Registro Civil das Pessoas Jurídicas. O referido é verdade e dou fé. Porto Alegre, 3 de outubro de 2022.

Ana Paula da Silva-Escrevente Autorizada

Emolumentos:
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R$ 173,90
ISS: R$ 9,14
Total: R$ 203,30

1º Títulos e Documentos
Pessoas Jurídicas

[illegible]

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PROCURAÇÃO *ad judicia*

Fórum Ong aids RS, pessoa jurídica de direito privado, sem fins lucrativos, constituída na forma da lei, registrada no CNPJ sob o n° 07.959.716/0001-60, com sede Rua Uruguai, 300/1º andar, Centro Histórico - CEP 90010-140, Porto Alegre/RS, na pessoa de sua Coordenadora Executiva, nos termos de seu Estatuto Social, **Márcia de Avila Berni Leão**, brasileira, solteira, maior, advogada, inscrita na OAB/RS 32459, RG 1044601753 – SSP/RS e CPF 549329630-68, vem pelo presente instrumento outorgar procuração *ad* judicia à advogada **SUSANA RODRIGUES CAVALCANTI VAN DER PLOEG**, inscrita no CPF 013.497.154-63 e na **OAB/MG 181.599**, com escritório na Avenida Presidente Vargas, 446, 13º andar, Centro - Rio de Janeiro - RJ, CEP 20071-907, concedendo-lhe poderes da cláusula *ad judicia et extra*, inclusive substabelecer com reserva de poderes, especificamente para apresentação de subsídio ao exame técnico e/ou processo administrativo de nulidade perante o INPI - Instituto Nacional da Propriedade Industrial relacionado à patente de invenção **BR112019022972-8**.

Porto Alegre, 19 de maio de 2025.

Márcia de Avila Berni Leão
Fórum Ong aids RS

Registro Civil das Pessoas Jurídicas
CARTÓRIO DO 1º OFÍCIO
NOVA IGUAÇU
Margarida Maria Gaspar Gomes
Oficial

ESTATUTOS SOCIAIS

AGANI- ASSOCIAÇÃO DE GAYS E AMIGOS DE NOVA IGUAÇU

CAPÍTULO I - DA DENOMINAÇÃO, SEDE E FINS

Artigo 1º - A AGANI - Associação de Gays e Amigos de Nova Iguaçu é uma associação civil, sem fins lucrativos, por tempo indeterminado, fundado em 17 de dezembro de 1988, com sede em Nova Iguaçu.

Parágrafo Único - Qualquer pessoa poderá tornar-se membro da AGANI conforme este estatuto, não havendo nenhuma forma de discriminação ou preconceito.

Artigo 2º - A AGANI não pertence a nenhuma facção politica, não se furtando, contudo, a apoiar qualquer partido ou candidato que defenda os direitos dos homossexuais e que, ao mesmo tempo, não contrarie o presente Estatuto.

Artigo 3º - A AGANI não tem qualquer vinculo a qualquer partido politico ou credo religioso, não sendo entretanto apolitico, e tem como objetivo a defesa e conscientização dos direitos e liberdades individuais das "minorias" oprimidas, dedicando especial atenção aos homossexuais, constituindo assim um Movimento de Emancipação Homossexual.

Parágrafo Único - Apesar de priorizar a defesa e conscientização dos direitos dos homossexuais a AGANI há de se manter atento e solidarizar-se com todas as iniciativas de combate ao preconceito e discriminação de que são alvo as " minorias".

Artigo 4º - Para atingir seus objetivos a AGANI propõe-se a:

A - Divulgar da maneira mais ampla possivel todos os pronúnciamentos cientificos, politicos, religiosos, etc., pelo fim do preconceito aos homossexuais.

B - Esforçar-se para conseguir junto a universidades, escolas, associações, de classe ou civis, partidos politicos, meios de comunicação de massas, etc., a promoção de debates, palestras e pronúnciamentos que visem o fim da discriminção aos homossexuais.

C - Combater a legislação discriminatória contra os homossexuais.

D - Protestar contra qualquer propaganda preconceituosa em relação aos homossexuais e outros segmentos estigmatizados.

E - Protestar contra qualquer arbitrariedade pública contra os homossexuais.

F - Manter intercâmbio com outros grupos homossexuais e demais instituições públicas e privadas do Brasil e do exterior.

Artigo 5º - Para viabilizar a consecução de seus objetivos a AGANI propõe-se, ainda, a criar e fortalecer os laços de solidariedade entre os homossexuais através de eventos culturais, educacionais e sociais.

CAPÍTULO II - DA ADMINISTRAÇÃO

Artigo 6º - A AGANI é constituido dos seguintes órgãos de administração:

- ASSEMBLÉIA GERAL
- DIRETORIA
- CONSELHO FISCAL

SESSÃO I - DA DIRETORIA

Artigo 7º - A AGANI será representado ativa e passivamente, em Juizo ou fora dele, pelo seu Presidente que responderá pessoalmente pela omissão ou negligência na defesa dos interêsses da associação. Para esse efeito, qualquer diretor da AGANI ou 1/3 de seus membros terá legitimidade para adotar as medidas legais compatíveis.

Artigo 8º - A Diretoria da AGANI com mandato de 01 (um) ano é composta de:

PRESIDENTE, VICE PRESIDENTE, SECRETÁRIO GERAL, TESOUREIRO, 1ª VOGAL e 2ª VOGAL. As delibrações serão tomadas em comum acordo, exigindo-se para isso o quorum minimo de 1/2 da Diretoria.

Parágrafo Primeiro - Qualquer membro do a AGANI poderá ser eleito para Diretoria, vetado a utilização do cargo para politica eleitoral (partidária) sob pena de destituição do cargo. A candidatura a cargo politico ou o seu exercicio implicará no afastamento automático do cargo que exercer na AGANI.

Parágrafo Segundo - A Diretoria da AGANI será eleita por Assembléia Geral Ordinària convocada com este fim especifico.

Parágrafo Terceiro - O mandato da Diretoria da AGANI poderá ser alterado por Assembléia Geral extraordinária com este fim especifico.

Artigo 9º - A Diretoria do poderá criar, em qualquer momento, ou dissolver, qualquer departamento que julgue necessário ao seu desenvolvimento e finalidades de acordo com os principios do presente Estatuto.

Parágrafo Primeiro - Tais departamentos terão um Diretor e um Suplente.

Parágrafo Segundo - O mandato dos departamentos se incorpora a Diretoria original.

Parágrafo terceiro- O(s) Diretor(s) e Suplente(s) passam a integrar a Diretoria em igualdade de direitos e deveres.

Artigo 10º- Compete à Diretoria da AGANI:

A - Procurar resolver por todos os meios legais ao seu alcance os problemas de interesse dos associados.

B - Convocar as Assembléias Gerais.

C - Cumprir e fazer cumprir os Estatutos e as decisões das Assembléias Gerais.

D - Providenciar para que a Tesoraria prepare os balancetes mensais e anuais para prestação de contas.

E - Preparar o relatório anual sobre as atividades da AGANI.

F - Reunir-se mensalmente para resolver as questões que lhe competem.

SESSÃO II - DAS ATRIBUIÇÕES DOS MEMBROS DA DIRETORIA

Artigo 11º- São atribuições dos membros da Diretoria da AGANI:

PRESIDENTE - A) Presidir e coordenar as reuniões da Diretoria e das Assembléias Gerais.
B) Assinar juntamente aos outros membros da Diretoria os livros da Secretaria e da Tesouraria.
C) Superintender em caráter geral todas as atividades da Diretoria e de seus membros respeitando sempre as funções de cada um.

D) Delegar poderes para representá-lo a outro membro da Diretoria ou Associado em seus impedimentos.

VICE - PRESIDENTE - A) Substituir o Presidente em todos os seus impedimentos.
B) Cooperar com o Presidente em todas as suas atribuições.

SECRETÁRIO GERAL - A) Redigir as atas de Assembléias Gerais e reuniões da Diretoria, como também todos os eventos promovidos

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pela AGANI ou nos quais o mesmo participe.

B) Dirigir os trabalhos da Secretaria Geral e redigir toda a correspondência da AGANI.

C) Fornecer ao Presidente os dados necessários para a confecção de relatórios da AGANI.

TESOUREIRO - A) Promover a arrecadação da receita.

B) Efetuar pagamento de todas as despesas da AGANI.

C) Apresentar à Diretoria da AGANI mensalmente e à Assembléia Geral anualmente, o balanço finaceiro e material.

D) Dirigir todo o serviço de escrita da Tesouraria em livros apropriados.

1ª VOGAL e 2ª VOGAL - A) Substituir as vacâncias da Diretoria.

B) Cooperar com a Diretoria em todas as suas atribuições.

Artigo 12º- Nenhum dos cargos que compõem a Diretoria da AGANI será remunerado.

CAPÍTULO III - DAS ASSEMBLÉIAS GERAIS

Artigo 13º- A ASSEMBLÉIA GERAL é o órgão máximo de deliberação da AGANI, constituida por todos os seus membros admitidos e legalmente registrados no mesmo e somente a eles cabe o direito a voto.

Artigo 14º- A ASSEMBLÉIA GERAL poderá ser convocada em caráter Ordinário ou Extraordinário, com ampla divulgação com antecedência minima de 15 dias.

Parágrafo Único - Em primeira convocação a ASSEMBLÉIA GERAL delibera com a presença minima de 1/3 de seus associados e com qualquer número na segunda convocação.

Artigo 15º- A ASSEMBLÉIA GERAL Ordinária será trimestralmente para conhecimento, discussão e deliberação dos relatórios da Diretoria.

Parágrafo Único - A convocação das ASSEMBLÉIAS GERAIS Ordinárias e Extraordinárias serão de competência da Diretoria da AGANI ou de 1/5 de seus membros.

Artigo 16º - Compete à ASSEMBLÉIA GERAL Ordinária:

A) Anualmente eleger a Diretoria bem como o seu programa.

B) Conhecer, discutir e julgar relatórios sobre as atividades da Diretoria da AGANI.

C) Traçar planos comuns de ação.

D) Decidir sobre casos omissos deste Estatuto.

E) Alterar o Estatuto conforme o disposto no artigo 30º.

Artigo 17º- A ASSEMBLÉIA GERAL Extraordinária será convocada para atendimento de situação de emergência sempre que necessário.

Artigo 18º- Qualquer pessoa poderá participar das Assmbléias e Reuniões de Diretoria, com direito à voz mas sem direito a voto.

Artigo 19º- É vetado o voto por procuração.

Artigo 20º- O Conselho Fiscal é composto de:

A) Presidente
B) Dois Conselheiros

Parágrafo Único- O Conselho Fiscal terá mandato e 01 (um) ano coincidente com o da Diretoria, permitindo a recondução de seus membros.

Artigo 21º- Compete ao Conselho Fiscal fiscalizar e indicar aprovação dos reletórios financeiros da Diretoria da AGANI na Assembléia Ordinária.

SESSÃO I - DOS MEMBROS

Artigo 22º- Serão considerados membros da AGANI todos aqueles maiores de 18 (dezoito) anos que estejam de acordo com o presente Estatuto e Programa de Ação, e que tenham participado de pelo menos duas atividades promovidas pela AGANI, preenchendo a ficha de filiação individual.

Parágrafo Único - Em quaisquer circunstâncias somente terão acesso às fichas de filiação a Diretoria da AGANI, resguardando assim o direito a inviolabilidade concernente à pessoa humana.

Artigo 23º- São direitos dos Membros da AGANI:

A) Propor e discutir e votar em ASSEMBLÉIAS GERAIS.

B) Votar e ser votado para cargos de direção segundo as restrições estabelecida no presente estatuto.

C) Usufruir dos serviços prestados pela AGANI.

Artigo 24º - São deveres dos membros da AGANI:

A) Respeitar e fazer respeitar o presente estatuto.

B) Propagar as vantagens decorrentes da união em torno da AGANI.

C) Promover por todos os meios ao seu alcance o progresso da AGANI.

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D) Participar na medida do possível de todas as atividades da AGANI.

E) Manter suas contribuições mensais em dia sujeitando-se os faltosos a perda do direito a voto.

F) Aceitar, salvo motivo de força maior, qualquer cargo para o qual tenha sido designado ou eleito e desempenhá-lo com dedicação e proficiência.

Artigo 25º - Poderão ser aplicadas sanções aos membros da AGANI que firam os intereses e objetivos do mesmo, devendo Assembléia Geral.

CAPÍTULO IV - DO PATRIMÔNIO E FUNDO SOCIAL

Artigo 26º - O patrimônio e fundo social da AGANI será constituido de:

A) Conribuições regulares dos membros decididas em Assembléia Geral

B) Doações e legados.

C) Bens e valores adquiridos

D) Rendas provenientes de qualquer atividade promovida pela AGANI.

E) Quaisquer valores adventícios (empréstimos e financiamentos).

Parágrafo Único - Ainda que sem fins lucrativos a AGANI constituir renda visando a sua aplicação na sequência dos objetivos do mesmo.

CAPITULO V - DAS DISPOSIÇÕES GERAIS E TRANSITÓRIAS

Artigo 27º - Os membros associados não responderão ainda que subsidiáriamente pelas obrigações contraidas pela AGANI.

Artigo 28º - Nenhum membro da AGANI poderá utilizar o patrimônio ou fundo social para benefício próprio.

Artigo 29º - A reforma estatutária somente será possivel em Assembléia Geral Ordinária que elege a Diretoria da AGANI, ou Assembléia Geral Extraordinaria convocada com este fim especifico.

Artigo 30º - Dar-se à a dissolução da AGANI somente por Assembléia Geral Extraordinária com este fim especifico exigindo quorum especial de 2/3 dos seus membros, e que tal decisão seja por maioria absoluta, isto é, 2/3 do total dos votantes.

Artigo 31º - Em caso de dissolução da AGANI seus bens imóveis e móveis serão doados a critério da Assembléia Geral que o dissolve.

Artigo 32º - O presente estatuto entrará em vigor na data de sua aprovação.

Nova Iguaçu, 17 de dezembro de 1988.

PRESIDENTE: Manoel Ferreira da Cunha

VICE-PRESIDENTE: Sérgio Serafim Pinto

SECRETÁRIO GERAL: Rosa Maria Campos Paiva

TESOUREIRO: Solange Wandt

1º VOGAL: Karin Bárbara Amorim Menezes

2º VOGAL: Odair, Souza da Silva

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REGISTRO DE PESSOAS JURÍDICAS
Nova Iguaçu
CGC 30.651.640/0001-22
CARTÓRIO 3.º OFÍCIO
Oficial - Margarida Maria Gaspar Gomes
Oficial Substituta - Divanice Rezende Soares da Silva
Apresentada hoje para registro e apontado sob o n.º de ordem 8957 do PROTOCOLO do livro n A-1 Registro sob o n.º de ordem 8957 do livro A-13 DE REGISTRO DE PESSOAS JURÍDICAS.
Nova Iguaçu 31 Agosto 1995
JACYRA DE OLIVEIRA COSTA - Sub oficial

Cartório do 3.º Ofício
Nova Iguaçu
Jacyra de O. Costa
Sub Oficial
Matr. 06 1889

ASSOCIAÇÃO DE GAYS E AMIGOS DE NOVA IGUAÇU E MESQUITA

ELEIÇÃO DA DIRETORIA 30/07/2024 – 30/07/ 2028

Aos 30 (trinta) dias do mês de julho de 2024, na cidade de Mesquita, Estado do Rio de Janeiro, com a presença do seu fundador, membro efetivo: seu Presidente, Manoel Ferreira da Cunha, técnico em relações públicas, solteiro, inscrito no RG sob o nº 27.391.408-5, expedida pelo DETRAN-RJ, em 27/11/2017, e inscrito no CPF sob o nº 876.553.737-87, residente e domiciliado na Rua Marcial, 42, Juscelino, Mesquita, Rio de Janeiro e demais membros da diretoria a seguir: Vanessa Aparecida Coelho da Silva, artesã, casada, inscrita no RG sob o nº 21.069.559-9, Vice-presidente, expedida pelo DETRAN RJ, inscrita no CPF sob o nº 111.010.517-79, Vice-presidente, residente e domiciliada na Rua Tenente Audir Soares Adriano, 118, casa 8, Mesquita, RJ e Regina Célia de Oliveira Bueno, advogada aposentada, solteira, inscrita na OAB-RJ sob o nº 156.275 e no CPF 510.502.267-04, Diretora Geral e de Articulação, residente e domiciliada na Rua Cinco de Julho, 335, ap. 301, Copacabana, RJ, foi realizada a Assembleia Ordinária da AGANI - Associação de Gays e Amigos de Nova Iguaçu, conhecida pelo nome fantasia de INSTITUTO AGANIM – ASSOCIAÇÃO DE GAYS E AMIGOS DE NOVA IGUAÇU E MESQUITA e RIO DE JANEIRO, CNPJ nº 00.790.968/0001-69, entidade civil sem fins lucrativos, com sede na Rua Marcial, 42, o Bairro Juscelino, Mesquita, RJ, tendo essa apenas a seguinte pauta: ELEIÇÃO DA NOVA DIRETORIA para cobrir a gestão de 2024 a 2028 e deliberado, a seguir: dado início aos trabalhos do pleito eleitoral da AGANIM 2024-2028 , foi convidado a presidir a assembleia, o Senhor Presidente da organização, Manoel Ferreira da Cunha, que aceitou o encargo e como secretária a Sra. Regina Celia de Oliveira Bueno, que igualmente aceitou o encargo dessa. Diretamente ao encaminhamento da presente pauta o Senhor Presidente informa sobre as competências necessárias para atendimento aos preceitos estabelecidos pelo estatuto vigente da organização quanto aos cargos, para que ninguém tivesse dúvida sobre os compromissos ora assumidos. Ato contínuo, informa a saída da Sra. Vanessa Aparecida Coelho da Silva que deixa o cargo de Vice-Presidente sendo substituída pela Sra. Regina Célia de Oliveira Bueno, acima qualificada e a saída da Sra. Adriana Vieira Ferreira que deixa o cargo de Diretora Executiva e Financeira não sendo substituída por nenhum dos integrantes da ONG e que no cargo da Sra. Regina Célia de Oliveira Bueno ficará a Moacyr

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Cajueiro da Silva, solteiro, brasileiro, portador da RG nº 08.619.410-7 e inscrito no CPF sob o nº 004.528.777-50, residente e domiciliada na Rua Mercúrio, 559,casa 3, Mesquita, RJ, para o cargo de Diretor Geral e de Articulação. Em seguida submeteu aos/as presentes os nomes escolhidos para a nova diretoria, que foi totalmente votada e aprovada, por unanimidade, inclusive seu Conselho Fiscal, que será formado por: Presidente: Manoel Ferreira da Cunha, Vice-presidente: Regina Célia de Oliveira Bueno e a Diretora Geral e de Articulação: Moacyr Cajueiro da Silva.

Nada mais havendo a se pronunciar o presente ato foi firmado entre as partes e ora apresenta ata firmado sendo, posteriormente, registrado no Cartório de Registro Civil de Pessoas Jurídicas.

Mesquita, RJ, 30 de julho de 2024.

MANOEL FERREIRA DA CUNHA

Presidente

MOACYR CAJUEIRO DA SILVA

Diretor Geral e de Articulação

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RUA PREFEITO JOSE MONTES PAIXAO, 1623
CNS: 154302
REGISTRO CIVIL DE PESSOA JURIDICA
Apres. no dia 07/02/2025 p/ Reg.Int. e Prot. 2717, Lv. A1
Reg.N.2717 no livro A-40,Fls.167/168.
No dia de hoje. MESQUITA, 13/02/2025.
Emol.: R$325,11. Fetj: R$65,02. Fund: R$16,25. Funp: R$16,25.
Funa.: R$19,50. Pmcmv: R$6,50. Iss: R$16,25. Selo: R$2,71.
Dist.: R$0,00. Total: R$467,59
EDEF 23471 WCZ Consulte www4.tjrj.jus.br/Portal-Extrajudicial/consultaselo/

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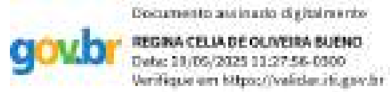
<u>**PROCURAÇÃO *ad judicia***</u>

AGANI – ASSOCIAÇÃO DE GAYS E AMIGOS DE NOVA IGUAÇU, conhecida pelo nome fantasia de AGANIM-ASSOCIAÇÃO DE GAYS E AMIGOS DE NOVA IGUAÇU, MESQUISTA E RIO DE JANEIRO, pessoa jurídica de direito privado, sem fins lucrativos, constituída na forma da lei, registrada no CNPJ sob o n° 00.790.968/0001-69, com sede na Rua Marcial, nº 42, Bairro Juscelino, Mesquita, RJ, na pessoa de seu representante nos termos de seu Estatuto Social, por seu Regina Célia de Oliveira Bueno, brasileira, advogada, solteira, Diretora Geral e de Articulação, inscrita na OAB/RJ sob o nº 156.275, e no CPF sob o nº 510.502.267-04, vem pelo presente instrumento outorgar procuração *ad* judicia à advogada **SUSANA RODRIGUES CAVALCANTI VAN DER PLOEG**, inscrita no CPF 013.497.254-63 e na **OAB/MG 181.599**, com escritório na Avenida Presidente Vargas, 446, 13º andar, Centro - Rio de Janeiro - RJ, CEP 20071-907, concedendo-lhe poderes da cláusula *ad judicia et extra*, inclusive substabelecer com reserva de poderes, especificamente para apresentação de subsídio ao exame técnico e/ou processo administrativo de nulidade perante o INPI - Instituto Nacional da Propriedade Industrial relacionado à <u>patente de invenção **BR112019022972-8**</u>.

Mesquita (RJ), 09 de maio de 2025.



REGINA CÉLIA DE OLIVEIRA BUENO
Diretora Geral e de Articulação