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Señor Director

NUEVAS CREACIONES

Superintendencia de Industria y Comercio

E. S. D.

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
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Trámite: 011 **Evento:** 378 **Actuación:** 523

Referencia: Expediente No. 14.170.731

Asunto: Solicitud de patente para: "*FORMULACIONES ESTABILIZADAS QUE CONTIENEN ANTICUERPOS ANTI-ANG2*".

Solicitante: REGENERON PHARMACEUTICALS, INC.

Fecha de solicitud: 05 de Agosto de 2014

RESPUESTA A PRIMER EXAMEN DE PATENTABILIDAD

Yo, Luz Clemencia de PAEZ, identificada como aparece al pié de mi firma, en mi condición de apoderada de la sociedad solicitante de la patente de la referencia, doy respuesta al Oficio **15111**, notificado por fijación en lista el 22 de Diciembre de 2015.

I. MODIFICACIONES AL PLIEGO REIVINDICATORIO

En respuesta al examen de patentabilidad el solicitante decide aportar un nuevo capítulo reivindicatorio conformado por 33 reivindicaciones en las que se han incluido las siguientes modificaciones:

1. Se ha modificado la reivindicación 1 de manera que se especifican las regiones variables del anticuerpo es anti-Ang2 y adicionalmente se especifican los componentes de la formulación y sus concentraciones.

2. Se han eliminado las reivindicaciones 42 y 43, de manera que el nuevo pliego reivindicatorio no hace referencia a materia no patentable.

3. Se han modificado las reivindicaciones 4, 32 y 34, de manera que las expresiones referidas a "peso molecular" y "punto isoeléctrico" han sido eliminadas.

4. Se han modificado las reivindicaciones 12 y 13, de manera que las expresiones referidas a "detergente no-iónico" han sido eliminadas.

5. Se han modificado las reivindicaciones 32 a 34 (y también la 35) con el fin de definir los anticuerpos en función de sus secuencias de aminoácidos de las regiones variables de las cadenas ligeras y pesadas. Los otros parámetros relacionados con los anticuerpos han sido eliminados de dichas reivindicaciones.

6. Adicionalmente se modificó el título de la presente solicitud así: "*FORMULACIONES FARMACÉUTICAS QUE CONTIENEN ANTICUERPOS ANTI-ANG2*" de manera que ahora permite identificar la materia de la cual se trata la presente solicitud.

7. Se han eliminado las reivindicaciones 36 a 41 referidas a una composición farmacéutica y a un kit de partes que lo contenía.

Estas modificaciones se ajustan al artículo 34 de la Decisión 486 de 2000 y no constituyen una ampliación de la protección que correspondería a la divulgación contenida en la solicitud inicial.

A pesar de eliminar materia del pliego reivindicatorio originalmente presentado, mi representada no renuncia a la protección que pueda obtener sobre dicha materia y se reserva el derecho de presentar una solicitud divisional que la contenga durante el trámite de la presente solicitud.

II. EN CUANTO A LA SUPUESTA FALTA DE CLARIDAD

1. Con respecto a la objeción formulada a la reivindicaciones 12, 32 y 33, por repetir la materia a proteger de manera innecesaria, cabe resaltar que en la reivindicación 12 está definida las concentraciones de un anticuerpo y los componentes de la formulación establecidos en la reivindicación 1. Por otra parte, la reivindicación 32 discute una formulación que comprende una combinación específica de concentraciones del anticuerpo y los

excipientes, en la cual el polisorbato está definido como polisorbato 20, mientras que en la reivindicación 33 discute una formulación que consiste en una combinación específica concentraciones de anticuerpo y excipientes. Por lo tanto, es claro que cada una de las anteriores reivindicaciones define un diferente alcance de la invención reivindicada en la presente solicitud.

2. Con respecto a la objeción formulada a la reivindicaciones 12, 34 y 35, por repetir la materia a proteger de manera innecesaria, considerando las razones anteriormente expuestas, queda claro que las reivindicaciones citadas definen un diferente alcance de la presente invención, por lo que esta objeción se debe tener por superada.

4. Con respecto a la objeción formulada a las reivindicaciones 4, 12, 13, 32 y 34 por no ser claras, debido a que los términos "peso molecular", "punto isoeléctrico" y "detergente no iónico" fueron eliminados del pliego reivindicatorio la objeción formulada a este respecto se debe tener por superada.

5. Con respecto a la objeción formulada a las reivindicaciones 36 a 41 por no ser claras, debido a que las mismas fueron eliminadas del pliego reivindicatorio la objeción formulada a este respecto se debe tener por superada.

6. Con respecto a la objeción formulada a las reivindicaciones 42 a 43 por incluir materia no patentable, debido a que las mismas fueron eliminadas del pliego reivindicatorio, la objeción formulada a este respecto se debe tener por superada.

III. RESPECTO A LA FALTA DE UNIDAD DE INVENCIÓN

Considerando que el Examinador establece dos grupos inventivos en la presente solicitud, es importante resaltar que el nuevo pliego reivindicatorio de la presente solicitud sólo hace referencia a la formulación que comprende el anticuerpo/histidina/polisorbato, identificado por el Examinador como el grupo inventivo (1). Por lo tanto, la objeción presentada por falta de unidad de invención se debe tener por superada.

IV. ESTADO DE LA TÉCNICA

Su despacho determinó dos documentos como relevantes para realizar el análisis de patentabilidad de la presente patente de invención:

1. D1, referido al documento US 7521053 titulado *"Angiopoietin-2-specific binding agents"* que se relaciona con composiciones farmacéuticas que comprenden un anticuerpo anti-ANG-2, un amortiguador, un surfactante no iónico y un estabilizador.

2. D2, referido al documento WO 2011014469 titulado *"High affinity human antibodies to human angiopoietin-2"* que divulga composiciones que comprenden anticuerpos de alta afinidad angiopoyetina-2.

3. D3, referido al documento WO 2006044908 titulado *"Antibody formulations"* que divulga composiciones estables que comprenden anticuerpos VEFG, tampón histidina-fosfato, polisorbato 20 y sacarosa.

EN CUANTO A LA SUPUESTA FALTA DE NOVEDAD

1. En virtud del nuevo pliego reivindicatorio presentado adjunto con este documento, es necesario aclara que las modificaciones realizadas sobre la reivindicación 1 son suficientes para superar la objeción presentada por falta de novedad, puesto que actualmente dicha reivindicación está referida al anticuerpo anti-Ang2 con especificidad definida en las regiones variables mientras que D1 no discute dicho anticuerpo. Por tanto, la presente invención es novedosa sobre el arte previo citado por el Examinador.

EN CUANTO A LA SUPUESTA FALTA DE NIVEL INVENTIVO

1. La formulación reivindicada en la presente solicitud sí presenta efectos técnicos inesperados, como por ejemplo, la estabilización del anticuerpo reivindicado, anti-Ang2, la cual no podría haber sido predicha por una persona normalmente versada en la materia a partir de los documentos citados como parte del arte previo. De hecho, la presente invención está directamente relacionada con formulaciones que comprenden un anticuerpo anti-Ang2 a una concentración desde 5 mg/ml hasta 150 mg/ml y comprende una región variable de cadena pesada que contiene una secuencia de amino ácidos SEQ ID N° 18 y una región variable de la cadena liviana que contiene una secuencia de amino ácidos SEQ ID N° 20, histidina a una concentración desde 5 mM hasta 15 mM, polisorbato a una concentración desde 0.1% p/v hasta

0.3% p/v, sacarosa a una concentración desde 3% p/v hasta 20 % p/v, y un pH desde 5.5 hasta 6.5.

2. D2 discute anticuerpos que se unen a la angiopoyetina-2 y métodos para usar dichos anticuerpos para el tratamiento de enfermedades y desórdenes asociados la actividad biológica de uno o dos Ang-2, incluyendo la angiogénesis. D2 discute preparaciones inyectables del anticuerpo en combinación, por ejemplo con solución salina fisiológica y un agente solubilizante como un alcohol (por ejemplo, etanol), un polialcohol (por ejemplo, propilenglicol, polietilenglicol), o un surfactante no iónico (por ejemplo, polisorbato 80, HCO-50), etc (ver párrafo 0093 de D2). Sin embargo, D2 no provee ninguna formulación específica y tampoco menciona el uso de sacarosa o una solución tampón o reguladora de histidina.

3. D3 discute la formulación de anticuerpos en una solución tampón con un sacárido y un surfactante. De este modo, D3 identifica diversas soluciones tampón, sacáridos y surfactantes para su uso en formulaciones. Por ejemplo, D3 identifica acetato, succsinato, gluconato, histidina, citrato, glicilglicina y otros ácidos orgánicos como posibles soluciones tampón (ver página 12, párrafo 8 de D3). Adicionalmente, D3 identifica glucosa, sucrosa, trehalosa, lactosa, fructosa, maltosa, dextrano, glicerina, eritritol, glicerol, arabitol, silitol, sorbitol, manitol, celobiosa, melezitosa, rafinosa, manotriosa, estaquiosa, maltosa, lactulosa, maltulosa, glucitol, maltitiol, lactitol, iso-maltulosa, etc, como posibles sacáridos (ver página 12, párrafo de D3). Además, D3 reconoce polisorbatos, poloxámeros, polietilglicol, polipropilglicol, Pluronic F68 y otros numerosos posibles surfactantes para su uso en formulaciones (ver páginas 12 a 13 de D3). Aunque supuestamente útil para la formulación de anticuerpos en general, D3 es un ejemplo de la formulación de solamente dos anticuerpos, un anticuerpo anti-HER2 (Ejemplos 1-11) y un anticuerpo anti-DR5 (Ejemplo 12). Aunque supuestamente la información divulgada en D3 es útil para la formulación de anticuerpos en general, D3 sólo ejemplifica la formulación de solamente dos anticuerpos, un anticuerpo anti-HER2 (Ejemplos 1-11) y un anticuerpo anti-DR5 (Ejemplo 12).

4. La literatura científica del arte previo relacionada con formulación de anticuerpos establece claramente que la formulación de un anticuerpo particular requiere componentes específicos teniendo en cuenta las diferencias estructurales en las proteínas. De hecho, se ha establecido que cada anticuerpo presenta requerimientos únicos para su estabilización derivados de las diferencias en la secuencia de amino ácidos que determinan la especificidad a un antígeno en particular (ver adjunto: Daugherty et al., *Advanced Drug Delivery Reviews* 58, 686-706 (2006) at p. 690, segunda columna, primer párrafo) y que un excipiente presente en

una formulación con el fin de estabilizar un anticuerpo específico, puede no ser útil para otro anticuerpo dadas las diferencias en su secuencia (ver adjunto: Wang et al., *Journal of Pharmaceutical Sciences*, 96(1):1-26 (2007) segunda columna, primer párrafo). Partiendo de lo anterior, y considerando lo establecido en los artículos anteriormente mencionados, un excipiente de una formulación dada que estabiliza un anticuerpo en especial, podrá no ser necesariamente apropiado para estabilizar otro anticuerpo, por lo tanto, no hay razón alguna para seleccionar D3 como punto de partida para identificar posibles excipientes adecuados y mucho menos poder predecir que dichos excipientes estabilizarían un anticuerpo diferente (por ejemplo, un anticuerpo anti-HER2) y que podrían ser utilizados para estabilizar un anticuerpo anti-Ang2, como se indica en la formulación reivindicada en la presente solicitud.

5. Considerando que D2 no discute ninguna formulación específica y tampoco menciona sacarosa o una solución tampón de histidina en ninguna de las formas preferidas, la combinación de D2 y D3 no provee ninguna enseñanza que le indique a una persona versada en la materia el uso de un componente en particular para la formulación que contiene un anticuerpo, por encima de otros componentes que permita estabilizar un anticuerpo anti-Ang2, como sí lo señala la presente invención. En vista de la necesidad de emplear un estabilizante específico para cada anticuerpo, no existe ninguna razón para que una persona versada en la materia escoja a partir de D3, los componentes específicos descritos en la presente solicitud con el fin de preparar una formulación que contenta el anticuerpo anti-Ang2. Así como los componentes, por separado, de la formulación reivindicada han sido utilizados en la formulación de otros anticuerpos, lo mismo puede decirse de cientos o miles de otros excipientes, algunos de los cuales son conocidos, que por el contrario podrían desestabilizar el anticuerpo anti-Ang2.

6. Dado que en el estado del arte se advierte que la formulación de anticuerpos es impredecible, una persona normalmente versada en la materia se habría visto motivada a cuestionar la veracidad de las afirmaciones del arte previo citado, que indican que las formulaciones son aplicables a anticuerpos en general. En particular, D3 solo revela formulaciones de anticuerpos monoclonales, con discretos ejemplos de formulaciones, no provee información relevante para una persona medianamente versada en la materia interesada en la preparación de una formulación de un anticuerpo anti-Ang2. Por lo tanto, las formulaciones reivindicadas para un anticuerpo anti-Ang2 presenta un resultado inesperado en vista de los componentes de la formulaciones en general, discutidas en el arte previo citado. Dicho resultado inesperado se refiere a que cada componente en las formulaciones reivindicadas contribuye a la estabilidad de dicha formulación.

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7. Las formulaciones reivindicadas incluyen excipientes seleccionados de forma cuidadosa y a concentraciones estrechamente definidas para un anticuerpo con regiones variables específicamente definidas, la cual corresponde a una característica del anticuerpo que el estado de la técnica enseña como la definición de su estabilidad en relación con los excipientes particularmente elegidos (véase *Daugherty, supra*). La persona normalmente versada en la materia no habría tenido ninguna motivación para emplear los excipientes divulgados en D3 con la expectativa de que pudieran elegir al azar componentes para una formulación de anticuerpos, partiendo de los divulgados en D3 y razonablemente esperar que se llegue a una formulación estable de un anticuerpo anti-Ang2.

8. Asumiendo que una persona medianamente versada en la materia tomara en consideración el documento D3, ésta hubiese estado motivada a elegir cualquiera de los excipientes identificados en dicho documento que como ha sido aquí discutido, pueden desestabilizar el anticuerpo anti-Ang2. Por ejemplo, D3 identifica una solución tampón de citrato de pH 5.5 ó 6.0 (página 12, párrafo 8), glicerol o manitol (página 12, párrafo 10), polietienglicol, polipropilglicol, Pluronics F68 (página 13, párrafo 1), y NaCl (página 63, ejemplo 1) entre las listas de los componentes de formulación adecuados. Por el contrario, en la presente invención, se demostró que una solución tampón a pH 5.5 ó 6.0 incrementa la formación de agregados (ejemplo 2, tabla 1), y en donde en presencia de polietilenglicol, propilenglicol y Pluronic F68 se observó que decrece significativamente la estabilidad térmica de en anticuerpo hAng2 (ejemplo 3, párrafo 91). Igualmente, NaCl y glicerol mostraron que incrementan la formación de agregados (ejemplo 4, tabla 4), mientras que el manitol desestabiliza el anticuerpo hAng2 en el proceso de congelación/descongelación (ejemplo 4, párrafo 96). Cualquier resultado negativo en los estudios de estabilidad mostrados en los ejemplos de la presente invención, está relacionado con un indicio de que un excipiente en particular puede desestabilizar, impidiendo de ese modo su selección como un componente de formulación para el anticuerpo particular.

9. Con base en los argumentos anteriormente expuestos, no habría sido obvio para una persona versada en la materia, a partir de los componentes divulgados en D3, o en algún otro documento del estado de la técnica, decidir cuáles componentes podrían ser útiles en la formulación de un anticuerpo anti-Ang2 con unas regiones variables específicamente definidas y qué efectos perjudiciales podrían tener en la estabilización de dicho anticuerpo. Además, sólo con un análisis en retrospectiva podría alguien elegir, a partir de los excipientes "adecuados"

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indicados en el arte previo, exactamente los mismos excipientes de la presente invención reivindicada.

V. PETICIÓN

1. Puesto que se ha dado respuesta a todas las objeciones formuladas por la Dirección de Nuevas Creaciones, se solicita proceder al otorgamiento de la solicitud de patente.

2. De manera subsidiaria, y en caso de que la Dirección de Nuevas Creaciones tenga observaciones adicionales a la patentabilidad del invento, solicito emitir un nuevo requerimiento en el marco del artículo 45 de la Decisión 486 de 2000.

VI. ANEXOS

1. Nuevo pliego reivindicatorio conformado por 33 reivindicaciones.

2. Artículo académico Daugherty et al., *Advanced Drug Delivery Reviews* 58, 686-706 (2006).

3. Artículo académico Wang et al., *Journal of Pharmaceutical Sciences*, 96(1):1-26 (2007).

Señor Director,

Luz Clemencia de Páez

LUZ CLEMENCIA DE PÁEZ

C.C. 35.456.344 de Usaquén

T.P.A. No. 23.555

COLO-16813-0841-0018-3
DPG\DPG\ID-287052\WPC16813
No. M-2016-287052\DPG

REIVINDICACIONES MODIFICADAS

1. Una formulación farmacéutica que comprende: (i) un anticuerpo a una concentración desde 5mg/ml hasta 150 mg/ml que se une específicamente a angiopoyetina 2 humana (Ang-2 humana), en donde el anticuerpo comprende una región variable de cadena pesada (HCVR) que tiene la secuencia de aminoácidos SEQ ID N° 18 y una región variable de cadena ligera (LCVR) que tiene la secuencia de aminoácidos SEQ ID N°20; (ii) histidina a una concentración desde 5 mM hasta 15 mM; (iii) polisorbato a una concentración desde 0,1% p/v hasta 0,3% p/v; y (iv) sacarosa a una concentración desde 3% p/v hasta 20% p/v,

en donde la formulación tiene un pH desde 5.5 hasta 6.5.

2. La formulación farmacéutica de la reivindicación 1, en donde el anticuerpo está presente en una concentración desde 20 ± 3 mg/ml hasta 60 ± 9 mg/ml.

3. La formulación farmacéutica de la reivindicación 1 o de la reivindicación 2, , en la que desde alrededor del 90% hasta alrededor del 92% de los anticuerpos están fucosilados.

4. La formulación farmacéutica de cualquiera de las reivindicaciones 1-3, en la que la concentración de anticuerpo es de 25 mg/ml ± 3,75mg/ml.

5. La formulación farmacéutica de cualquiera de las reivindicaciones 1-3, en la que la concentración del anticuerpo es 50 mg/ml ± 7,5 mg/ml.

6. La formulación farmacéutica de la cualquiera de las reivindicaciones 1-5 , en la que la concentración de histidina es 10 mM ± 1,5 mM.

7. La formulación farmacéutica de cualquiera de las reivindicaciones 1-6, en la que el polisorbato es polisorbato 20.

8. La formulación farmacéutica de la reivindicación 7, en la que la concentración de polisorbato 20 es de 0,2% p/v ± 0,03%.

9. La formulación farmacéutica de cualquiera de las la reivindicaciones 1- 8, en la que la concentración de sacarosa es de 10% ± 1,5% p/v.

10. La formulación farmacéutica de la reivindicación 1 , en la que la concentración de anticuerpo es 25 mg/ml ± 3,75 mg/ml, la concentración de histidina es 10 mM ± 1,5 mM, pH 6 ± 0,3, la concentración de polisorbato es 0,2% p/v ± 0,03% p/v, y la concentración de sacarosa es 10% p/v ± 1,5% p/v.

11. La formulación farmacéutica de la reivindicación 1, en la que la concentración de anticuerpo es 50 mg/ml ± 7,5 mg/ml, la concentración de histidina es 10 mM ± 1,5 mM, pH 6 ± 0,3, la concentración de polisorbato es 0,2% p/v ± 0,03% p/v, y la concentración de sacarosa es 10% p/v ± 1,5% p/v.

12. La formulación farmacéutica de cualquiera de las reivindicaciones 1-11, en la que al menos un 93% del anticuerpo tiene una conformación nativa después de 28 días a 45 °C.

13. La formulación farmacéutica de cualquiera de las reivindicaciones 1-12, en la que al menos un 32% del anticuerpo es la variante de carga principal del anticuerpo después de 28 días a 45 °C.

14. La formulación farmacéutica de cualquiera de las reivindicaciones 1-13, en la que al menos un 97% del anticuerpo tiene una conformación nativa después de 28 días a 25 °C.

15. La formulación farmacéutica de cualquiera de las reivindicaciones 1-14, en la que al menos un 53% del anticuerpo es la variante de carga principal del anticuerpo después de 28 días a 25 °C.

16. La formulación farmacéutica de cualquiera de las reivindicaciones 1-15, en la que al menos un 96% del anticuerpo tiene una conformación nativa después de 28 días a 37 °C.

17. La formulación farmacéutica de cualquiera de las reivindicaciones 1-16, en la que al menos un 45% del anticuerpo es la variante de carga principal del anticuerpo después de 28 días a 37 °C.

18. La formulación farmacéutica de cualquiera de las reivindicaciones 1-17, en la que al menos un 97% del anticuerpo tiene una conformación nativa después de seis meses a 5 °C.

19. La formulación farmacéutica de cualquiera de las reivindicaciones 1-18, en la que al menos un 55% del anticuerpo es la variante de carga principal del anticuerpo después de seis meses a 5 °C.

20. La formulación farmacéutica de cualquiera de las reivindicaciones 1-19, en la que la potencia relativa en porcentaje del anticuerpo después de seis meses a 5 °C es al menos un 100% de la potencia del anticuerpo antes del almacenamiento.

21. La formulación farmacéutica de cualquiera de las reivindicaciones 1-20, en la que al menos un 97% del anticuerpo tiene una conformación nativa después de seis meses a -80 °C.

22. La formulación farmacéutica de cualquiera de las reivindicaciones 1-21, en la que al menos un 55% del anticuerpo es la variante de carga principal del anticuerpo después de seis meses a -80 °C.

23. La formulación farmacéutica de cualquiera de las reivindicaciones 1-22, en la que la potencia relativa en porcentaje del anticuerpo después de seis meses a -80 °C es al menos un 85% de la potencia del anticuerpo antes del almacenamiento.

24. La formulación farmacéutica de cualquiera de las reivindicaciones 1-23, en la que al menos un 96% del anticuerpo tiene una conformación nativa después de seis meses a -30 °C.

25. La formulación farmacéutica de cualquiera de las reivindicaciones 1-24, en la que al menos un 55% del anticuerpo es la variante de carga principal del anticuerpo después de seis meses a -30 °C.

26. La formulación farmacéutica de cualquiera de las reivindicaciones 1-25, en la que la potencia relativa en porcentaje del anticuerpo después de seis meses a -30 °C es al menos un 84% de la potencia del anticuerpo antes del almacenamiento.

27. La formulación farmacéutica de cualquiera de las reivindicaciones 1-26, en la que al menos un 96% del anticuerpo tiene una conformación nativa después de seis meses a -20 °C.

28. La formulación farmacéutica de cualquiera de las reivindicaciones 1-27, en la que al menos un 55% del anticuerpo es la variante de carga principal del anticuerpo después de seis meses a -20 °C.

29. La formulación farmacéutica de cualquiera de las reivindicaciones 1-28, en la que la potencia relativa en porcentaje del anticuerpo después de seis meses a -20 °C es al menos un 90% de la potencia del anticuerpo antes del almacenamiento.

30. Una formulación farmacéutica que comprende (a) 25 mg/ml ± 3,75 mg/ml de un anticuerpo anti-Ang-2, (b) histidina 10 mM ± 1,5 mM, pH 6 ± 0,3, (c) 0,2% p/v ± 0,03% de polisorbato 20, y (d) 10% p/v ± 1,5% de sacarosa, en la que:
(a) el anticuerpo comprende un HCVR que tiene una secuencia de aminoácidos de SEQ ID Nº: 18 y un LCVR que tiene una secuencia de aminoácidos de SEQ ID Nº: 20;

31. La formulación farmacéutica de la reivindicación 30 que consiste en (a) 25 mg/ml ± 3,75 mg/ml del anticuerpo, (b) histidina 10 mM ± 1,5 mM, pH 6 ± 0,3, (c) 0,2% p/v ± 0,03% de polisorbato 20, y (d) 10% p/v ± 1,5% de sacarosa, en agua.

32. Una formulación farmacéutica que comprende (a) 50 mg/ml± 7,5 mg/ml de un anticuerpo anti-Ang-2, (b) histidina 10 mM ± 1,5 mM, pH 6 ± 0,3, (c) 0,2% p/v ± 0,03% de polisorbato 20, y (d) 10% p/v ± 1,5% de sacarosa, en la que:

(a) el anticuerpo comprende un HCVR que tiene una de secuencia de aminoácidos de SEQ ID Nº: 18 y un LCVR que contiene una de secuencia de aminoácidos de SEQ ID Nº: 20;

33. La formulación farmacéutica de la reivindicación 32 que consiste en (a) 50 mg/ml ± 7,5 mg/ml del anticuerpo, (b) histidina 10 mM ± 1,5 mM, pH 6 ± 0,3, (c) 0,2% p/v ± 0,03% de polisorbato 20, y (d) 10% p/v ± 1,5% de sacarosa, en agua.

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MINIREVIEW

Antibody Structure, Instability, and Formulation

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ABSTRACT: The number of therapeutic monoclonal antibody in development has increased tremendously over the last several years and this trend continues. At present there are more than 23 approved antibodies on the US market and an estimated 200 or more are in development. Although antibodies share certain structural similarities, development of commercially viable antibody pharmaceuticals has not been straightforward because of their unique and somewhat unpredictable solution behavior. This article reviews the structure and function of antibodies and the mechanisms of physical and chemical instabilities. Various aspects of formulation development have been examined to identify the critical attributes for the stabilization of antibodies. © 2006 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 96:1–26, 2007

Keywords: biotechnology; stabilization; protein formulation; protein aggregation; freeze drying/lyophilization

INTRODUCTION

Protein therapies are entering a new era with the influx of a significant number of antibody pharmaceuticals. Generally, protein drugs are effective at low concentrations with less side effects relative to small molecule drugs, even though, in rare cases, protein-induced antibody formation could be serious.[1] Therefore, this category of therapeutics is gaining tremendous momentum and widespread recognition both in small and large drug firms. Among protein drug therapies, antibodies play a major role in controlling many types of diseases such as cancer, infectious diseases, allergy, autoimmune diseases, and inflammation. Since the approval of the first monoclonal antibody (MAb) product -OKT-3 in 1986, more than 23 MAb drug products have entered the market (Tab. 1). The estimated number of antibodies and antibody derivatives constitute 20% of biopharmaceutical products currently in development (about 200).[2] The global therapeutic antibody market was predicted to reach $16.7 billion in 2008.[3]

There are several reasons for the increasing popularity of antibodies for commercial development. First, their action is specific, generally leading to fewer side effects. Second, antibodies may be conjugated to another therapeutic entity for efficient delivery of this entity to a target site, thus reducing potential side effects. For instance, Mylotarg is an approved chemotherapy agent composed of calicheamicin conjugated to humanized IgG4, which binds specifically to CD33 for the treatment of CD33-positive acute myeloid leukemia. Another example is the conjugation of immunotoxic barnase with the light chain of the anti-human ferritin monoclonal antibody F11 as potential targeting agents for cancer immunotherapy.[4] Third, antibodies may be conjugated to radioisotopes for specific diagnostic purposes. Examples include CEA-Scan for detection of colorectal cancer and ProstaScint for detection of prostate cancer. Lastly, technology advancement has made complete human MAb available, which are less immunogenic.

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Table 1. Commercial Monoclonal Antibody Products

#	Brand name	Molecule	MAb	Year	Company	Route	Indication	MAb Conc	Buffer	Excipients	Surfactant	pH
1	Avastin	Bevacizumab	Humanized IgG1, 149 kDa	2004	Genetech and BioOncology	IV infusion	Metastatic carcinoma of colon or rectum, binds VEGF	100 mg and 400 mg/vial (25 mg/mL) solution	5.8 mg/mL monobasic NaPhos H_2O; 1.2 mg/mL dibasic NaPhos anhydrous (4 mL, 16 mL fill in vial)	60 mg/mL α-Trehalose dihydrate (4 mL, 16 mL fill in vial)	0.4 mg/mL PS20 (4 mL, 16 mL fill in vial)	6.2
2	Bexxar	Tositumomab and I-131Tositumab	Murine IgG2λ	2003	Corixa and GSK	IV Infusion	CD20 positive follicular nonHodgkins lymphoma	Kit: 14 mg/mL MAb solution in 35 mg and 225 mg vials; 1.1 mg/mL *I131-MAb* solution	10 mM phosphate (MAb vial)	145 mM NaCl, 10 % w/v Maltose; *I131-MAb*: 5–6% Povidone, 1–2, 9–15 mg/mL Maltose, 0.9 mg/mL NaCl, 0.9–1.3 mg/mL Ascorbic acid		7.2
3	Campath	Alemtuzumab	Humanized, IgG1k, 150 kDa	2001	Ilex Oncology; Millenium and Berlex	IV infusion	B-cell chronic lymphocytic leukemia, CD52-antigen	30 mg/3 mL solution	3.5 mg/3 mL dibasic NaPhos, 0.6 mg/3 mL monobasic KPhos	24 mg/3 mL NaCl, 0.6 mg/3 mL KCl, 0.056 mg/3 mL Na2EDTA	0.3 mg/3 mL PS80	6.8–7.4
4	CEA-Scan (lyo)	Arcituomab; Tc-99	Murine Fab, 50 kDa	1996	Immunomedics	IV injection or infusion	Imaging agent for colorectal cancer	1.25 mg/vial Lyophilized MAb. Reconstitute w 1 mL Saline w Tc99m	0.29 mg/vial Stannous chloride, potassium sodium tartrate tetrahydrate, NaAcetate.$3H_2O$, NaCl, glacial acetic acid, HCl	Sucrose		5.7
5	Erbitux	Cetuximab	Chimeric human/ mouse IgG1k, 152 kDa	2004	ImClone and BMS	IV infusion	Treatment of EGFR-expressing colorectal carcinoma	100 mg MAb in 50 mL; 2 mg/ mL solution	1.88 mg/mL DibasicNaPhos · $7H_2O$; 0.42 mg/mL MonobasicNaPhos · H_2O	8.48 mg/mL NaCl		7.0–7.4
6	Herceptin (lyo)	Trastuzumab	Humanized IgG1k	1998	Genetech	IV infusion	Metastatic breast cancer whose tumor overexpress HER2 protein	440 mg /vial, 21 mg/mL after reconstitution	9.9 mg/20 mL L-HistidineHCl, 6.4 mg/20 mL L-Histidine	400 mg/20 mL α-Trehalose Dihydrate	1.8 mg/20 mL PS20	6
7	Humira	Adalimumab	Human IgG1k, 148 kDa	2002	CAT and Abbott	SC	RA patients not responding to DMARDs. Blocks TNF-alpha	40 mg/0.8 mL solution (50 mg/mL)	0.69 mg/0.8 mL Monobasic NaPhos · $2H_2O$; 1.22 mg/0.8 mL DibasicNaPhos · $2H_2O$; 0.24 mg/0.8 mL NaCitrate, 1.04 mg/ 0.8 mL Citric acid · H_2O	4.93 mg/0.8 mL NaCl; 9.6 mg/0.8 mL Mannnitol	0.8 mg/0.8 mL PS80	5.2
8	Lucentis	Ranibizumab	Humanized IgG1k fragment	2006	Genentech	Intravitreal injection	Age-related macular degeneration (wet)	10 mg/mL solution	10 mM HistidineHCl	10% α-Trehalose-Dihydrate	0.01% PS20	5.5

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9	Mylotarg (lyo)	Gemtuzumab ozogamicin	Humanized IgG4k conjugated with calicheamicin	2000	Celltech and Wyeth	IV infusion	Humanized Ab linked to calicheamicin for treatment of CD33 positive acute myeloid leukemia	5 mg protein-equivalent lyophilized powder/20-mL vial	Monobasic and dibasic NaPhosphate	Dextran 40, Sucrose, NaCl		
10	OncoScint	Satumomab pendetide	Murine IgG1k conjugated to GYK-DTPA	1992	Cytogen	IV injection	Imaging agent for colorectal and ovarian cancer	0.5 mg conjugate/mL solution (2 mL per vial)	Phosphate buffer saline			6.0
11	Orthoclone OKT	Muromomab-CD3	Murine, IgG2a, 170 kDa	1986	Ortho Biotech	IV injection	Reversal of acute kidney transplant rejection (anti CD3-antigen)	1 mg/mL solution	2.25 mg/5 mL monobasic NaPhos, 9.0 mg/5 mL dibasic NaPhos	43 mg/5 mL NaCl	1 mg/mL PS80	7 ± 0.5
12	ProstaScint	Indium-111 capromab pendetide	Murine IgG1k-conjugated to GYK-DTPA	1996	Cytogen	IV injection	Imaging agent for prostate cancer	0.5 mg conjugate/mL solution (1 mL per vial)	Phosphate buffer saline			5–7
13	Raptiva (lyo)	Efalizumab	Humanized IgG1k	2003	Xoma and Genentech	SC	Chronic moderate to severe plaque psoriasis, binds to CD11a subunit of LFA-1	150 mg MAb/vial; 125 mg/ 1.25 mL (100 mg/mL) after reconstitution with 1.3 mL SWFI	6.8 mg/vial L-HistidineHCl · H_2O; 4.3 mg/vial L-Histidine	123.2 mg/vial Sucrose	3 mg/vial PS20	6.2
14	Remicade (lyo)	Infliximab	Chimeric human/ murine MAb against TNFalpha (app. 30% murine, 70% corresponds to human IgG1 heavy chain and human kappa light chain constant regions)	1998	Centocor	IV infusion	RA and Crohn's disease (anti TNFalpha)	100 mg / 20-mL Vial, 10 mg/mL on reconstitution	2.2 mg/10 mL MonobasicNaPhos H_2O, 6.1 mg/10 mL DibasicNaPhos · $2H_2O$	500 mg/10 mL Sucrose	0.5 mg/10 mL PS80	7.2
15	ReoPro	Abciximab	Fab, Chimeric human-murine, 48 kDa	1994	Centocor / Lilly	IV injection and infusion	Reduction of acute blood clot related complications	2 mg/mL solution	0.01 M NaPhosphate	0.15 M NaCl	0.001% (0.01 mg/ mL) PS80	7.2
16	Rituxan	Rituximab	Chimeric mouse/ human IgG1k with murine light and heavy chain variable region (Fab domain), 145 kDa	1997	IDEC and Genentech	IV infusion	NonHodgkin's lymphoma. (anti CD20-antigen)	10 mg/mL solution	7.35 mg/mL NaCitrate · $2H_2O$	9 mg/mL NaCl	0.7 mg/mL PS80	6.5

(Continued)

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Table 1. (*Continued*)

#	Brand name	Molecule	MAb	Year	Company	Route	Indication	MAb Conc	Buffer	Excipients	Surfactant	pH
17	Simulect (lyo)	Basiliximab	Chimaric IgG1k, 144 kDa	1998	Novartis	IV injection and infusion	Prevention of acute kidney transplant rejection, IL-2 receptor antagonist	10 mg and 20 mg/ vial, 4 mg/mL on reconstitution	3.61 mg, 7.21 mg Monobasic KPhos; 0.50 mg, 0.99 mg Na_2HPO_4	0.8 mg, 1.61 mg NaCl; 10 mg, 20 mg Sucrose; 40 mg, 80 mg Mannitol; 20 mg 40 mg Glycine		
18	Synagis (lyo)	Palivizumab	Humanized IgG1k, CDR of murine MAb 1129, 148 kDa	1998	MedImmune	IM injection	Prevent replication of the Respiratory syncytial virus (RSV)	50 mg and 100 mg / vial, 100 mg/mL on reconstitution	47 mM Histidine, 3.0 mM Glycine	5.6 % Mannitol		
19	Tysabri	Natalizumab	Humainzed IgG4k	2004	Biogen IDEC	IV Infusion	MS relapse	300 mg/15 mL solution	17.0 mg MonobasicNa Phos · H_2O, 7.24 mg diBasicNaPhos · $7H_2O$ for 15 mL	123 mg/15 mL NaCl	3.0 mg/15 mL PS80	6.1
20	Verluma	Nofetumomab	Murine Fab	1996	Boehringer Ingelheim and DuPont Merck	IV injection	Imaging agent for lung cancer	10 mg/mL solution	Phosphate buffer saline			?
21	Xolair (lyo)	Omalizumab	Humanized IgG1k, 149 kDa		Genentech w Novartis and Tanox	SC	Asthma, inhibits binding of IgE to IgE receptor FCeRI	202.5 mg/vial, Deliver 150 mg/ 1.2 mL on reconstitution with 1.4 mL SWFI	2.8 mg LHistidineHCl · H_2O; 1.8 mg LHistidine	145.5 mg Sucrose	0.5 mg PS20	
22	Zenapax	Daclizumab	Humanized IgG1, 144 kDa	1997	Roche	IV infusion	Prophylaxis of acute organ rejection in patients receiving renal transplants. Inhibits IL-2 binding to the Tac subunit of IL-2 receptor complex	25 mg/5 mL MAb Solution	3.6 mg/mL MonobasicNa Phos · H_2O; 11 mg/mL DibasicNaPhos · $7H_2O$	4.6 mg/mL NaCl	0.2 mg/mL PS80	6.9
23	Zevalin	Ibritumomab-Tiuxetan	Murine IgG1k-thiourea covalent linkage to Tiuxetan		IDEC	IV infusion	CD20 antigen. (Kit with Ytterium-90 induces cellular damage by beta emission)	3.2 mg/2 mL solution		09% NaCl		7.1

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Development of commercially viable antibody pharmaceuticals has, however, not been straightforward. This is because the behavior of antibodies seems to vary, even though they have similar structures. In attempting to address some of the challenges in developing antibody therapeutics, Harris et al.[5] reviewed the commercial-scale formulation and characterization of therapeutic recombinant antibodies. In a different review, antibody production and purification have been discussed.[2] Nevertheless, the overall instability and stabilization of antibody drug candidates have not been carefully examined in the literature. This article, not meant to be exhaustive, intends to review the structure and functions of antibodies, discuss their instabilities, and summarize the methods for stabilizing/formulating antibodies.

ANTIBODY STRUCTURE

Antibodies (immunoglobulins) are roughly Y-shaped molecules or combination of such molecules (Fig. 1). Their structures are divided into two regions—the variable (V) region (top of the Y) defining antigen-binding properties and the constant (C) region (stem of the Y), interacting with effector cells and molecules. Immunoglobulins can be divided into five different classes –IgA, IgD, IgE, IgM, and IgG based on their C regions, respectively designated as α, δ, ε, μ, and γ (five main heavy-chain classes).[6] Most IgGs are monomers, but IgA and IgM are respectively, dimmers and pentamers linked by J chains. IgGs are the most abundant, widely used for therapeutic purposes, and their structures will be discussed as antibody examples in detail.

Primary Structure

The structure of IgGs have been thoroughly reviewed.[6] The features of the primary structure of antibodies include heavy and light chains, glycosylation, disulfide bond, and heterogeneity.

Heavy and Light Chains

IgGs contain two identical heavy (H, 50 kDa) and two identical light (L, 25 kDa) chains (Fig. 1). Therefore, the total molecular weight is approximately 150 kDa. There are several disulfide bonds linking the two heavy chains, linking the heavy and light chains, and residing inside the chains (also see next section). IgGs are further divided

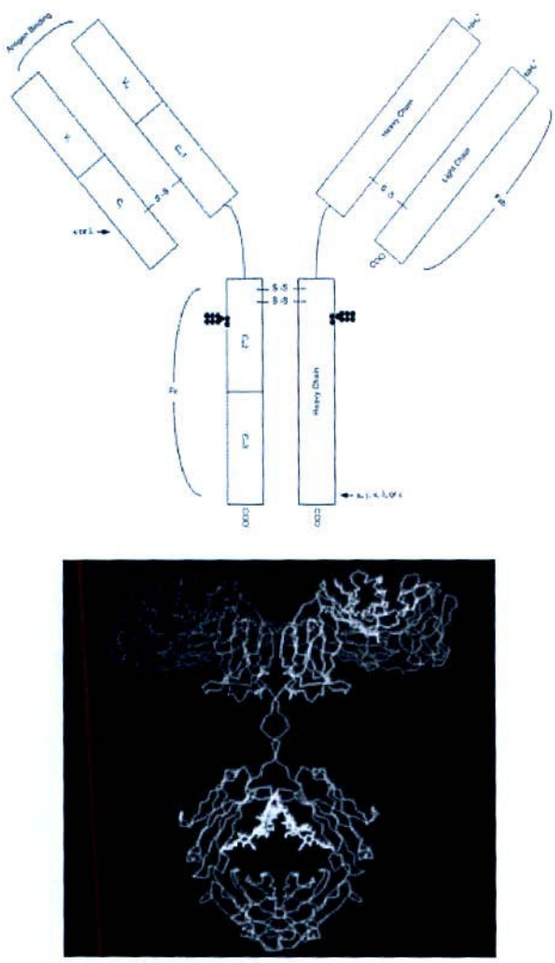


Figure 1. Linear (upper panel) and steric (lower panel) structures of immunoglobulins (IgG).

into several subclasses—IgG1, IgG2, IgG3, and IgG4 (in order of relative abundance in human plasma), with different heavy chains, named γ1, γ2, γ3, and γ4, respectively. The structural differences among these subtypes are the number and location of interchain disulfide bonds and the length of the hinge region. The light chains consist of two types—lambda (λ) and kappa (κ). In mice, the average of κ to λ ratio is 20:1, whereas it is 2:1 in humans.[6] The variable (V) regions of both chains cover approximately the first 110 amino acids, forming the antigen-binding (Fab) regions, whereas the remaining sequences are constant (C) regions, forming Fc (fragment crystallizable) regions for effector recognition and binding.[6] The N-terminal sequences of both the heavy and light chains vary greatly between different antibodies. It was suggested that the conserved sequences in human IgG1 antibodies

are approximately 95% and the remaining 5% is variable and creates their antigen-binding specificity.[5]

The V regions are further divided into three hypervariable sequences (HV1, HV2, and HV3) on both H and L chains. In the light chains, these are roughly from residues 28 to 35, from 49 to 59, and from 92 to 103, respectively.[6] Other regions are the framework regions (FR1, FR2, FR3, and FR4). The HV regions are also called the complementarity determining regions (CDR1, CDR2, and CDR3). While the framework regions form the β-sheets, the HV sequences form three loops at the outer edge of the β barrel (also see Section 2.2).

Disulfide Bonds

Most IgGs have four interchain disulfide bonds—two connecting the two H chains at the hinge region and the other two connecting the two L chains to the H chains.[6] Exceptions do exist. Two disulfide bonds were found in IgG1 and IgG4 linking the two heavy chain in the hinge region but four in IgG2.[7] In IgG1 MAb, HC is linked to the LC between the fifth Cys (C217) of HC and C213 on the LC. In IgG2 and IgG4 MAbs, it is the third Cys of HC (C123) linking to the LC.[7] A disulfide bond between HC C128 and LC C214 was found for mouse catalytic monoclonal antibodies (IgG2a).[8]

IgGs have four intrachain disulfide bonds, residing in each domain of the H and L chains, stabilizing these domains. The intrachain disulfide bonds in V_H and V_L are required in functional antigen binding.[9] Native IgG MAbs should not have any free sulfhydryl groups.[7] However, detailed examination of the free sulfhydryl groups in recombinant MAbs (one IgG1, two IgG2, and one IgG4) suggests presence of a small portion of free sulfhydryl group (approximately 0.02 mol per mole of IgG2 or IgG4 MAb and 0.03 for IgG1.[7] In rare cases, a free cysteine is found. A nondisulfide-bonded Cys at residue 105 was found on the heavy chain of a mouse monoclonal antibody, OKT3 (IgG2a).[10]

Oligosaccharides

There is one oligosaccharide chain in IgGs.[6] This N-linked biantennary sugar chain resides mostly on the conserved Asn 297, which is buried between the C_H2 domains.[5,11] For example, the oligosaccharide resides on Asn-297 of the C_H2 domain of chimeric IgG1 and IgG3 molecules[12] but on Asn 299 in a monoclonal antibody, OKT3 (IgG2a).[10] The oligosaccharide, often microheterogeneous, is typically fucosylated in antibodies produced in CHO or myeloma cell lines[5] and may differ in other cell lines.[2,11] There are many factors that dictate the nature of the glycan microheterogenity on IgGs. These include cell line, the bioreactor conditions and the nature of the downstream processing. An additional oligosaccharide can be found in rare cases. A human IgG produced by a human-human-mouse heterohybridoma contains an additional oligosaccharide on Asn 75 in the variable region of its heavy chain.[13] In addition, O-linked carbohydrates could also exist in this antibody.

Proper glycosylation is critical for correct functioning of antibodies.[11] It was demonstrated that removal of the oligosaccharide in IgGs (IgG1 and IgG3) made them ineffective in binding to C1q, in binding to the human FcγRI and activating C; and generally more sensitive to most proteases than their corresponding wild-type IgGs (one exception).[12] This is because the binding site on IgG for C1q, the first component of the complement cascade, is localized in the C_H2 domains.[11] Furthermore, the glycosylation can affect the antibody conformation.[12]

Oligosaccharides in other regions can also play a critical role. Removal of an oligosaccharide in a Fv region of the CBGA1 antibody resulted in a decreased antigen-binding activity in several ELISA systems.[13] In addition, this oligosaccharide might play critical role in reducing the antigenicity of the protein.[14]

The sugar composition of the oligosaccharide is also critical in antibody functions. It has been shown that a low fucose (Fuc) content in the complex-type oligosaccharide in a humanized chimeric IgG1 is responsible for a 50-fold higher antibody-dependent cellular cytotoxicity (ADCC) compared with a high Fuc counterpart.[15]

Heterogeneity

Purified antibodies are heterogeneous in structure. This is true for all monoclonal antibodies (MAbs) due to differences in glycosylation patterns, instability during production, and terminal processing.[5] For example, five charged isoforms were found in recombinant humanized monoclonal antibody HER2 as found by capillary isoelectric focusing (cIEF) and sodium dodecyl sulfate–capillary gel electrophoresis (SDS–CGE).[16] Six separate bands were focused under

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IEF for two mouse monoclonal antibodies IgG2a (κ) and IgG1 (κ).[17] A mature monoclonal antibody, OKT3 (IgG2a), contain cyclized N-terminus (pyroglutamic acid, −17 D) in both H and L chains, processed C-terminus (no Lys, −128 D) of the H chains, and a small amount of deamidated form.[10] Similar observation was also reported for a humanized IgG1 (κ).[18] In rare cases, gene cross-over may lead to formation of abnormal heavy chains. For example, a purified monoclonal anti-IgE antibody contains a small amount of a variant H chain, which had 16 fewer amino acid residues than the normal H chain (position is between Arg108 of the L chain and Ala124 of the H chain).[19]

Secondary and Higher-Order Structure

The basic secondary and higher-order structural features of IgGs have been reviewed.[6] Only a small portion of the three-dimensional structures of IgGs has been solved.[20] The antibody's secondary structure is formed as the polypeptide chains form anti-parallel β-sheets. The major type of secondary structure in IgGs is these β-sheets and its content is roughly 70% as measured by FTIR.[21] The light chain consists of two and the heavy chain contains four domains, each about 110 amino acid long.[6,20] All these domains have similar folded structures—β barrel, also called immunoglobulin fold, which is stabilized by a disulfide bond and hydrophobic interaction (primary). These individual domains (~12 kDa in size) interact with one another (V_H and V_L; C_H1 and C_L; and between two C_H3 domains except the carbohydrate-containing C_H2 domain) and fold into three equal-sized spherical shape linked by a flexible hinge region. These three spheres form a Y shape (mostly) and/or a T shape.[22]

The less globular shape of IgGs is maintained both by disulfide bonds and by strong noncovalent interactions between the two heavy chains and between each of the heavy-chain/light-chain pairs.[23] Through noncovalent interactions, a less stable domain becomes more stable, and thus, the whole molecule can be stabilized.[24] A detailed study indicates that the interaction between two CH3 domains are dominated by six contact residues, five of these residues (T366, L368, F405, Y407, and K409) forming a patch at the center of the interface.[25] These noncovalent interactions are spatially oriented such that variable domain exchange (switching V_H and V_L; inside-out IgG; ioIgG) induces noncovalent multimerization.[26]

The six hypervariable regions in CDR (L1, L2, L3, H1, H2, and H3) form loops of a few predictable main-chain conformations (or canonical forms), except H3 loop, which has too many variations in conformation to be predicted accurately.[27,28] There is a slight difference in the loop composition and shape between the two types of light chains.[20] However, no functional difference was found in antibodies having λ or κ chain.[6]

Basic Functions of Antibodies

The basic functions of antibodies have been reviewed.[6] There are two functional areas in IgGs—the V and C regions. The V regions of the two heavy and light chains offer two identical antigen-binding sites. The binding of the two sites (bivalent) can be independent of each other and does not seem to depend on the C region.[29] The exact antigen-binding sites are the CDR regions with participation of the frame work regions.[30] Binding of antigens seems through the induced-fit mechanism.[31,32] The induced-fit mechanism allows multispecificity and polyreactivity. It has been suggested that about 5–10 residues usually contribute significantly to the binding energy.[32]

The C regions of antibodies have three main effector functions (1) being recognized by receptors on immune effector cells, initiating antibody-dependent cell cytotoxicities (ADCC), (2) binding to complement, helping to recruit activated phagocytes, and (3) being transported to a variety of places, such as tears and milk.[6] In addition, C domains also modulate *in vivo* stability.[23,29,33] The function of Fc is affected by the structure of Fab. Variable domain exchange (switching V_H and V_L; inside-out IgG; ioIgG) affected Fc-associated functions such as serum half-life and binding to protein G and FcγRI.[26]

The hinge region provides flexibility in bivalent antigen binding and activation of Fc effector functions.[26] Two chimeric IgG3 antibodies lacking a genetic hinge but with Cys residues in CH2 regions was found to be deficient in their intermolecular assembly, and both IgG3 ΔH + Cys and IgG3 ΔH + 2Cys lost greatly their ability to bind FcγRI and failed to bind C1q and activate the complement cascade.[34]

Alternative Forms of Antibodies

In addition to species-specific antibodies, other antibody forms are generated to meet various needs. In the early development of antibody therapies, antibodies were made from murine

sources. However, these antibodies easily elicit formation of human anti-mouse antibody (HAMA). Therefore, humanized chimeric antibodies were generated. Chimeric monoclonal antibodies (60–70% human) are made of mouse variable regions and human constant regions.[2] Such antibodies can still induce formation of human anti-chimeric antibody (HACA). Highly humanized antibodies, CDR-grafted antibodies, are made by replacing only the human CDR with mouse CDR regions (90–95% human).[2] These antibodies are almost the same in immunogenicity potential as completely human antibodies, which may illicit formation of human anti-human antibody (HAHA).

Other alternative forms of antibodies have also been generated and these different forms have been reviewed.[35] Treatment with papain would cleave the N-terminal side of the disulfide bonds and generate two identical Fab fragments and one Fc fragment. Fab's are 50 kDa ($V_H + C_H1$)/($V_L + C_L$) heterodimers linked by a single disulfide bond. Treatment with pepsin cleaves the C-terminal side of the disulfide bonds and produces a $F(ab)'_2$ fragment. The remaining H chains were cut into several small fragments.[6] Cleavage by papain occurs at the C-terminal side of His-H228[36] or His-H227.[37] Reduction of $F(ab')_2$ will produce two Fab'.[23]

Fv fragments are noncovalent heterodimers of V_H and V_L. Stabilization of the fragment by a hydrophilic flexible peptide linker generates single-chain Fv (scFvs).[2] Fragments without constant domains can also be made into domain antibodies (dAbs). These scFvs are 25–30 kDa variable domain ($V_H + V_L$) dimers joined by polypeptide linkers of at least 12 residues. Shorter linkers (5–10 residues) do not allow pairing of the variable domains but allow association with another scFv form a bivalent dimer (diabody) (about 60 kDa, or trimer: triabody about 90 kDa).[38] Two diabodies can be further linked together to generate bispecific tandem diabody (tandab).[39] Disulfide-free scFv molecules are relatively stable and useful for intracellular applications of antibodies—"intrabodies."[38] The smallest of the antibody fragments is the minimal recognition unit (MRU) that can be derived from the peptide sequences of a single CDR.[2]

ANTIBODY INSTABILITY

Antibodies, like other proteins, are prone to a variety of physical and chemical degradation pathways, although antibodies, on the average, seem to be more stable than other proteins. Antibody instabilities can be observed in liquid, frozen, and lyophilized states. The glycosylation state of an antibody can significantly affect its degradation rate.[40] In many cases, multiple degradation pathways can occur at the same time and the degradation mechanism may change depending on the stress conditions.[41] These degradation pathways are divided into two major categories—physical and chemical instabilities. This section will explore the possible degradation pathways of antibodies and their influencing factors.

Physical Instability

Antibodies can show physical instability via two major pathways—denaturation and aggregation.

Denaturation

Antibodies can denature under a variety of conditions. These conditions include temperature change, shear, and various processing steps. Compared with other proteins, antibodies seem to be more resistant to thermal stress. They may not melt completely until temperature is raised above 70°C,[21,42,43] while most other mesophilic proteins seem to melt below 70°C.[44] Shear may cause antibody denaturation. For example, the antigen-binding activity of a recombinant scFv antibody fragment was reduced with a first-order rate constant of 0.83/h in a buffer solution at a shear of approximately 20,000/s.[45]

Lyophilization can denature a protein to various extents. An anti-idiotypic antibody (MMA 383) in a formulation containing mannitol, saccharose, NaCl, and phosphate was found to loose its *in vivo* immunogenic properties (only 10–20% of normal response rate) upon lyophilization.[46] Since the protein showed no evidence of degradation after lyophilization, no change in secondary structure by CD (29% β-sheet, 14% α-helix, and 57% "other"), the loss of activity was attributed to the conformational change. Indeed, tryptophan fluorescence properties were different between the lyophilized and unlyophilized antibodies.[46]

Aggregation

Antibody aggregation is a more common manifestation of physical instability. The concentration-dependent antibody aggregation was considered the greatest challenge to developing protein formulations at higher concentrations.[47] This is

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because protein aggregates generally have reduced activity and more importantly, greater immunogenicity potential because of the multiplicity of epitopes and/or conformational changes.[14,48] Immunoglobulin aggregates have been shown to cause serious renal failure[49] and anaphylactoid reactions such as headache, fever, and chills.[50] Therefore, the aggregate level in commercial intravenous immunoglobulin products is limited to less than 5% based on the WHO standards.

Aggregates can form easily both in liquid and solid states under a variety of conditions (Tab. 2). Since protein aggregation is often the consequence of protein–protein interactions, a process influenced by diffusion rate and geometric constraints of the interaction sites, such factors would influence significantly the aggregation rate, including protein concentration change, viscosity, ionic strength, pH, and temperature.[51] Other conditions may also affect protein aggregation, including shaking, long-term storage, freeze-thaw process, lyophilization process, etc.

Increasing the concentration of antibodies often increases the aggregation tendency of the protein. It was demonstrated that increasing the IgG1 concentration (in 10 mM citric acid, 100 mM NaCl pH 5.5) from 2.7 mg/mL to 50 mg/mL almost linearly increased the Nephelometric Unit (NU) from 2 to 40.[52] Since EP defines a clear solution as having equal or less than three NU, most antibody solutions in the study are opalescent except those at 5 mg/mL or less. However, as the weight average molecular weight was found to be 0.9–1.3 times that expected of a monomer (about 149 kDa), minimal protein–protein association (and readily reversible) is suggested.[52] Shaking can accelerate antibody precipitation and the shape of the precipitates may depend on the sample volume in a container.[53] It appears that protein solutions exhibiting lower surface tension are more susceptible to protein denaturation and precipitation.[53]

Low-temperature treatment may induce aggregation of antibodies. The reversible low-temperature-induced aggregation (below 37°C) of serum cryoglobulins is well known.[54] Human IgM cryoglobulin preparations easily precipitate or gel at temperatures below 10–12°C and the process is reversible at a higher temperature.[20] In a recent study, aggregation of IgG1 at low temperature at above 18 mg/mL was reversible as measured by light scattering.[52] The low-temperature-induced aggregation of cryoglobulins is poorly understood and was thought to involve sites within both Fab and Fc.[20] The authors of this article believe that low temperature reduces the hydrophobic interaction, which is the major force in protein folding. Without enough hydrophobic interaction at low temperature, hydrophobic regions of antibodies become more exposed to solvent and lead to increased intermolecular hydrophobic interaction, leading to aggregation.

In close relation to the low-temperature effect, freeze-thaw process often induces protein aggregation. However, freeze-thaw-induced antibody aggregation does not seem to be a major issue, partly due to the reversibility of antibody aggregates. Freeze-thawing of a chimeric (L6) antibody solution at pH 7.2 for as many as 35 times led to formation of dimers (not larger aggregates).[41] Maximum amount of dimers (about 20%) was observed at pH 6.5 and minimum was below 5.5 or above 8.5 (less than 2%). The freeze-thaw-induced aggregation is apparently reversible after treatment at 37°C for a few hours.[41] Freeze-thawing of rhuMAb anti-CD20 three times (freezing to either −20 or −70°C, thawing to 5°C) did not lead to significant change in aggregation, no significant loss of monomer by RP-HPLC or SEC-HPLC.[55] On the other hand, isolated Fabs seems to be more prone to freeze-thaw-induced aggregation relative to full-length antibodies. For example, Lee[56] found that freeze-thawing of a scF_v (MW 27,000 d) at 1.45 mg/mL in sodium phosphate buffered saline (PBS) at pH 7.3 dropped the %monomer content (due to aggregation) from 95% to 84%, 59%, 44%, and 37%, respectively after 1–4 cycles.

Lyophilization may induce antibody aggregation to a variable degree. It was demonstrated that lyophilization of a recombinant humanized monoclonal antibody (rhuMAb HER2), in the absence of sugars, led to a slight increase in aggregate (less than 1.2%).[57] Lyophilization caused detectable aggregation of a human monoclonal antibody in PBS or physiological saline as measured by OD600,[58] and aggregation of a mouse monoclonal antibody (MN12) [IgG2a (κ)] in the absence or presence of 5% sucrose, dextran, or hydroxypropyl-beta-cyclodextrin (HP-β-CD), as measured by visual examination.[59] In a different study, lyophilization of a monoclonal antibody-vinca conjugate in PBS at pH 7.4 caused 6.2% aggregation.[60] The lyophilization-induced aggregates could be non-covalent-linked monomers and/or even disulfide-linked.[61,62]

Antibody aggregation can easily occur during storage in a liquid state. Storing of a fully human anti-IL8 monoclonal antibody (ABX-IL8; IgG2)

Table 2. Instabilities of Antibodies

Instability	Antibody	Formulation	Condition	Effect	References
Denaturation	scFv	3 μg/mL in 10 mM phosphate-buffered saline containing 0.02% sodium azide	Shaking at a mean velocity gradient approximately 20,000/s	Loss of activity at a first-order rate of 0.83/h	45
Aggregation	Chimeric mouse/human IgG1	2 mg/mL in PBS at pH 7.2	Stirring for 48 h with Teflon bar at 600 RPM	Increased turbidity Formation of >15 k particles (>10 μ)	91
	Human Anti-IL-8 (IgG2)	55 mg/mL, 5 mM His, pH 6	Freeze/thaw three times (room/−70°C)	About 0.6% aggregates (by SEC-HPLC)	21
	Anti-IL-8 (IgG2)	100 mg/mL, 200 mM Sucrose, 15 mM Arg, 15 mM His, pH 6	Incubation at 40°C for 6 months	About 11% aggregates (by SEC-HPLC)	21
	rhuMAbE25	10 mg/mL in excipient-free solution	Spray drying Tin = 105°C, Tout = 50–55°C	5–6% aggregate (by SEC-HPLC)	65
	MAb (type not specified)	MAb at 40 mg/mL, 85 mM sucrose, 5 mM His at pH 6, 0.01% Tween 20	Lyophilization	Cloudy (OD350 > 0.2)	1
	Human Anti-IL-8 (IgG2)	50 mg/mL, 20 mM Gly, 4 mM His, 0.2% mannitol, 16 mM Glu, 0.03% Tween 20 pH 5.7	Incubation of lyophilized form at 37°C for 1 month	4.8% aggregates (by SEC-HPLC)	21
	RhuMAb HER2	50 mg/mL in 100 mM trehalose, 5 mM His at pH 6.0	Incubation of lyophilized form at 40°C for 23 months	9.7% aggregates (by SEC-HPLC)	57
Deamidation	OKT3 (IgG2a)	1 mg/mL, PBS at pH 7.0 + Tween80	Incubation at 37°C for 2 months	90% deamidation of Asn386	10
	LA298 (IgG1)	0.5 mg/mL in 10 mM citrate at pH 7	Incubation at 25°C for 6 weeks	~12% increase in deamidation at Asn55	78
Isomerization	MAb	Lyophilized at 40 mg/mL, 85 mM sucrose, 5 mM His, 0.01% Tween 20, pH 6.0, containing 8.4% moisture	Incubation at 5°C	First-order rate constant $\sim 0.33 \times 10^{-4}$/day	1
Oxidation	rhuMAb HER2	5 mg/mL, 147 mM NaCl, 0.01% polysorbate 20, 5 mM sodium acetate at pH 5.0	5, 30, and 40°C for 2 weeks	10, 17, and 52% (total peak area)	81

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in aqueous solutions at 2–8°C or 25°C led to formation of high-molecular-weight (HMW) species.[21] Incubation of two mouse monoclonal antibodies IgG2a (κ) and IgG1 (κ) at 37°C for 32 days caused precipitation in pH 3 and 4 solutions for both antibodies.[17] Aggregation can also occur to an antibody conjugate. Storing a monoclonal antibody-vinca alkaloid conjugate (murine IgG2a) in phosphate-buffered saline solutions at 5°C between pH 4.5 and 7.4 for 1.5 months caused precipitation regardless of solution pH and aggregation was accelerated at 30°C throughout the pH range.[63]

Antibody aggregation is a common phenomenon during storage in a lyophilized state. Aggregation (OD350 > 0.2) was observed of a high concentration, lyophilized, monoclonal antibody (type not specified) by SEC under accelerated conditions.[1] So did a lyophilized chimeric monoclonal antibody during storage at 5°C (0.4% after 2 months).[64] In the absence of sugars, the extent of rhuMAb HER2 aggregation increased from about 1% to >10% after 3 months at 40°C and the rate of aggregation for these formulations was about 0.2% per month at 40°C.[57]

Antibody aggregation can readily occur in other solid forms during storage.[5] It was found that the primary degradation upon storage of a spray-dried anti-IgE (protein:mannitol = 80:20) is aggregation, and the amount of aggregates increased with increasing temperature and relative humidity (Maa et al., 1998a).[65] As much as 52% aggregate was observed by SEC-HPLC of a spray-dried anti-IgE (protein:mannitol = 80:20) after 1 year at 40°C and 38% RH (Maa et al., 1998b).[66]

Antibody aggregation is usually accelerated upon thermal treatment. In the study of chimeric (human/mouse) monoclonal antibody (BR96), Alexander and Hughes demonstrated that thermal treatment at 60°C for 166 h generated noncovalently linked aggregates.[67] In the evaluation of the relative stability of murine IgG2a (κ), chimeric IgG1(κ), trioma IgG1 (λ), and murine IgG1 (κ), Hartmann et al. demonstrated that IgG2a (κ) aggregated and precipitated after heating at 55°C for 8 h and the half-life for the loss of monomer for those four IgGs is 1, 18.1, 3.4, and 8.2 h with different aggregate species (dimer, trimer...) by SEC-HPLC.[68]

Surface Adsorption

Antibodies, like other proteins, can easily adsorb to a variety of surfaces. Surface adsorption can significantly reduce the antibody concentration in a solution. For example, a mouse monoclonal IgG1 has been shown to be adsorbed on the surface of glass shake flasks, causing a loss of protein in plant culture media.[69] Such a loss was minimized by coating the glass vessels or by addition of Pluronic F127.

Chemical Instability

A variety of chemical degradation pathways have been observed. These chemical degradants may or may not loose protein activity depending on the site of changes. For examples, deamidation of a monoclonal antibody, OKT3 (IgG2a) as measured by change in IEF pattern does not correlate with loss of antigen binding potency (90% deamidation of Asn386 vs. 80% activity in the potency assay).[10] In comparison, the activity of light chain deamidated recombinant humanized monoclonal antibody HER2 (rhuMAb HER2, Herceptin, trastuzuMAb) at Asn30 (to aspartate) is only 70% of the main species and that of the isomerized (heavy chain) asp102 species is only 9–21% of major species.[37] The major chemical degradation pathways in an antibody include cross-linking, deamidation, isomerization, oxidation, and fragmentation.

Disulfide Formation/Exchange

Disulfide bond formation/exchange is probably the most common cross-linking pathway, leading to chemical aggregation. This degradation pathway can occur readily in various processing steps. For example, lyophilization of a murine monoclonal antibody (IgG2a) without additives reduced the average number of –SH groups per molecule from 6.65 to 6.28, suggesting disulfide formation.[70] Since the thiolate ions are often the initiating species for this chemical degradation, increasing formulation pH usually leads to increased formation of disulfide formation. This is the case for an antifebrin T2G1s Fab′ (IgG1 (κ)), containing free –SH groups, where the percentage of dimers increased with increasing the pH from 5.8 to 9.5.[61]

Storage often leads to disulfide-based aggregation. Storage of a lyophilized excipient-free antibody (IgG1, containing 12 intra and 4 interchain disulfides) formulation for 1 year at 30°C resulted in formation of reducible dimer and trimers.[62] Similarly, reducible aggregates of a monoclonal antibody (type not specified) were detectable

by SDS–PAGE after storage of the lyophilized formulation at 50°C for 6 months.[1] Such disulfide-linked covalent dimers and trimers were also observed during storage of spray-dried anti-IgE humanized monoclonal antibody.[71]

Nonreducible Cross-Linking

Nonreducible cross-linking was observed in several studies. Incubation of a human monoclonal antibody (C23) in solution at a basic pH at 37°C for 14 days resulted in formation of nonreducible bands above the light chains at pH 4 and 10.[72] Incubation of two mouse monoclonal antibodies—IgG2a (κ) and IgG1 (κ), between pH 3.0 and 10 at 37°C for 32 days led to formation of a nonreducible 88 kDa species by SDS–PAGE.[17] A species with the same molecular weight was observed after incubation of another monoclonal antibody, OKT3 (IgG2a), at 37°C for 2 months.[10,73] Further examination suggests that this species is a cross-linked product of H and L chains between the L46-52 and H99-121 regions. Although the exact chemical nature was not determined, an oxidative step is apparently involved as inhibition of oxidation delayed the formation of this species.[10,73]

A recent study shows that a nonreducible thioether bond can be formed between C223 of the heavy chain and the C-terminal Cys residue of the light chain in humanized IgG1 antibodies. This cross-linked product has a molecular weight of 75 kDa by MS but shows an apparent molecular weight of 92 kDa by SDS–PAGE.[74]

Nonreducible cross-linked products were also observed in solid dosage forms. Incubation of a lyophilized mouse monoclonal antibody [IgG2a (κ)] without lyoprotectant or in the presence of 5% sucrose, dextran, or hydroxypropyl-beta-cyclodextrin (HP-β-CD) at 56°C for 18 days led to formation of 100-kDa band under reduced conditions by SDS–PAGE.[59]

Deamidation

Antibody deamidation has been extensively reported in the literature. This common protein degradation pathway is so prevalent that purified antibody preparations may contain many deamidated forms. For example, several deamidated forms of recombinant humanized monoclonal antibody HER2 (rhuMAb HER2, Herceptin, trastuzuMAb) were found by cation-exchange chromatography, including (1) deamidated Asn30 (to aspartate) on one light chain, (2) deamidated Asn30 (to aspartate) on two light chains, and (3) deamidated Asn55 on the heavy chain (to isoaspartate).[37] The sole conversion of Asn30 to aspartate (not isoaspartate) suggests that the deamidation mechanism in cell culture may be different from that during storage.

Storage can easily generate large amounts of deamidated products. Incubation of a monoclonal antibody, OKT3 (IgG2a) in a PBS solution at pH 7 containing Tween 80 at 37°C for 2 months led to more than 90% deamidation at Asn H386.[10] By monitoring the ammonia generation, Poborji et al.[41] estimated that 4% of the total number of Asn and Gln residues in a chimeric antibody (L6) was deamidated after storing the solution at pH 7.2 at 50°C for 3 weeks.

Deamidation also occurs in solid state. In the evaluation of storage stability of a model recombinant humanized monoclonal antibody (rhuMAb HER2), Cleland et al. reported that incubation of a high-moisture formulation (8.4% moisture) for 12 months at 40°C resulted in a 19% drop in the main peak by IEX due to deamidation.[57]

Deamidation in proteins goes mostly through the intermediate of succinimide at Asn (more readily) and Gln (see reviews[75,76]).This seems to be the case for antibodies, even though alternative mechanisms were reported, such as deamidation of Asn30 on the light chain of recombinant humanized monoclonal antibody HER2 (rhuMAb HER2, Herceptin, trastuzuMAb).[37] A method has been developed to detect the formation of succinimide.[77]

The rate of deamidation via succinimidation in proteins can be affected by a variety of factors, such as pH, sequence, and steric effect (see review[44]). A recent report demonstrated a strong pH-dependency of deamidation at Asn55 (pH 7 > pH 6 > pH 5 > pH 4) in a fully human IgG1 in the pH range of 4–7 during storage at 25°C.[78] Using small peptide as a model, Tyler-Cross and Schirch[79] found that deamidation of Asn residues depends on the amino acid residue on the carboxyl side of Asn in neutral and alkaline solutions. Increasing the size and branching of these amino acids decreased the rate of deamidation by as much as 70-fold compared with the N–G sequence (greatest rate of deamidation) but under acidic conditions, these amino acids lost their protective effect.

Isomerization

The most common isomerization in antibodies is iso-aspartic acid formation, which results not only

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from direct isomerization of Asp but also, mostly, from hydrolysis of succinimide intermediate. The pH-dependent formation of succinimide intermediate can occur either from Asn deamidation or Asp dehydration.[36] In neutral and alkaline condition, the isoAsp and Asp products formed in a peptide were at a ratio of about 3:1.[79]

Isomerization is also prevalent and difficult to control. Again, many purified proteins contain significant amount of isomerized products, either from deamidation or direct isomerization. A typical example is recombinant humanized monoclonal antibody HER2 (rhuMAb HER2, Herceptin, trastuzuMAb).[37] In addition, since isomerization occurred at Asp H102 in CDR regions, the activity of the isomerized species is only 9–21% of the major species.[37] Similarly, isomerization of Asp L32 on one light chain in a recombinant monoclonal anti-IgE antibody (E25) reduced the relative binding activity of $F(ab)'_2$ to 42% and the activity of isomerized form on both chains (iso-Asp-iso-Asp) to 15%.[36]

Since formation of the succinimide intermediate does not need participation of water, it can readily occur in solid state. Asp isomerization is one of the major degradation pathways for a lyophilized, monoclonal antibody (type not specified), and increases with increasing storage temperatures both above and below their Tg values.[1]

Like deamidation, the relative rate of isomerization can be influenced strongly by steric effect. For example, a recombinant monoclonal anti-IgE antibody (E25) contains two Asp-Gly sequences within its CDRs, but only one site was found to be labile to isomerization (Asp L32). The unreactivity of one Asp residue is suggested to be due to hydrogen bonding.[36]

Oxidation

The oxidizable residues in proteins include Met, Tyr, Trp, His, and Cys. Although oxidation is not as prevalent as deamidation and isomerization in antibodies, it can easily occur during storage of antibodies. For example, the major route of degradation of a monoclonal antibody, OKT3 (IgG2a), in solution during storage at 5°C is oxidation of a nondisulfide Cys and several Met residues.[10] Oxidation was also observed in rhuMAb anti-CD20 in solution during storage at 40°C for 2 months.[55]

Oxidation of CDP870, a pegylated one-half antibody (one light and one heavy chain) at a Met residue (M3) was also observed after incubation of the protein at 25°C or 40°C for 12 weeks (14% at pH 4.1 and 4.2% at pH 5.5).[80] Light exposure can accelerate baseline oxidation. For example, light exposure (20,000 lux for 2 weeks at 27°C) of recombinant humanized monoclonal antibody HER2, rhuMAb HER2 caused 5–10% increase in oxidation in various liquid formulations at Met H255 (primary site) and Met H431 at temperatures 30 and 40°C.[81]

Formation of Acidic or Basic Species

Antibodies can form acidic or basic species, which are often monitored by IEF or ion exchange chromatography. Among all the possibilities, deamidation is the most likely degradation pathway, leading to formation of acidic species. Therefore, formation of such species is used loosely as an indication of deamidation in the absence of other confirmatory studies. Strictly speaking, formation of such species can only be considered as an indication of chemical instability (see below). A pH-dependency is almost certain for this kind of degradation. In the stabilization of CDP870, a one-half antibody (one light and one heavy chain), which is linked through Cys to a branched (20 kDa each) PEG, Johnson et al.[80] found that the protein generated more acidic species (up to 17% at pH 4.1 and 41% at pH 5.5) by IEF as the solution pH was increased from 4.1, 4.5, 5.0, to 5.5 during storage at 25°C for 12 weeks.[80]

Another possible mechanism for generating acidic species in antibodies is the Maillard reaction, where reducing sugars can react with lysine residues. Such a reation will result in loss of positive charges and formation of apparently more acidic species. This is the case for an spray-dried anti-IgE humanized monoclonal antibody with lactose during storage.[71]

Formation of basic species was also observed in antibodies. Its formation can result from several possibilities, most likely as a result of succinimide formation, removal of sialic acids, or pyroGlu formation. Such a basic species was observed after incubation of a human monoclonal antibody (C23) at 37°C for 14 days in a solution at an acidic pH by IEF.[72]

C-Terminal Clipping

Terminal cleavages can easily occur during normal production of antibodies. For example, the majority of a purified chimeric protein rCD4-IgG lacks the heavy chain C-terminal Lys.[82]

This is also the case for recombinant humanized monoclonal antibody HER2 (rhuMAb HER2, Herceptin, trastuzuMAb)[37] and a recombinant monoclonal anti-IgE antibody (E25).[36] It was suggested that the cleavage was due to the action of basic carboxypeptidases during production.[37]

Fragmentation

The most likely sequences leading to fragmentation in proteins are Asp-Gly and Asp-Pro,[83] although other sequences can also experience cleavages, such as Asn-Ser.[79] Fragmentation can easily occur in antibodies even during production processes. A small amount of fragment (MW < LC) was detectable under reduced condition in a purified recombinant MAb IgG4 produced in Chinese hamster ovary (CHO) cells.[7] Half molecules should be monitored routinely for IgG4s.[84]

A variety of processing conditions may accelerate fragmentation of antibodies, such as acidic or basic treatment,[72] thermal treatment,[41,67] freeze-thaw,[41] and storage.[17,73,78] Fragments can simply include masses with a loss of a light chain (M-LC), a loss of a Fab arm (M-Fab), and separated heavy-chain (HC) and light-chain (LC), as well as species less than common antibody subunits, resulting from both peptide and/or disulfide bond cleavage.[67] Antibodies are also subject to radiation-induced cleavages.[85]

Maillard Reaction

Proteins may form adducts with sugars. In an investigative study, mouse MAbs were found to form glucose adducts after incubation with 0.5 M glucose at pH 7.4 for 14–21 days at 37°C and the glycated antibodies significantly increased the dissociation of the antibody-antigen binding complex.[86]

ANTIBODY FORMULATION

As mentioned above, antibodies, like other proteins, may generate a variety of degradants during production, processing, and storage both in liquid and solid states. Their tendency to generate such degradants depends very much on their individual sequence, pI, hydrophobicity, and carbohydrate content.[23] The antibody degradation products may have reduced activity and more importantly, increased immunogenicity.[14,87] A strong immune response (antibody formation) may lead to severe neutralization of the protein drug product and even endogenous proteins.

The major purpose of formulating antibodies as therapeutic agents is to control the rate of antibody degradation to provide an acceptable shelf life for worldwide shipping and storage. This can be done by choosing proper formulation excipients and other formulation conditions. It should be stressed that one formulation excipient stabilizing a specific antibody may not be suitable for another because of the differences in their sequence. A proper formulation composition should not only maximally preserve the activity of the antibody but also minimize the potential immune response, as excipients could alter the immune response by changing the presentation of the protein (such as the spacing of epitopes).[14] The following section will discuss critical factors in formulating both liquid and lyophilized antibody products. Advanced formulations will also be briefly described.

Liquid Formulation

Liquid dosage form is usually preferable to lyophilized products as it is easier to administer and less expensive to manufacture. Among all the commercial antibody products, about half are stable enough to be formulated in a liquid form (Tab. 1). Formulating a successful liquid product needs consideration of at least the following aspects.

Protein Concentration

Since the EC50 values of antibodies are often 10–1,000 times higher than for other proteins such as hormones and cytokines,[35] a relatively large amount of antibodies needs to be dosed to achieve any therapeutic effect. A large dose requires that an antibody product needs to be formulated/reconstituted at a high concentration to reduce dose volume, especially for subcutaneous injections; otherwise, a large volume of antibody preparation has to be infused. Since the aggregation process is usually concentration-dependent, antibodies at high concentrations have at least two potential issues: high tendency to aggregate during storage and likely high viscosity, leading to more difficulty during injection (injection time correlated with viscosity).[47] It has been suggested that the concentration-dependent protein aggregation is the greatest challenge to developing high-concentration

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protein formulations.[47] On the other hand, moderate increase in protein concentration may not have a significant effect. In fact, increasing the concentration of antifebrin T2G1s Fab′ (IgG1 (κ)), from 0.05 to 5.0 mg/mL in solution resulted in a decrease in dimer formation during storage (73.2% to 22.9% after 13 weeks at 22°C).[61]

An effective way to reduce the viscosity of an antibody solution is to add sufficient amount of NaCl.[5,88] Adjustment of formulation pH could potentially reduce the viscosity of an antibody solution.[88] Although not very effective, histidine has been shown to reduce the solution viscosity of a fully human anti-IL8 monoclonal antibody (ABX-IL8; IgG2) from 20 to about 9 centistokes when its concentration was increased from 0 to 60 mM.[21] However, use of such excipients in reducing viscosity can be limited, as the excipient can adversely affect the stability of the antibody, such as MAb anti-CD20.[55]

Another potential issue in formulating high-concentration antibody products is their solubility limits, as presence of undissolved proteins are generally not acceptable for intravenous injection. Fortunately, the solubility of antibodies seems to be relatively high and solubility-limited formulation difficulty has rarely been reported to our knowledge. To increase the solubility of antibodies, excipients such as surfactants may be used. In a recent report, Golovanov[89] found that a combination of L-Arg and L-Glu at an equimolar concentration (50 mM) significantly increased the solubility of several proteins (either alone does not have a significant effect). In addition, the room-temperature storage stability (fragmentation) of proteins was significantly improved as monitored by SDS–PAGE.

One potential advantage of formulating a protein product at higher concentrations is the greater freeze-thaw stability. Often, proteins at high concentrations are more resistant to freeze-thaw-induced aggregation, as the interface-induced protein denaturation can easily reach a saturation point. However, it does not seem to be the case for antibodies. Increasing the concentration of a chimeric antibody (L6) did not inhibit freeze-thaw-induced aggregation.[41] In addition, surfactants such as Pluronic F68 and Tween 80 did not appreciably prevent antibody aggregation. In a different study, Tween 80 alone (or Pluoronic F68) at 0.1% failed to offer any protection against freeze-thaw-induced aggregation of a single-chain antigen-binding protein CC49/218 sF_v (MW 27,000 d) at 1.45 mg/rnL in sodium PBS at pH 7.3.[56] These results suggest that freeze-thaw-induced antibody aggregation may not be a surface phenomenon.

Effect of Formulation pH

Formulation development often starts with determination of a stable formulation pH. Like other proteins, the pH effect on the stability of antibodies depends on the formulation composition,[55] stress conditions,[41] and even antibody concentration.[84] Formulation pH may affect the physical stability of antibodies as it alters the number and distribution of charges on the protein surface. For example, low pH (below 3) caused a loss of tertiary structure in rhuMAb anti-CD20.[55] Low pH (pH 3 and 4) is also responsible for the precipitation of two mouse monoclonal antibodies IgG2a (κ) and IgG1 (κ) during incubation at 37°C for 32 days.[17] Susceptibility to pH stress varies among different IgG molecules.[68]

Regarding chemical stability, formulation pH may play a critical role in controlling many degradation pathways, such as disulfide bond formation/exchange, deamidation, fragmentation, isomerization, etc. Due to variable effects on different degradation pathways, the optimum pH value varies depending on both the sequence of an antibody and the analytical methods used in monitoring the stability. By IEF, Lam et al. found that rhuMAb anti-CD20 is most stable at pH 5 within the pH range of 3–6 irrespective of protein concentration in formulations containing 10 mM citrate, 8% trehalose, and 0.05% Tween 20.[55] By SEC-HPLC, Usami et al. found that pH 6 was the most stable condition during incubation of a human monoclonal antibody (C23) at 37°C for 14 days in a solution containing isotonic sodium chloride between pH 4 and 10.[72] Sometimes, the optimum pH values reside outside a certain range. In the study of the stability of a chimeric antibody (L6), minimum amount of dimmer formation was observed at pH below 5.5 or above 8.5 (less than 2%) during freeze-thaw.[41]

It needs to be mentioned that oxidation could be inhibited by adjusting formulation pH. In a formulation containing 40 mg/mL MAb anti-CD20, 150 mM trehalose, 0.9% benzyl alcohol, 0.02% Tween 20, and 50 mM histidine, reducing the pH from 7.5 to 5 virtually eliminated the oxidation during storage at 40°C for 2 months.[55] In a different study, oxidation (M3) of PEG-CDP870, a one-half antibody, in a formulation containing 160 mg/mL CDP870, 10 mM sodium acetate, and

125 mM NaCl was slower at pH 5.5 than at pH 4.1 (4.2% vs. 14% after 12 weeks at 40°C).[80]

Due to the simultaneous presence of multiple antibody degradation pathways, the formulation pH has to be adjusted to balance all the potential degradations. Generally, a weakly acidic condition seems to be the optimum for most antibodies (see Tab. 1).

Effect of Buffering Agents

It has been long recognized that both the type and concentration of a buffering agent may affect the stability of proteins (see review[44]). For example, phosphate buffer accelerated deamidation of an IgG1 relative to citrate buffer at both pH 6.5 and 7.0 during storage at 25°C.[78] Therefore, proper choice of a buffering agent can potentially achieve both pH control and stabilization of antibodies. In rare cases, a buffering agent good for one antibody may be harmful to another. One of these agents is histidine, which has a pKa of pH 6 and potentially good in controlling a weakly acidic condition. It has been shown to provide better protection against precipitation for a fully human anti-IL8 monoclonal antibody (ABX-IL8; IgG2) upon thermal incubation both at 50 and 40°C than citrate at pH 6.[21] Addition of a small amount of histidine (0.4 mM) also increased the freeze-thaw (4×) recovery of a single-chain antigen-binding protein CC49/218 sF_v (MW 27,000 d) at 1.45 mg/rnL in PBS solution at pH 7.3 from 37% to 90%.[56] On the contrary, histidine was found not only to increase the rate of aggregation of rhuMAb anti-CD20 during storage but also to induce coloration of the solution compared to a acetate-buffered antibody solution at pH 5.[55] In addition, oxidation of Fc was also faster in histidine solution.

The concentration effect of a buffering agent was demonstrated in a study where freeze-thaw-induced aggregation of a chimeric antibody (L6) was higher with increasing phosphate buffer concentration (0.01 vs. 0.05).[41] Therefore, both the type and concentration of buffering agents need to be examined.

Effect of Formulation Excipients

Use of formulation excipients remains a major and convenient approach in stabilizing antibodies in solutions. It is so at least in reducing antibody aggregation.[47] A variety of formulation excipients have been shown to stabilize antibodies under different processing conditions and during storage, including sugars,[41] polyols,[41] amino acids,[41,84] surfactants,[84] and polymers.[45,84]

Formulation excipients are effective generally in protecting the physical stability of an antibody. Among these excipients, sugars are the most commonly used. A variety of sugars, such as sucrose, trehalose, glucose, have been shown to reduce the freeze/thaw-induced aggregation of rhuMAb anti-CD20,[55] a chimeric antibody (L6),[41] a human anti-IL8 monoclonal antibody (ABX-IL8; IgG2),[21] and a single-chain antigen-binding protein CC49/218 sF_v (27 kDa).[56] Other excipients can be equally or even more effective in protecting antibodies. Histidine was shown to be equally effective as sucrose in reducing freeze-thaw induced aggregation of a human anti-IL8 monoclonal antibody (ABX-IL8; IgG2) and combination with Arg resulted in a better protection than sucrose in solution stability at different incubation temperatures.[21] Sugars are not always effective. In the evaluation of freeze-thaw stability of a single-chain antigen-binding protein CC49/218 sF_v (MW 27 kDa) at 1.45 mg/mL in sodium phosphate buffered saline (PBS) at pH 7.3, addition of 10 mM trehalose, lactose, glucose, or mannitol did not offer any protection.[56] In a rare case, sucrose has been shown to promote agitation-induced aggregation of an IgG1 antibody.[90]

Formulation excipients may inhibit chemical degradation of antibodies. Replacing 0.14 M NaCl with 8% trehalose or 4% mannitol in a rhuMAb anti-CD20 increased the chemical stability as measured by IEX during storage at 40°C.[55] Such an effect is likely a result of stabilization of antibody conformation. Some excipients may protect antibodies against oxidation. Replacing NaCl with 4% mannitol and 1% benzyl alcohol protected recombinant humanized monoclonal antibody HER2, rhuMAb HER2, against oxidation (primarily Met-255) at 30 or 40°C.[81] This effect is likely due to a function of mannitol as a free radical scavenger. Some commonly used antioxidants, such as thiosulfate and methionine, are effective in inhibiting antibody oxidation. The minimum effective levels (molar ratios of antibody to antioxidant) required to inhibit temperature-induced oxidation of rhuMAb HER2 were 1:5 and 1:25 for methionine and thiosulfate, respectively.[81] However, use of thiosulfate has led to formation of a thiosulfate adduct of rhuMAb HER2.[81] The safety of such a thio-containing adduct has not been reported.

One of the main excipients often used in antibody formulation is surfactants. They are usually

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effective in reducing shaking/stirring-induced aggregation[91] but may have negative effect during long-term storage.[92] One obvious concern is that such compounds may contain a residual level of peroxides, which have been shown to cause protein oxidation.[93] Therefore, use of surfactants should be kept to a minimum in an antibody formulation if possible. For example, removing Tween 20 in a recombinant humanized monoclonal antibody HER2, rhuMAb HER2 solution reduced the oxidized antibody from 52% to <10% at 40°C.[81]

Effect of Shaking/Shearing

Both shaking and shearing can denature antibodies. It was found that shaking murine antibody-based products caused antibody precipitation and the shape of the precipitates are different depending on the fill volume.[53] In the evaluation of shear effect on the antigen-binding activity of a recombinant scFv antibody fragment in a buffer solution, Harrison et al.[45] found that the binding activity decayed at a mean rate constant of 0.83/h at a shear of approximately 20,000/s.

Effect of Preservatives

A limited number of studies have been conducted on the compatibility of preservatives with antibodies. Compatibility depends both on the antibody and the preservative. In the evaluation of the effect of several preservatives, including benzyl alcohol, chlorobutanol, methylparaben, propylparaben, phenol, and m-cresol, on the stability of a humanized monoclonal antibody, Gupta and Kaisheva[94] found that the antibody was most stable in the presence of methylparaben and propylparaben, compatible with benzyl alcohol and chlorobutanol at low concentrations, and not compatible with phenol and m-cresol, as measured by a variety of assays, including SEC-HPLC, DSC, light scattering, UV, and potency assay.

Effect of Processing Equipment

Another important issue in antibody formulation is the choice of proper processing equipment/containers. A few studies have shown that antibody stability can be significantly affected if an inappropriate choice is made. In the study of the stability of recombinant humanized monoclonal antibody HER2, rhuMAb HER2, in liquid formulations, Lam et al.[81] found that replacing the stainless steel filler with a nonstainless steel filler reduced the oxidized antibody (primarily Met-255) from 52% to 18% after storage for 2 weeks at 40°C. It was suggested that Fe ions, released from erosion of stainless steel by chloride ion at low pH, catalyzed the oxidation. In a similar case, Chen et al.[21] demonstrated that a fully human anti-IL8 monoclonal antibody (ABX-IL8) in aqueous formulations containing 60 mM His had a higher level of aggregates after freeze-thaw in a stainless steel vessel and subsequent storage at 40°C, possibly due to the effect of Fe ions generated from a similar mechanism. In addition, proteins have been shown to facilitate metal corrosion.[95]

Effect of Product Containers

The effect of product containers was clearly demonstrated in a recent study. McCormick et al.[96] reported that Tween 80, intended to replace human albumin in the original formulation, interacted with uncoated rubber stoppers in single-use Eprex syringes. The stoppers leached a small amount of plasticizers in the drug solution, which acted as an adjuvant, and caused a strong IgG response to the recombinant human erythropoietin. The immune response was significantly minimized later by switching to PTFE-coated rubber stoppers. In addition, headspace oxygen can accelerate oxidation of antibodies and replacement with an inert gas could minimize such a reaction.[73]

Accelerated Stability Studies

To accelerate formulation development process, accelerated stability studies are usually conducted. However, estimation of room temperature or 5°C stability based on accelerated stability studies for antibodies, like other proteins, may or may not be possible or accurate. The unpredictability is due to the presence of complex and multiple degradation pathways, which may have different degree of temperature dependencies.[10] Kroon et al.[10] found that relative to other degradation pathways of a monoclonal antibody, OKT3 (IgG2a), Asn deamidation was preferentially accelerated with increasing temperature. Therefore, caution needs to be applied in interpreting such high-temperature-induced deamidation. On the other hand, Poborji et al.[41] found that loss of monomeric chimeric antibody (L6) at pH 7.2 followed Arrhenius behavior in the temperature range of 30–50°C and extrapolation of the high temperature SEC data to 2–8°C at pH 7 and

5.5 resulted in comparable stability estimates with the available real-time data.

Lyophilized Formulation

Preparation of lyophilized protein formulations has been extensively reviewed.[97–101] Like most proteins, some antibodies are not stable enough in a liquid form and lyophilized dosage forms will have to be considered. Lyophilization was suggested to be one of the two approaches in solving antibody aggregation.[47] This section will discuss some critical issues in formulating a lyophilized antibody product.

Amorphous Versus Crystalline State

It has been commonly believed that proteins are more stable in an amorphous state.[103] This is mainly because proteins need another excipient(s) to replace water molecules to from the required H-bonds as water molecules are removed during drying and a crystalline matrix does not provide intimate H-bonding between the excipient and the protein.[104,105] It appears that if the protein itself can be crystallized during the lyophilization process (may be difficult practically), the protein could be more stable and might not need as much stabilizing agent as in the amorphous state. In a recent report, Shenoy et al.[106] compared the stability of two model proteins, glucose oxidase (GO) and lipase, and demonstrated that dry crystalline formulations are significantly more stable than their amorphous counterparts as monitored by size-exclusion chromatography. It remains to be seen whether this can be applicable to antibodies.

Effect of Formulation Excipients

To make a lyophilized antibody product, at least one formulation excipient, a bulking agent, is needed to confer an acceptable cake. If the antibody is not stable enough in the presence of a bulking agent during lyophilization or storage, a stabilizer(s) is included. The relative amount of formulation excipients (bulking and stabilizing agents) should be carefully chosen as these agents may affect each other's behavior, the tonicity, and stability of the lyophilized products.

Commonly used bulking agents include mannitol and glycine. Crystallization of these agents during lyophilization makes them wonderful bulking agents but poor stabilizing agents.[57] Although mannitol could stabilize an antibody as in the case for a monoclonal antibody-vinca conjugate in a PBS solution at pH 7.4 upon lyophilization,[60] many studies showed that mannitol could not stabilize effectively or even destabilizes antibodies. Such examples include the aggregation of a lyophilized model recombinant humanized monoclonal antibody (rhuMAb HER2) in the presence of mannitol during storage.[3,57] In the lyophilization of a human monoclonal antibody at 1 mg/mL, it was demonstrated that addition of 5% mannitol has no effect on the lyophilization-induced aggregation in PBS or physiological saline as measured by OD600 and addition of 10% mannitol actually accelerated aggregation during the lyophilization process.[58] On the other hand, when used in combination with sucrose or trehalose, similar degree of stabilization to that for sugar alone may be achieved such as the case for model recombinant humanized monoclonal antibody (rhuMAb HER2).[57] The disaccharide/mannitol formulations also inhibited deamidation of this antibody during storage to a greater extent than the lyoprotectant formulations alone.[57]

Sugars are the most common stabilizing agents used in lyophilized formulations. Among these, two nonreducing sugars, sucrose and trehalose, are usually the first choice. Both sugars protected the stability of a few lyophilized antibodies equally well. These antibodies include lyophilized recombinant humanized monoclonal antibody, rhuMAb HER2,[57] IgG1 (containing 12 intra and 4 interchain disulfides),[62] anti-IgG,[107] and chimeric monoclonal antibody.[64] However, sucrose seemed to be more effective in protecting a lyophilized IgG1 against aggregation during storage at 40°C at a sugar:protein weight ratio of 1:1 (Chang et al. 2005b).[102] In another study, sucrose at 5% failed to offer any protection to a lyophilized mouse monoclonal antibody (MN12) (IgG2a (κ)) during storage at 56°C for 18 days.[59] Again, as mentioned in the liquid formulation section, even the most commonly used stabilizers may not stabilize all antibodies in the solid state and antibodies need to be treated as individually different molecules.

When sugars are used as stabilizing agents (lyoprotectants), a sufficient quantity needs to be used to achieve a significant effect depending on the protein concentration. It has been suggested that a molar lyoprotectant:protein ratio of 300:1 or greater should be used.[47] Indeed, the available literature data indicate a ratio higher than 300 is needed for better stabilization. It was shown that sugars protect the stability (aggregation and Asp isomerization) of a lyophilized, monoclonal

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antibody (type not specified) to a significant level when the sugar to protein molar ratio was increased to 500:1, which happens to the theoretical number of water binding sites in the molecule.[1] Similarly, Andya et al.[62] demonstrated that use of sucrose or trehalose inhibited the aggregation of a lyophilized antibody (IgG1—containing 12 intra and 4 interchain disulfides) during storage at 30°C to a maximum degree when the sucrose/trehalose:protein ratio was 500 or higher, which is roughly equivalent to the number of water-binding sites on the surface of the protein (550 estimated). Increasing the trehalose/anti-HER2 ratio from 60 mM (360 molar ratio) to 200 mM (1,200 molar ratio) significantly increased the stability (monomer by SEC-HPLC) of lyophilized formulation at 25 mg/mL at 40°C.[107] In a recent report, it was found that at least a weight ratio of 2:1 (molar ratio of about 800) was needed to achieve a maximum protection of a lyophilized sucrose:IgG1 formulation against both aggregation and chemical degradation during storage at 40°C (Chang et al. 2005a).[101]

Other formulation excipients were also used and some of these are effective in stabilizing certain antibodies. A recent study demonstrated that addition of sorbitol to a sucrose or trehalose formulation resulted in further enhancement of storage stability of a lyophilized IgG1 antibody (Chang et al. 2005b).[102] Either dextran or HB-β-CD (better) protected the activity of a lyophilized mouse monoclonal antibody (MN12) (IgG2a (κ)) during storage.[59] Glucose was shown to inhibit gamma irradiation-induced fragmentation of humanized monoclonal antibodies (hr3).[85]

Effect of Formulation "pH" and Buffering Agents

The pH of the liquid formulation can have a potential effect on the stability of the lyophilized formulation, even though the solid-state "pH" could not be well defined. In the evaluation of the stability of a lyophilized monoclonal antibody-vinca conjugate, aggregation seems to be lower at a neutral pH 7.1 than at either pH 8.5 or 6.1 when the lyophilized formulation was stored at 25°C.[60] In adjusting the formulation pH, use of a buffering agent may have effect on the antibody stability both during lyophilization and storage of the lyophilized products. For example, in the stability study of a fully human anti-IL8 monoclonal antibody (ABX-IL8; IgG2), Chen et al.[21] demonstrated that addition of histidine inhibited the formation of HMW species upon lyophilization and during storage at 37°C in a concentration-dependent manner. Among several excipients including His, Gly, mannitol, Glu, and polysorbate 20, His is the most critical in protecting solid-state stability of the antibody (minimizing aggregation). In a different study, the aggregation rate of a lyophilized anti-IgE at 5°C at 5 mg/mL in 10 mM buffer is affected both by pH and buffers in the following order: Na Phosphate, pH 7 > K Phosphate, pH 7 > Na Succinate, pH 6 or 5 > Histidine, pH 7.[107] It should be noted that some buffering agents may induce significant pH change due to selective crystallization during lyophilization, such as sodium phosphate. Such buffering agents should be avoided in preparing pH-sensitive antibody formulations.

Effect of Protein Concentration

In many cases, increasing the protein concentration increases the stability of the protein during lyophilization (see review).[98] However, antibodies do not seem to follow this trend and many antibodies have been shown to be less stable both during lyophilization and storage at high concentrations. For example, lyophilization of a human monoclonal antibody caused significant aggregation of MAb formulated in PBS or physiological saline as measured by OD600 and increasing the protein concentration from 1 to 2.5 or 5 mg/mL increased the OD600 from 0.194 to 0.311 or 0.427, respectively.[58] Similar trend was also observed during storage of lyophilized formulation. Increasing the concentration of a chimeric monoclonal antibody from 2.5 mg/mL to 25 mg/mL in a lyophilized formulation, containing 31 mg/mL sucrose, 15 mM NaCl, 0.02% Tween 80, 10 mM citrate, increased the aggregate level from 0.4% to 0.6% after 2 months at 5°C.[64] In the evaluation of the stability of a model recombinant humanized monoclonal antibody (rhuMAb HER2), Cleland et al.[57] demonstrated that increasing the protein concentration from 25 mg/mL to 50 mg/mL in a lyophilized formulation (containing 60 mM trehalose, 5 mM His at pH 6) increased the aggregate level from 8.5% to over 30% by SEC-after storage at 40°C for 44 months. Such behavior was also observed of anti-HER2 from 5 to 21 mg/mL containing 250 mannitol.[107]

Effect of Moisture Content

The effect of moisture on the stability of lyophilized antibodies has not been consistent and seem

to depend on the antibody and the degradation pathways being monitored.[57] Generally, increasing moisture content of a lyophilized antibody will gradually increase the rate of degradation during storage. Such negative effect of moisture was clearly demonstrated in a variety of degradation pathways, including dimerization of T2G1s Fab′ (IgG1 (κ)) in a moisture content range of 1.5–12.3%,[61] aggregation of TNF-MAb between 0.68% and 8%,[108] formation of acidic/basic species of rhuMAb HER2 between 1% and 8.4%[57] and fragmentation of a mouse monoclonal antibody (MN12) with and without secondary drying.[59]

In other cases, the effect of moisture is either complex or not significant. For example, Breen et al.[1] found that high-moisture formulations (8%) of a lyophilized monoclonal antibody (type not specified) showed a higher rate of aggregation, fragmentation, and Asp isomerization during storage than those containing 2% or 5% moisture but formulations containing intermediate moisture levels (2–3%) were less prone to aggregation than those containing a lower moisture levels (1%) as measured by SEC under accelerated storage conditions. Similarly, the aggregation of a lyophilized IgG1 was found to be minimal at an intermediate moisture level around 3% at different storage temperatures. (Chang et al. 2005b).[102] In a different study, Cleland et al.[57] found that using a model recombinant humanized monoclonal antibody (rhuMAb HER2), a residual moisture between 1 and 8.4% had no impact on the rate of aggregation during storage at 2–8 or 40°C for 12 months.

Accelerated Stability Studies

Like antibodies in a liquid state, those in a solid state may or may not undergo degradation that is characteristic of Arrhenius behavior. It is generally believed that stability studies should be conducted at least below the glass transition temperature of the formulation. Duddu et al.[64] found that accelerated stability data generated in the glassy state (40°C) seems to be a better predictor of the relative stability of formulations than the data generated at a higher temperature (60°C). In the study of the stability of a high-concentration, lyophilized, monoclonal antibody (type not specified), Breen et al.[1] demonstrated that chemical and physical degradation pathways followed Arrhenius kinetics during storage in the glassy state. Only Asp isomerization followed the Arrhenius model above the Tg value.

Advanced Formulations

In addition to the liquid and lyophilized dosage forms on the market, other dosage forms have been tried and limited success has been reported.

Spray-Dried Formulations

Spray drying is an efficient way of preparing solid dosage forms because of the rapid removal of a solvent(s) from a drug solution. It is a very attractive alternative to lyophilization in preparing solid protein formulations. So far, a number of studies have been conducted for proteins and partly because of the high-temperature exposure in the process, albeit very short, limited success has been achieved. Protein aggregation during spray drying seems to be the major challenge.[65,66,109,110]

Spray drying of antibodies has also been tried. As for other proteins, process-induced protein aggregation seems to be the major issue.[71,110] Careful selection of proper excipients could minimize this problem. In the evaluation of a spray-dried formulation for a rhuMAbE25, it was demonstrated that trehalose or lactose minimized the aggregation during spray drying (aggregate dropped from 5–6% to ≤1%). For trehalose, the molar ratio for significant inhibition of aggregation is at or above 300:1 to 500:1 (excipient: protein).[71] Such a ratio seems to be comparable with that used in the lyophilized formation. The addition of mannitol also reduced aggregation, but only up to a molar ratio of 200:1 and further increase in mannitol content resulted in crystallization, which had a detrimental effect on protein stability and aerosol performance.[71] In a different study, either trehalose or sorbitol could inhibit IgG aggregation during spray drying.[110]

Stabilized Antibodies via Mutagenesis

Antibody fragments, such as scFv, are alternative antibody therapeutics. However, since their stability is not as good as full-length antibodies, mutagenesis, or chemical modifications have been tried to improve its stability. For example, R71A mutagenesis in the heavy chain of an unstable scFv dramatically increased its stability when incubated in human serum while having only a minor effect on the binding affinity of the molecule.[111] A more stable heavy chain, made through mutation, can increase the stability of

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the light chain, and thereby confer more stability to the whole scFv.[24]

Stabilized Antibodies via Chemical Modifications

The clinical performance of an antibody can be desirably changed by conjugation of the antibody to another entity. For example, McIntosh et al.[112] demonstrated that conjugation of carboxymethyl dextran to a tumor-antigen-specific monoclonal antibody (A5B7) increased the clearance of the antibody in mice but conjugation of cisplatin (*cis*-dichlorodiammine platinum II) with the antibody complex restricted its tissue distribution and reduced its systemic clearance.

PEGylated Antibodies

A common method of increasing plasma half-life of proteins is to increase the size of the protein by attaching a PEG moiety. Currently, there are at least four PEGylated commercial protein products on the market. However, the long plasma half-lives (as high as 7–23 days) of antibodies made much less appealing the development of such an antibody product. Since the long half-life was due to the binding of Fc to the Fc-receptor (neonatal Fc receptor, FcRn) in various tissues,[33,35] antibody fragments, such as Fab′ lacking the Fc portion have a much shorter half-life and could be good candidates for PEGylation. Such a modification was made on a humanized IgG Fab′ fragment via site-specific attachment to an additionally engineered Cys residue, as modification via the lysine residues led to substantial losses in binding activity.[29]

Controlled Release Systems

Controlled release systems have been extensively investigated for proteins in the past two decades.[113,114] However, limited work has been conducted in this area for antibodies, partly due to their relatively long plasma half-lives compared with other proteins. To achieve long-term effect, a controlled released system consisting of biodegradable poly (lactic-glycolic) acid (PLGA) was prepared for an anti-cocaine catalytic monoclonal antibody and an *in vivo* release of the antibody for up to 10 days was demonstrated in mice following subcutaneous injection.[115] It is anticipated that such systems could be useful if a minimum of 1-week duration of release is achieved.

SUMMARY

Antibodies have similar tertiary structures. The presence of a significant number of disulfide bonds and intimate domain–domain interactions in antibodies make them relatively stable and more resistant to moderate thermal stress compared to other proteins. Nevertheless, antibodies do experience a variety of instabilities similar to most proteins. These physical and chemical instabilities include denaturation, aggregation, surface adsorption, deamidation, oxidation, isomerization, fragmentation, etc. Due to the significant difference in the primary sequence among different antibodies, the relative severity of these degradation pathways can be significantly different. On the average, aggregation, deamidation, and isomerization seem to be more prevalent in antibodies.

Partly due to the stability difference, some antibody products are made into a liquid form while others are lyophilized (Tab. 1). The solution pH of these commercial products is either neutral or weakly acidic, suggesting that such a pH range is probably optimal for most antibodies. Surfactants have been used in most of these antibody products, presumably to inhibit antibody aggregation, and will likely be used in other antibody drug candidates. Two most commonly used formulation excipients in these antibody formulations are sucrose and NaCl. For liquid formulations, either one can be used if they offer the same level of stability protection. For lyophilized formulations, sucrose is preferable as NaCl does not form intimate H-bonds with the antibody and also reduce the glass transition temperature of formulation, making lyophilization less efficient. All the formulation excipients and buffering agents used in commercial antibody products or discussed in the article should be evaluated individually for each antibody drug candidate through stability studies before they are chosen as part of the product, as antibodies are structurally different.

The available stability data in the literature and the information on the commercial antibody products suggest that antibody formulation seems challenging but relatively manageable, compared to other protein drugs. Three major issues in antibody formulation are apparently challenging and need significant attention in the coming years: (1) development of stable high-concentration formulations, (2) management of the high-concentration-associated viscosity of these formulations,

and (3) minimization of antibody-formulation-induced immune response.

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Formulation and delivery issues for monoclonal antibody therapeutics☆

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Abstract

Antibodies can have exquisite specificity of target recognition and thus generate highly selective outcomes following their systemic administration. While antibodies can have high specificity, the doses required to treat patients, particularly for a chronic condition, are typically large. Fortunately, advances in production and purification capacities have allowed for the exceptionally large amounts of highly purified monoclonal antibodies to be produced. Additionally, genetic engineering of antibodies has provided a stable of antibody-like proteins that can be easier to prepare. Together, these advances have made antibody-based therapies one of the most commonly pursued pharmaceuticals in biotechnology pipelines. With this success, however, has come a series of technical challenges in the formulation of antibody-based materials to maintain sufficient stability in a variety of configurations and sometimes at particularly high concentrations. This review focuses on issues related to identifying and verifying stable antibody-based formulations.
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Keywords: Antibody; Stability; Delivery; Formulation

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1. Introduction

Therapeutic and diagnostic antibodies have become the fastest growing area of biopharmaceutical applications. There are now 18 monoclonal antibodies on the market and over 100 in clinical trials, which highlights the significance of these therapeutics and the advances made in antibody engineering. Further, by 2008, engineered antibodies are projected to be the source of over a third of the revenues from biotechnology [1,2].

A few seminal events have led to the current and projected prominence of antibodies as biopharmaceuticals include the identification of methods to generate murine monoclonal versions of antibodies [3], the cloning of human antibody sequences to allow for humanization of murine monoclonal antibodies through complementary-determining region (CDR) grafting [4] and even the establishment of fully humanized systems to generate monoclonal antibodies [5]. With the sequential identification of these technological advances, antibodies for therapeutic and prophylactic indications have moved from fully murine, to partially murine (mostly human) to completely human constructions. Added to these events, dramatic advances in production have led to the ability to prepare monoclonal antibodies at scales that can provide sufficient material at costs that make this area appealing to pharmaceutical companies. One important outcome of these various advances is a greater potential to use antibody drugs in chronic settings, tremendously expanding their biopharmaceutical applications.

The majority of currently approved antibody drugs address previously unmet medical needs in the areas of cancer and inflammation. These indications came from practical considerations related to earlier forms of murine and partially murine antibody therapeutics; the first monoclonal to be approved (OKT-3®, muromonab-CD3) was approved in 1986 for a single treatment regimen to protect from life-threatening tissue rejection following kidney transplant [6]. Adverse reactions due to acute immunogenicity problems due to delivery of a non-self protein limited the use of OKT-3® to only acute application [7,8]. Subsequent partially murine antibodies such as Panorex® (17-1A), Zevalin® (ibritumomab tiuxetan) and Bexxar® (tositumomab) were approved for a limited number of administrations to cancer patients who had failed frontline therapies [2]. With the advent of fully humanized antibodies, chronic therapeutic or prophylactic applications for infectious agents have become a realistic clinical option [2]. Another critical aspect to the success of antibodies as therapeutic agents involves improved methods to express, purify and characterize these proteins [9]. In general, antibody therapeutics are

large (typically >150 kDa), complex in nature (most are glycosylated) and must be administered in stoichiometric rather than catalytic quantities (nearly a gram per dose is required for many antibodies to be effective). Production and purification scales have thus reached levels of production that were previously assumed impossible in biotech companies [10]. Novel concerns then related to the development of stable formulations and delivery strategies for such large amounts of a complex molecule. Understanding product instability with regard to physicochemical and thermodynamic events becomes more and more critical to ensure market success [11]. The required high concentrations of antibodies in these formulations reduced the need to add a carrier protein, as is the case where serum albumin is commonly added in formulations of highly potent proteins such as erythropoietin alpha (Epogen®) and Factor VIII (Kogenate®) to enhance stability and reduce loss to vial surfaces. Simultaneously, the large amounts of an antibody protein used in these formulations allowed degradation products to be more readily detectable particularly when using the highly sensitive methods designed for evaluating hormone biotherapeutics.

Finally, the ability to prepare large quantities of an antibody that could be administered for chronic therapy resulted in a plethora of new and novel applications [9,10] involving the delivery of chemotherapeutics, radionuclides and imaging agents — to name a few. New issues, related to identifying stable formulations such as high concentration formulations that might reduce the volume of antibody administrations, arose in the move from intravenous infusions to the realm of subcutaneous injection strategies. In some cases the combination of agents with antibodies produced conflicting stability profiles. Additionally, potential new indications for antibody drugs increased the desire to find novel methods for sustained release, intracellular targeting [12] or delivery via methods not involving an injection. Thus, as is the case in most scientific advances, technological advances opened up possibilities for therapeutic opportunities that were not possible previously as well as a totally new set of problems that required solving. In this review we have attempted to identify the major issues associated with formulating and delivering antibody drugs, to recognize challenges already identified accompanied when possible by solutions, as well as to discuss concerns that will need to be addressed in the future as the use of antibody drugs continues to evolve.

2. Antibody characteristics relevant for formulation and delivery

The human body produces several classes of antibodies that appear to perform specific functions under different conditions and at discrete anatomic sites. Most of the currently approved antibody-based drugs are of the IgG1 class [13] and are glycosylated on the Fc (fragment crystallizable) domain. Glycosylation has been shown to participate in specific cell response events mediated by antibodies [14–16]: antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cell-mediated cytotoxicity (CDCC) through receptor recognition events. Individual glycoforms of antibodies can provide improved efficacies for certain clinical outcomes [17]. In general, IgG1 and IgG3 antibodies are effective at initiating ADCC while IgG2 readily fixes complement but is poor at inducing ADCC. IgG4 appears to be inefficient at both ADCC and CDCC [13]. Interestingly, antibodies can be re-engineered to increase their capacity to recruit complement [18] or to adjust their affinities to the various classes of Fc receptors and thus ADCC function through mutation of specific amino acids within the Fc domain [19]. Molecular engineering has also provided a spectrum of new antibodies (Fig. 1). Other chapters in this issue delve deeply into the many characteristics of antibodies that make them remarkable and diversely applicable treatment agents: ligand binding properties, effector functions and potential for modification in their design. Other chapters will also address applications of newly engineered antibody-like molecules that move beyond the realm of intact, full-length IgG antibodies. In the current review, attention will be focused primarily on IgG1 since this is where the majority of published information exists for antibody drug formulation and delivery.

Antibodies, due to their large size (being greater than the filtration cut-off size of the kidney) and their ability to be sequestered for periods of time from the blood, via interactions with FcRn receptors present on

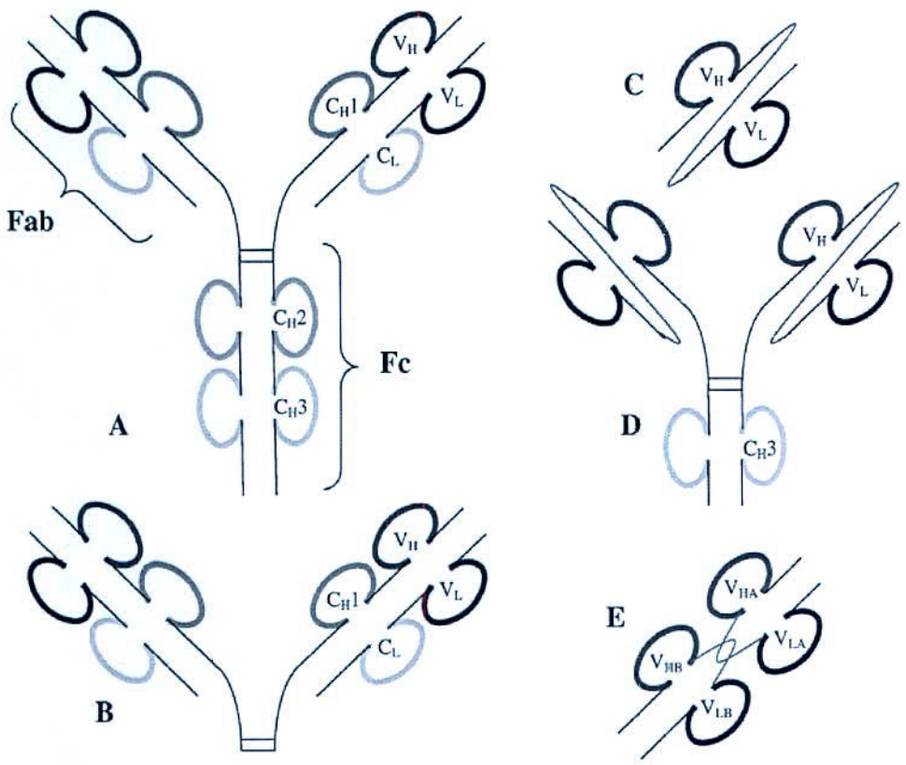


Fig. 1. General antibody structure and some antibody-based protein constructions. A) Human antibodies have a common framework that can be described as Fc (fragment crystallizable) and Fab (fragment antigen binding) domains that are derived from the folded structure of heavy (H) and light (L) chain proteins. Multiple constant (C) regions on both the H and L chain proteins are donated as are the variable (V) segments involved in antigen-specific recognition. B) Removal of the Fc domain produces a F(ab)2 structure. C) Single chain structures of the antigen binding region (scFv) can be prepared by themselves or D) in a construct that includes Fc domain elements. E) Bi-specific antibody elements (diabodies) can be prepared to bring two different antigens into close proximity. C_H, heavy chain constant regions; C_L, light chain constant region; V_H, heavy chain variable region; V_L, light chain variable region.

endothelial cells, can have extended serum profiles in the range of weeks. A number of engineered antibody-like molecules are now being examined for therapeutic applications [20]. Some engineered fragments, such as diabodies and minibodies, are much smaller than intact, full-length IgG1 and have very different properties of systemic distribution and clearance. Additionally, methods to prepare other antibody types such as dimeric secretory IgA [21] and methods to prepare antibodies using non-mammalian cell systems, such as plants [22], from the eggs of chimeric chickens [23] and from transgenic animals [24], are now being explored by academic and industrial groups. As these new approaches gain prominence, more information will be acquired about their unique requirements for successful formulation and delivery. At present, however, there is little information on formulation approaches for antibodies produced using these production processes.

3. General concerns for antibody formulations

Due to their proteinacous nature, antibodies present generic formulation issues that are similar for most protein therapeutics [11]. Proteins, in general, can be degraded under conditions when they are exposed to heat, freezing, light, pH extremes, agitation, sheer-stress, some metals and organic solvents. With ever advancing analytical technologies, the ability to assess the physicochemical and thermodynamic instability of antibody drugs has also led to the identification of several events that are more specific to the unique nature of this particular class of proteins: variations in

Fc glycosylation, partial heavy chain C-terminal Lys processing, Fc methionine oxidation, hinge-region cleavage and glycation of Lys residues [25]. In addition to these issues is the difficulty of preparing dosing materials that might have protein concentrations in the range of 100 mg/ml or greater. Since early preclinical assessment is so critical to successful identification of a new biotherapeutic product, general pre-formulation strategies are usually employed for initial studies with new antibody drugs. An initial formulation effort will likely employ an isotonic, non-hemolytic, slightly acidic solution that can be administered by intravenous infusion or subcutaneous injection in studies employing a relevant animal model. In some cases, some stability information for the antibody drug can be obtained from initial in silico and in vivo testing during this pre-clinical phase that aids in the selection of a formulation that is used in clinical trials [26].

Since most early pre-clinical studies attempt to emulate the delivery strategy that will be used in the clinic, initial studies frequently identify problems with either the initial pre-formulation approach and/or stability issues with the antibody drug itself. In the case of the formulation, protein precipitation can occur with refrigerated storage or with repeated freeze–thaw cycles; events that may be facilitated by an inappropriate pH or buffer selection. Analysis of antibody drugs stored in this initial pre-formulation system may also identify unstable sites within the protein or its linkage to attached molecules (chemotherapeutics, imagining agents, etc.). In the case of the antibody, this information is frequently evaluated to determine if re-engineering of the protein could be performed, for example, to provide greater thermodynamic stability or folding efficiency [27]. Such changes in the Fc region are typically conservative, but might act to modify Fc receptor binding properties to provide a different biological outcome [28,29]. Amino acid substitutions can also be made within the Fv region to improve thermal stability [30], but such changes might also alter antigen binding specificity [31].

4. Stability issues for antibody formulations

If one examines the amino acid sequences for currently approved IgG1-based antibody drugs, it becomes quite clear that only a small segment of these proteins are dramatically different from one another, being the Fv segments that are involved in antigen binding. It should be pointed out, however, that there are some minor differences in conserved regions as well. A priori, one might assume that by finding a stable formulation for one of these antibody drugs, that such a formulation would be good for most if not all, similar antibodies. If this were borne out by experience, there would be no need for a review such as this. Instead, each antibody seems to have a unique personality related to its requirements for stability; a phenomenon derived from the fact that the small differences between these antibodies are focused on surface-exposed amino acid differences that stipulate antigen specificity. Thus, the interfacial surface of each antibody drug is unique and thus requires specific formulation components to provide maximal stability and retention of activity.

Probably the most important aspect of identifying a successful antibody drug formulation is in the identification of truly detrimental alterations rather than all of the modifications that might occur. Although, from a regulatory standpoint, it may be beneficial to understand all of the potential degradation events associated with an antibody formulation, it is the recognition of a stability-indicating parameter (or several parameters) that allows the formulation scientist to focus in on what is critical to development of a successful product. Here, a series of physicochemical and bioanalytical assays can be used to identify these parameters [32]. Although in vivo bioassays may ultimately be required to validate a stability-indicating formulation parameter, the most critical tools at the disposal of the formulation scientist are rapid and reproducible assays to measure protein changes due to oxidation, deamidation, aggregation, fragmentation and other forms of chemical modification that could lead to a loss of function of the antibody drug.

4.1. Oxidation

Methionine and cysteine residues are frequently a site of oxidation in protein drugs and this is also the case for antibody drugs [33]. In the case of antibody-based drugs, specific methionine residues within the Fc domain may be prone to oxidation, resulting in the

production of methionine sulfoxide [25]. While cysteines are present in the Fc framework as disulfide pairs, unpaired cysteines in the variable region may also be sites of oxidation. Besides methionine and cysteine residues, oxidation of histidine, tyrosine, tryptophan and phenylalanine residues can also occur [34]. A number of methods can be used to monitor oxidation of amino acid residues. As a starting point, the number of reactive protein thiols can be monitored using reagents such as 5,5′-dithionitrobenzoic acid or iodoacethyl dansylcadaverine [35], providing a simple readout on the number of protein thiols per protein. Total amino acid analysis can also be used to monitor reductions in methionine, tyrosine and phenylalanine residues. Tryptic digestion and peptide separation by liquid chromatography followed by mass spectroscopy analysis (LC-MS) can be used to identify specific sites of amino acid oxidation.

A careful study of methionine oxidation has been performed for the humanized monoclonal antibody that recognizes the her2/neu gene product. For this protein it was determined that a methionine at position 255 in at heavy chain of the Fc region was the primary site of oxidation and that a methionine at position 431 could also oxidize under more aggressive conditions. In this study, a chromatography assay was used to detect oxidation events following papain digestion to separate Fab and Fc segments of the antibody [36]. Another study on the stability of this same protein provides a valuable template for dealing with antibody oxidation: identification of a critical oxidation event, validation of a robust assay for monitoring that event and comparison of formulation parameters to determine conditions that facilitate oxidation. Addition of antioxidants or stoichiometric levels of free methionine or thiosulfate were found to reduce the extent of oxidation in formulations of this antibody [37].

Issues associated with administration of oxidized forms of an antibody may extend beyond concerns of reduced efficacy. Systemic lupus erythematosus (SLE) is a disease characterized by the induction of anti-double-stranded DNA antibodies. Studies have shown that increased levels of markers of protein oxidation, including those of methionine sulfoxide and 3-nitrotyrosine, correlated with SLE disease activity [38]. Additionally, oxidative modifications of antibodies isolated from synovial fluid samples collected from patients with rheumatoid arthritis suggested alteration of histidine, methionine, tyrosine and cysteine residues [39]. While no direct causal association is shown or implied, these observations bring attention to the issue that we do not fully understand the clinical impact of antibody oxidation events.

4.2. Deamidation

Glutamine and asparagine residues show a propensity for deamidation [40]. Both light and heavy chains of antibodies can undergo deamidation of these amino acids [41] and deamidation events are considered to be one of the major sources of charge heterogeneity of monoclonal antibodies [42]. Deamidation events appear to be highly selective events for individual antibodies. For example, analysis of several monoclonal antibodies that bind the her2/neu gene product demonstrated asparagine 30 deamidation on one or both light chains and asparagine 55 on the heavy chain [43]. Deamidation events lead to more acidic forms of the antibody through the acquisition of additional carboxylic acid groups. Conversely, aspartic acid residues can also undergo modification to a succinimide moiety that results in a basic antibody isoform of the antibody by removal of a carboxylic acid group [43]. In general, deamidation events appear to be relatively slow but are accelerated by unfolding of the antibody [44]. Initial detection of deamidation in antibody preparations is typically identified by differences in charge distribution or content using methods such as isoelectric focusing (IEF) or high-performance cation-exchange chromatography [43].

High performance chromatography can then be used to separate succinimide, isoaspartic and aspartic acid isoforms of tryptic digest fragments of the antibody which can be unambiguously characterized by tandem mass spectroscopy (MS). Correlation between tryptic digest tandem MS results and some other more readily accessible method, such as IEF, can provide a rapid format to follow antibody deamidation events. Using these techniques, asparagine deamidation events residues appears to occur more frequently than glutamine deamidation and specific asparagine residues appear to be selectively targeted for this event [33]. Deamidation of asparagine is frequently associated with rearrangement

events that lead to the formation of isoaspartic acid [42] and this outcome is correlated to amino acids adjacent to the asparagine prone for deamidation [44] that provide a method for prediction of potential deamidation sites (BOX). In particular, asparagine–glycine sequences can demonstrate accelerated deamidation rates since glycine residues lack a side-chain structure that would otherwise act to decreased conformation interference for backbone-associated isoaspartyl formation [45].

Box 1

Amino acid* sequences predicted to deamidate
SNG, LNG, LNN and ENN
(Note these have a following amino acid of G or N)
and those that are less prone to deamidation
GNT, GNS, GNV, TNY, SNY, SNF, SNT, SNK, SNL,
WNS, WNS, FNW, FNS, FNR HNA, HNH, LNW,
CNV, NNF, DNA, and YNP
*standard single amino acid letter nomenclature.

Non-enzymatic parameters of asparagine and glutamine deamidation in proteins have been defined in detail for factors such as pH, temperature and ionic strength [46,47]. The primary sequence dependence of these deamidation events can be explained by a simple steric and catalytic model [46,47]. Prediction of deamidation events based upon three-dimensional structures have also been described but the data set for these studies is not yet sufficient to produce a general model [48]. That asparagine and glutamine deamidation appear to be a constant phenomenon of proteins and correlate with their turnover provides the basis for the hypothesis that the presence and distribution of these amino acids within a protein can act as a molecular clock for its biological turnover [49,50]. For the formulation scientist, empiric assessment of factors such as pH and ionic strength provide the best strategy to identify a formulation that minimizes deamidation events. In general, deamidation does not result in a decrease in potency and deamidation may be hard to detect as the charge difference is small. At times, however, there can be a detrimental effect on potency through the introduction of an unfavorable negative charge that can have further ramification on biological function, particularly when deamidation occurs in the binding regions [51]. As particularly reactive residues are identified, it may be possible to have the antibody engineered to remove the problematic amino acid or to change adjacent residues to reduce its reactivity.

4.3. Aggregation

Liquid formulations of protein therapeutics frequently exhibit a decrease in concentration due to adsorptivity to container walls at low concentration and can show aggregation events at high concentrations. Initial preparations of antibody drugs were administered in acute situations by IV infusion that could allow for medium protein concentration (1–10 mg/ml) formulations not dramatically affected by adherence or aggregation losses. As antibody-based drugs became more widely used in chronic care, high concentration formulations that would allow for clinically acceptable SC injection volumes became desirable and aggregation became an important concern for designing formulations. Along with aggregation events, protein solutions at concentrations > 50 mg/ml may become sufficiently viscous as to no longer readily pass through the gauge of a needle that would be comfortable for patients' use. Some antibodies demonstrate high concentration viscosity issues while others do not. For example, the anti-respiratory syncytial viral antibody Synagis® is formulated at 100 mg/ml protein and is administered successfully by IM injection. Thus, addressing viscosity, adsorptive loss and aggregation in any specific antibody formulation will likely be performed on a case-by-case basis.

As protein–protein contact frequency increases at high concentrations, the opportunity for stable aggregate formation increases proportionally. Proteins are folded in such a way as to internalize hydrophobic domains and surface-expose more hydrophilic domains in a thermodynamically stable situation. As folded proteins undergo normal molecular motion transient surface exposure of hydrophobic domains can lead to protein–protein contacts that hide these

surfaces in a more energetically favorable manner. The outcome of these events is the stabilization of these protein–protein contacts with the ultimate outcome of dimerization, trimerization and so on — leading to extended aggregation events [52]. As protein concentration and temperature goes up, so does the probability of energetically favorable contacts leading to aggregation with increased frequency and duration of reversible protein–protein contacts leading to a greater extent of stable (even irreversible) aggregation events. Aggregation (covalent, non-covalent, dissociable, non-dissociable) must be considered a likely degradation event for high concentration antibody formulations.

For some antibodies reduced temperatures can incite reversible self-associations; these molecules are known as cryoimmunoglobulins [53]. IgG3 cryoimmunoglobulins have been shown to self-associate through sugar structures of the Fc domain [54]. Self-association events involving IgG1 antibodies seem to occur primarily through weak ionic and hydrophobic interactions [55]. More recent studies have examined kinetic and thermodynamic aspects of antibody solution dimerization using a recombinant human monoclonal antibody that recognizes vascular endothelial growth factor (rhMab-VEGF) as a model [56]. It was found that for this IgG1 class antibody, aggregation rates were greater in slightly alkaline (pH 7.5–8.5) compared to slightly acidic (pH 6.5–7.5) conditions. A high salt environment (1 M NaCl) also enhanced dimerization. Not surprisingly, self-association events were accelerated as the storage temperature was increased. One of the most striking aspects of these dimerization studies on rhMab-VEGF is that trastuzumab, another genetically engineered antibody with the same IgG1 framework and a 92% sequence homology with rhMab-VEGF, fails to demonstrate aggregation events under the same conditions.

A number of additives have been identified that can reduce the rate of protein aggregation [52,57]. A rather wide spectrum of agents can reduce protein aggregation rates: urea, guanidinium chloride, amino acids (in particular glycine and arginine), various sugars, polyalcohols, polymers (including polyethylene glycol and dextrans), surfactants and even antibodies themselves. Studies have shown that different proteins benefit from different types of anti-aggregating factors, consistent with the notion that the interfacial surfaces that drive interactions leading to aggregation are protein-specific. Conceptually, anti-aggregating agents might fall into one of two categories: small agents that fit into channels or grooves and large agents that might interact with lower-curvature surfaces of the protein. In essence, the small agents would act to stabilize a protein from acquiring a conformation that might be more reactive toward aggregation while a large agent might act to reduce the frequency of surface contacts that lead to an aggregation event. In the case of antibody formulation, either or both may be important. As we will discuss, agents found to effectively protect an antibody from aggregation may differ for various stress conditions; antibodies in solution that are undergoing agitation and thermal stress may differ from those of the same antibody as it undergoes lyophilization and reconstitution.

Protection from aggregation can be limited in antibody formulations by the addition of surface-active agents such as detergents, both charged and uncharged. As an interesting aside, such surface-active agents can help reduce protein loss due to adherence to surfaces that can occur with low concentration protein formulations. Polysorbate 20 and 80 (now available from a vegetal source) are examples of detergents that are widely used to reduce the rate of protein aggregation in high concentration antibody drug formulations. A precautionary note must be sounded, however, as detergents perform this function optimally at low concentrations (0.01% to 0.05%) and can act detrimentally to participate in protein unfolding at higher concentrations, e.g., 1%.

That antibody aggregation can be accelerated by several physical conditions provides the potential for multiple methods to determine if a particular antibody drug is prone to aggregation and to rapidly test formulations for their ability to impede aggregation. Agitation studies are done, in addition to thermal stability studies, to determine if aggregation can occur in a given formulation. Different test methods are used, employing a shaker or a stirring apparatus. In one study done on an IgG1 antibody found sizes of aggregates produced by the two methods were comparable [58]. They concluded that a higher throughput test could be constructed using stirring analyzed by absorbance measurement. In their work,

the stirred samples exhibited much higher absorbance and second particle species in DLS, indicating that stirring stress produces a higher amount of smaller protein aggregates. While Mahler found polysorbate 80 protects against aggregation, the stirred samples had an increase in turbidity and more aggregate products were detected by DLS as compared to a surfactant-free formulation. Polysorbate 80 stabilizes small aggregates and prevents further progress in the aggregation process.

4.4. Fragmentation

Intact, full-length antibodies are composed of several segments that have natural flexure sites. One site, in particular, exists at the flexure point between the Fc and Fv domain. Cleavage at this hinge region is a common concern when assessing antibody drug stability [25]. Molecular sizing methods such as sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and size-exclusion high performance liquid chromatography (SE-HPLC) can be used to rapidly identify fragmentation events in antibody drugs [59]. Fragmentation of full-length antibody drugs is a common occurrence and invariably affects their function. In general, cleaved forms are present in such low amounts that it is likely that little change in efficacy would be seen [60]. At the very least, the antibody drug may have some small reduction in efficacy following fragmentation or may be more prone to proteases, degradation and clearance events that would limit the duration of its efficacy. At the worst, a fragmented antibody preparation may result in a different biodistribution and thus a different safety profile. This can be of particular concern if the antibody is being used to delivery a cytotoxic chemotherapeutic agent. Fortunately, the extent of fragmentation in an antibody can be easily limited in the formulated product.

5. Strategies to stabilize antibody formulations

While liquid antibody formulations are less expensive, faster to develop and generally easier to prepare for administration than alternative formulation approaches, liquid antibody formulations are prone to oxidation, deamidation, aggregation and fragmentation as discussed above. In each of these events, water is the common culprit: water mediates electron transfer during oxidation and deamidation events and its required addition across a peptide bond can be a critical step in fragmentation. The thermodynamic stresses that lead to protein aggregation are caused by hydrophobic protein surfaces being exposed to water and trying to find a lower energy state by non-native protein–protein interactions. Thus, water is a big problem for stabilizing antibody-based drugs. Lyophilization or introduction into hydrophobic polymer systems can reduce the impact of water on antibody drug formulations.

5.1. Lyophilization

Although some antibodies have demonstrated a propensity to undergo precipitation as they are cooled from 37 °C [53], antibodies are typically robust to freeze/thaw cycles [61]. Early studies reported that antibodies were stable to the freeze-drying (lyophilization) process in the absence of excipients that function as cryoprotectants [62], however, with the technical advancement of certain analytical methodologies, became evident that antibodies are generally damaged in some way as a result of lyophilization as demonstrated by extensive formation of insoluble aggregates [61]. More recently, studies have shown that freeze-drying antibodies to a specific percent of residual water (usually between 1 and 8%) allows for optimal stabilization in the dry state and stability during reconstitution [63]. Higher moisture content in lyophilized antibody preparations correlates with increased aggregation (as seen by SEC) and asparagine deamidation and isomerization (as measured by HIC). Studies have also been performed to identify optimized lyophilization schedules where the secondary drying phase is performed at an elevated temperature [64]. This technique allows the lyophylized cake to achieve optimum moisture content, allowing the final product to be conformationally stable (as measured by DSC) during extended storage. Solid-state structural studies can be used to monitor conformational changes and aggregation events during the storage of a lyophilized monoclonal antibody preparation [65]. Improved native-like structure and a reduction in aggregation can be obtained by incorporation of a carbohydrate excipient in sufficient

quantities to fulfill the hydrogen bonding requirements on the protein surface, suggesting a critical role for non-water molecules to act as placeholders for water molecules during drying and in the dry state. That the addition of excess carbohydrate failed to improve the stabilizing function of this excipient, reaffirms the conclusion that antibodies have a limited number of sites that must be protected in this fashion [65]. A variety of carbohydrates or polyol compounds such as sucrose, trehalose and mannitol have been shown to provide this stabilizing function [66,67].

The rate of rehydration when water is reintroduced during reconstitution of lyophilized formulations is a critical parameter. As water is removed during the different drying stages of the lyophilization cycle and replaced by a shell of non-water excipients, the antibody drug can undergo structural modifications that result in a non-native conformation as may be seen by differential scanning calorimetry [63]. If the rate of rehydration is sufficiently slow to allow recovery of native conformation as water replaces non-water excipient molecules, reconstitution will typically provide a satisfactory outcome for the antibody. If, however, the antibody does not have sufficient time or capacity to recover its native state during reconstitution, extensive aggregation can result. Efforts to protect monoclonal antibodies from the rigors of freeze-drying and reconstitution have been aided by a number of analytical methods that can examine protein modifications. In the solid state, circular dichroism (CD) and Fourier transform infrared (FTIR) spectroscopy may be used to evaluate the protein; after reconstitution in the liquid phase, SEC or SDS-PAGE have been utilized to gather information from lyophilization studies [68,69]. A microscopic apparatus to visually monitor freeze-drying events has even been described [70]. Excipients that have been reported to slow the reconstitution rate and thereby preventing such changes in the rehydrated antibody are glycerol [71,72] and polymeric materials such as polyethylene glycol [73].

Pharmaceutical scientists frequently use increased temperatures for accelerated formulation stability studies. Although such studies can be used to assess formulation parameters such as pH and ionic strength, antibodies lyophilized in the presence of a cryoprotective sugar excipient provide a more complicated picture. Lyophilized amorphous solids composed of a monoclonal antibody and a sugar cryoprotectant material such as sucrose or trehalose form glassy structures, liquids that are too viscous to flow. That antibody-excipient complexes exhibit a glass transition temperature (Tg) provides the basis for observations showing that these materials have unique properties above and below this value. Thus, in predicting shelf life in accelerated degradation studies, it is important to use testing temperatures that do not exceed the Tg threshold of the complex [67]. In general, lyophilized formulations containing trehalose may likely show less tendency toward antibody aggregation than those with sucrose, since trehalose preparations have a higher Tg [67]. With this in mind, a promising approach to assess secondary structural parameters in evaluating antibody formulation stability is the use of amide I band Raman spectroscopy. This method demonstrates a good correlation between structural protein perturbations immediately after lyophilization and the rate of aggregation during long-term storage under accelerated conditions [74].

5.2. Polymer delivery systems

Removal of water from antibody preparations, either by lyophilization or spray freeze-drying, provides a provides a starting point for incorporation of this dried material into a hydrophobic polymer matrix such as polylactide-co-glycolide (PLGA) that can be made into microspheres generated through a solid-in-oil-in-water (S/O/W) encapsulation process [75]. Although both mannitol and trehalose can stabilize human IgG during spray freeze-drying, the double-emulsion solvent evaporation method used to load spray freeze-dried human IgG into PLGA microspheres has been shown to produce extensive antibody aggregation [75]. Such an outcome is likely the result of thermodynamic stress experienced at the water/organic solvent interface and a variety of process methods and excipients can be use to minimize this concern [76].

One feature of PLGA microparticle preparations is their recognition by immune elements and their capacity to stimulate immunization against incorporated proteins [77–79]. While this has clear advantages for vaccines, it poses a serious concern for therapeutic protein delivery. Hydrophobic microparticles have been reported to incite local inflammation

at subcutaneous injection sites [80], events that may be partially mediated through macrophage recruitment [81]. Not only is the potential for antibody formation increased with local inflammation, the resulting injection site reaction may be clinically unacceptable.

In spite of some serious caveats, polymeric microspheres and delivery systems can be successful antibody formulations if administered to the appropriate sites, using certain types of polymers or preparing microspheres or polymeric formulations by methods that are more protective of the antibody. An example of a certain delivery site that may have less proclivity towards an inflammatory response is reported, where PLGA microspheres loaded with an anti-VEGF antibody were injected into the intravitreal humor of the eye, an immune-privileged site, for the treatment of age related macular degeneration (AMD) [82]. Resorbable polymers that show less potential to incite inflammation, e.g., have smoother surface characteristics, might be used to deliver antibodies in a sustained manner. Direct, local delivery of antibodies using carboxymethylcellulose aqueous gels can be an effective post-surgical anti-infective strategy [83]. Antibodies covalently coupled to a biodegradable hyaluronic acid hydrogel through a labile hydrazone linkage have been used for the sustained delivery of antibodies to sites within the central nervous system [84]. Introduction of dry powder IgG into ethylene–vinyl acetate copolymer (EVAc) has also been examined as a potential polymer-based antibody delivery system [85]. Similar to PLGA systems, EVAc microspheres can extend the time course of IgG release. This property makes polymers loaded with antibodies interesting opportunities for injectable sustained release delivery formats but also for topical application at mucosal surfaces. For example, long-term vaginal delivery of an antibody can be achieved in a mouse model using polymer vaginal rings [86,87]. Formulations of a biomedical grade polyurethane hydrogel with an antibody coating have also been prepared and shown to release bioactive protein [88]. It is important to note that one complicating aspect of many of these approaches is the requirement of a polymer solubilization step using a solvent, such as methylene chloride or isopropanol, which can compromise protein drug stability. Sustained release formulation approaches such as microspheres prepared using water, such as the PROMAXX microspheres (Epic Therapeutics) or crystallization of proteins [89] can bypass the use of solvents that are incompatible with proteins and antibodies.

6. Formulation issues for alternative forms of antibody delivery

To date, antibody development has focused primarily on injectable routes of administration: intravenous (IV), intramuscular (IM) and subcutaneous (SC). A long history of IV antibody infusions exists [90] and the advantages are clear for antibody-based drugs that are used in cancer treatments in order to reach tumor sites that may be diffuse or not otherwise accessible. In fact, the majority of the approved antibody-based drugs are administered by IV infusion (Table 1) with the total dose administered approaching one gram of protein per treatment in some cases. For many antibody-based therapies a critical safety concern involves the onset of acute or delayed systemic reactions following infusion [91,92]. Parameters and characteristics of the "infusion reaction" as well as treatment protocols have been well described with acute events commonly occurring within 10 min to 4 h and delayed events occurring 24 h to 4 days post dosing [93]. Although formation of antibodies to the administered antibody drug appear to be a risk factor for infusion reaction events [94], infusion reactions can occur following the initial exposure [93] and these reactions do not appear to be due to IgE responses or mast cell activation [95]. Most importantly for the patient, there appear to be treatment protocols to provide re-treatment of patients even after an infusion reaction event [93] with concomitant administration of immunomodulation molecules [94]. There are many unanswered questions about the mechanism and prevention of adverse infusion reaction events associated with IV infusion of antibody drugs. Certainly, one method is to explore IM and SC delivery when possible, although these delivery approaches may also have confounding aspects of tissue response and volume limitations.

Other routes are also amenable to antibody delivery. The increased number of antibody therapeutics on the market and the greater exploration of non-life threatening indications being treated by antibodies, drive efforts to deliver these agents by routes other than IV

Table 1
Formulations of approved products

Antibody product; company	Generic name; description	Delivery route	Formulation concentration	Buffer components; pH[a]	*Excipients*
Othroclone OKT3®; J & J-Ortho Biotech	Muromonab-CD3; Murine IgG2a, anti-CD3	IV	1 mg/ml	Na,K phosphate; pH 7	NaCl, polysorbate 80
Reopro®; Centocor-Lilly	Abciximab; Chimeric IgG1, anti-GPIIb/IIIa, Fab	IV	2 mg/ml	Na phosphate; pH 7.2	NaCl, polysorbate 80
Rituxan®; Idec-Genentech	Rituximab; Chimeric IgG1, anti-CD20	IV	10 mg/ml	Na citrate; pH 6.5	NaCl, polysorbate 80
Zenapax®; Roche	Daclizimab; Humanized IgG1, anti-CD25	IV	5 mg/ml	Na phosphate; pH 6.9	NaCl, polysorbate 80
Simulect; Novartis[b]	Basilixamab; Chimeric IgG, anti-CD25	IV	4 mg/ml	Na,K phosphate, glycine	NaCl, sucrose, mannitol, polysorbate 80
Synagis®; MedImmune[b]	Palivizumab; Humanized IgG1, anti-RSV	IM	100 mg/ml	Histidine, glycine	Mannitol
Remicade®; Centocor[b]	Infliximab; Chimeric IgG1, anti-TNFα	IV	10 mg/ml	Na phosphate; pH 7.2	Sucrose, polysorbate 80
Herceptin®; Genectech[b]	Trastuzumab; Humanized IgG1, anti-HER2	IV	21 mg/ml	Histidine; pH 6	Trehalose, polysorbate 20, benzyl alcohol[c]
Mylotarg®; Wyeth[b]	Gemtuzumab ozogamicin; Humanized IgG4, anti-CD33, immunotoxin	IV	4 mg/ml	Na phosphate	NaCl, sucrose, dextran 40
Campath®-1H; Millennium-ILEX	Alemtuzumab; Humanized IgG1, anti-CD52	IV	Unclear — 30 mg /vial	Na,K-phosphate pH 7	NaCl, KCl, NaEDTA, polysorbate 80
Zevalin®; Biogen Idec	Ibritumomab tiuxetan; Murine IgG1, anti-CD20; radiolabeled (^{90}Y or ^{111}In)	IV[d]	1.6 mg/ml	Na Acetate, Na, K-phosphate pH 7	NaCl, human albumin
Humira®; Abbott	Adalimumab; Human IgG1, anti-TNFα	IV	50 mg/ml	Na phosphate, Na Citrate; pH 5.2	Mannitol, polysorbate 80
Xolair®; Genentech[b]	Omalizumab; Humanized IgG1, anti-IgE	SC	125 mg/ml	Histidine	Sucrose, polysorbate 20
Bexxar®; Corixam-GSK	Tositumomab-I131; Murine IgG2a, anti-CD20, radiolabeled (^{131}I)	IV	14 mg/ml	Na phosphate; pH 7.2	NaCl, maltose
Raptiva®; Genentech	Efalizumab; Humanized IgG1, anti-CD11a	SC	100 mg/ml	Histidine; pH 6.2	Sucrose, polysorbate 20
Erbitux®; Imclone Systems	Cetuximab; Chimeric IgG1, anti-EGFR	IV	2 mg/ml	Na phosphate; pH 7.2	NaCl
Avasatin®; Genentech	Bevacizumab; Humanized IgG1, anti-VEGF	IV	25 mg/ml	Na phosphate; pH 6.4	Trehalose, polysorbate 20
Tysabri; Biogen Idec	Natalizumab; Humanized IgG4, anti-4α-integrin	IV	20 g/ml	Na phosphate; pH 6.1	NaCl; polysorbate 80

Ordered by approval dates in the US.
Abbreviations: RSV=respiratory syncytial virus; TNFα=tumor necrosis factor alpha; EGFR=epidermal growth factor receptor; VEGF=vascular endothelial growth factor; NaEDTA=sodium edentate.
[a] Some lyophilized formulations do not stipulate a pH (or range) following reconstitution.
[b] Lyophlized product, concentration, pH, etc. values are obtained after reconstitution.
[c] Added as a preservative to generate a multi-dose vial configuration.
[d] Kit where components are mixed just prior to administation.

infusion. These routes may be more effective, less invasive, provide easier delivery, permit self-administration and thereby be more convenient and possibly have lower costs if less active agent can be used, as in the case where the alternate route of administration delivers the therapeutic agent to the intended site directly. Interestingly, some studies have suggested that antibodies administered by IV infusion are more

susceptible to proteolysis than via IM injection [59] and sucrose-stabilized immunoglobulins have been associated with acute renal failure following IV infusion [96]. The antibody used to protect neonates from respiratory syncytial viral (RSV) infection (Synagis®) is one example of an antibody administered by IM injection. For reasons discussed above, the majority of antibody-based drugs have moved toward SC delivery with formulations efforts being focused to this end. Based upon the desired actions for a number of antibody-based drugs, however, routes of administration other than IV, IM or SC may be desirable. These alternative routes can provide unique formulation and stability issues.

6.1. Oral

Uptake of antibodies from the intestinal lumen can occur through fetal Fc receptors (FcRn) expressed at the apical surface of enterocytes [97]. This natural antibody uptake mechanism was initially identified in the gastrointestinal (GI) tract of neonatal rodents and its expression shown to vanish following "closure" of the intestine after weaning. It was only recently that the continued expression of this receptor in the adult intestine was demonstrated and this finding has prompted efforts to examine this receptor system as a means of delivery antibodies after oral administration. Although this pathway appears to be a viable route for the delivery of therapeutic antibodies, issues associated with antibody stability in the GI tract [98] and competition from endogenous antibodies can dramatically reduce the efficiency of uptake after oral delivery. Outside of this specific mechanism, no other transcellular mechanism of efficient antibody transport across epithelial cell of the GI tract has been demonstrated to date. Topical application of antibodies to produce a desired clinical outcome have been shown, however, with the oral delivery of a chicken-derived (IgY) antibodies for treating and preventing infectious diseases of the GI tract [98–100]. It should be noted that IgY antibodies have been shown to not bind to (or be transported by) FcRn [97].

6.2. Respiratory tract

Intranasal and pulmonary administration of protein therapeutics has shown more promising clinical outcomes than oral delivery. While the surfaces of both the respiratory tract and the gastrointestinal tracts are readily accessible, the respiratory tract is a far less hostile environment for an administered protein therapeutic. Nonetheless there are physical clearance mechanisms in place to remove foreign particles which act to reduce residence time dramatically following administration. In the case of nasal administration the use of a cream emulsion and a polymer delivery system has been shown to significantly decrease the clearance rate for a monoclonal antibody when applied to the nasal mucosa [101]. This study provides one approach for the topical application of a therapeutic antibody designed to provide clinical benefit at the mucosal surface. It does not, however, suggest that an appreciable amount of an antibody might be absorbed into the systemic circulation from this site. The nasal mucosa affords only a small absorptive surface area compared to the epithelial linings of the lung.

Aerosol delivery of antibodies to the lung has been explored as either a liquid or dry powder formulation, where in both cases the droplets or particles must be of sufficiently small size that they are respirable perhaps to the level of the deep lung but large enough that they are not readily exhaled or just impact the back of the throat [102]. The sizes of particles that provide this performance have a mass mean aerodynamic diameter and density such that they enter the alveoli and remain in the lung. This size window has been reported to be between 1 to 5 μm depending on the formulation used and in one case, far larger, 20 μm where the particles are dense (<0.4 g/cm^3) and highly porous [102].

Formulation efforts were made for producing fine dry powder particles of an anti-IgE monoclonal antibody (E25) in the size range desired for delivery to the lung [103]. These studies showed that sodium phosphate was capable of extending the usefulness of mannitol as a protective excipient in spray-dried antibody formulations and that mannitol can play a role both in maintaining protein stability as well as producing a suitable aerosol preparation. Further studies to assess carbohydrate excipients for spray-dried aerosol E25 formulations showed that excipient to protein ratios were critical. Degradation issues involved not only protein aggregation but also protein glycation or lactosylation, a protein modification

event where a reducing sugar can add covalently at a free amine [104]. The ability to formulate and delivery an antibody-based drug to the lung provides the potential for systemic delivery of that protein via uptake through the FcRn expressed at this mucosal surface. Elegant studies showing the distribution and function as well as the application of this transport route for systemic deliver of an Fc-containing protein chimera has been demonstrated [105,106]. Thus, the potential or systemic delivery of antibody-based drugs via the respiratory tract looks promising.

6.3. Miscellaneous delivery sites of the body

Unique aspects of antibody–polymer formulations designed for intravitreal injection [82] or topical vaginal delivery [86,87] have been discussed above. Additionally, antibody formulations using hyaluronic acid hydrogels for sustained brain delivery have been noted [84]. These methods focus on strategies to administer large amounts of an antibody at a specific site in order to provide a sustained level to improve efficacy and/or to minimize injection frequency. Other strategies have been described to deliver antibodies to miscellaneous sites throughout the body, For example, an anti-transferrin antibody (OX-26) has been described as a carrier for delivery of covalently attached therapeutic molecules across the blood–brain barrier following IV injection [107]. An anti-tumor necrosis factor antibody has been adsorbed onto cardiovascular stent wires for local neutralization of this cytokine in order to limit restenosis events following stent placement [108]. A CMC gel containing antibodies to prevent post-surgical infections has been administered to the intraperitoneal cavity [83].

6.4. Intracellular targeting

Some promising antibody drug targets are intracellular. Although techniques exist where a cell can synthesize an antibody and secrete it into the cell cytoplasm, successful use of this technology requires gene therapy. Instead, it is possible to chemically couple an antibody to a pH-sensitive polymer that functioned to disrupt membranes at acidic pH. Many antibodies are internalized by cells through a receptor-mediated uptake process that leads to their delivery into early and then late endosomes. During endosomal maturation after receptor–antibody internalization the pH of these vesicles will drop from near neutral to below pH 6. This approach was demonstrated in a format where the pH-responsive polymer poly(propylacrylic acid) was covalently attached to a monoclonal antibody to facilitate its delivery to the cytoplasm of target cells [12].

7. Other delivery issues

A wide range of novel drugs and drug conjugates are being developed using monoclonal antibodies and engineered antibody fragments [20]. For some of these novel therapeutic approaches to be successful, they require additional or non-traditional delivery strategies. A cursory discussion of some of these approaches as they relate to intracellular targeting, site placement and engineered antibodies fragments follows.

7.1. Antibody fragments

An entire spectrum of antibody fragments have been identified through protein engineering studies with many of these showing remarkable potential for unique and novel clinical opportunities [109]. These smaller recombinant antibody fragments, the monovalent antibody fragments, Fab and single chain Fv and the engineered variants, diabodies, triabodies, minibodies and single-domain antibodies are an emerging form of antibody therapy that are less costly to manufacture while still retaining the targeting specificity of full length antibodies. In fact some may have greater efficacy and more applications than whole monoclonal antibodies. It may be anticipated that this new subset of antibodies are posing new formulation challenges, however, to date, publications detailing stability studies and formulation development for these molecules are rare in the scientific literature so formulation data on antibody fragments is scant. The single V domains have been found to be poorly soluble and have a tendency to aggregate [109], but these problems are being overcome by the identification of mutants that minimize the hydrophobic interface and selected by phage display [110]. Five Fab molecules have been approved as of this writing with the one humanized

Fab (ranibizumab rhFab) awaiting approval in the very near future, but many are in early clinical trials and most in preclinical development. One study has been described that focused on Fab fragments. In general, the binding of an antibody to an effector protein does not necessarily inactivate that target's function [111]. When an antibody is effectively inactivating, however, the Fab fragments in the absence of an Fc domain can frequently function as highly potent binding agents capable of masking binding sites or enzymatic elements on macromolecules. For example, Fab fragments prepared from sheep anti-snake venom antibodies (Digibind, from GSK) can be an effective anti-venom. Storage of liquid formulations of such anti-venoms in sub-tropical countries where refrigeration may not be possible provides a significant formulation challenge. Although lyophilized products can perform well under these conditions, a pH 4.0 (acetate buffer) formulation was found to also be stable and potent for at least one year at room temperature [112], presumably room temperatures characteristic of the tropical and subtropical regions where the material would be used. Strikingly, this stable formulation did not require the presence of a protective carbohydrate excipient. To improve drug delivery, antibody fragments have been fused to many types of molecules, such as radionuclides, cytotoxic drugs, toxins, peptides and proteins, enzymes and liposomes [113]. An immunoliposome made with a Mab fragment is reported to deliver drugs to the brain as such a molecule is able to cross the formidable barrier of the blood brain barrier [114].

7.2. Immunoconjugates

Arming antibodies with materials useful for detection or cell killing has become a prominent approach to increase the utility of these proteins, reviewed by Wu and Senter, [20]. Antibodies provide a useful format for the production of radiopharmaceuticals for site- or tissue-specific localization using gamma scintigraphy [10,115] or for enhanced cell killing. There are currently three approved antibody–radionuclide drugs: two are murine radiolabeled Ab for B cell lymphomas ibritumomab tuixetan (^{90}Y, Zevalin) and tositumomab (^{131}I Bexxar) and the third is a humanized, gemtuzumab ozogamicin (calicheamicin, Mylotarg) for leukemia. The presence of a high energy emitting radionuclide, however, will compromise long-term stability of any antibody formulation; not only would the released energies act to damage the protein but there is strong preference to use short half-life isotopes for these procedures [116]. Thus, in these instances, a stable formulation based upon the principles discussed above can provide a product that can be radiolabeled and then used soon after this step. One example of this approach is a lyophilized antibody formulation that is reconstituted and prepared by a kit method prior to patient dosing [117].

Antibodies coupled with cytotoxins for targeted cell killing [118] are particularly useful in antibody-based cancer therapies [119]. Beyond the identification of a stable formulation for the antibody, such materials have several additional problems for the formulation scientist to solve: stability issues of the attached agent and stability of the linker used to join the attached agent to the antibody. In the case of a monoclonal antibody-vinca alkaloid conjugate, a pH range of 4.5–5.5 in a phosphate buffer showed improved solution stability compared to material stored at pH 6.5–7.4 as evidenced by antibody aggregation and hydrolysis of the chemical linker used to conjoin the alkaloid to the antibody [120]. In most cases, cytotoxic materials are coupled to antibodies using reversible chemistries to provide a release mechanism for maximal activity at the target site. Many of these antibody conjugates use a disulfide bond linkage that can be reduced by a novel redox enzyme present in endosomes and lysosomes of target cells [121]. Slightly oxidizing conditions that would provide added stability of this linker in the formulation could, over time, act to cause detrimental oxidation events in the antibody and/or the linked compound. Other conjugates use an acid-labile linker [122]. In this case, formulation pH must be restricted to either neutral or alkaline conditions. Enzymatic separation of antibody and a cytotoxic material could also be achieved by coupling the two through an amino acid linker that is recognized as a substrate by a specific protease or peptidase [123]. Formulation instability of this linker sequences can be corrected to some extent by selective changes of the amino acids used.

Thus, the challenges of immunoconjugates include maintaining both drug and antibody potency and stability. While some antibodies are efficacious by

themselves, many are much more therapeutically active after being armed with a toxin, drug and radionuclide [124]. These changes make the resulting product more difficult to prepare, stabilize and deliver. Yet, there is tremendous enthusiasm for these approaches as they provide the promise for some companies to transform their antibody into a potentially curative drug. For this reason many antibodies are combined with a generic delivery system, such as a liposome, that can be used to delivery a variety or even multiple agents simultaneously through antibody-directed targeting [125]. Examples of such an approach include studies performed using an antibody directed against the Her2/neu antigen to delivery liposomes containing a chemotherapeutic have been described [126], antibodies used to deliver cationic liposomes for the administration of nucleic acid material for gene therapy [127] and antibodies used in the targeted delivery of modified gelatin nanoparticles [128] that can be coupled through a disulfide linkage [129].

8. Conclusions — challenges and opportunities

As antibody-based drugs come to the forefront of therapeutics derived from biotechnology, they provide a range of new and exciting opportunities to treat a number of currently unmet medical needs. Successful clinical application of these novel agents requires the development of stable formulations that can be used for specific delivery methods. Antibodies, because of their endogenous nature, have built-in features that may pose problems for stability as biotherapeutics, that function in the normal clearance of these proteins from the body [40]. Thus, despite our best efforts to prepare stable antibody-based drug formulations, the ultimate stability of these products may be limited to inherent mechanisms of degradation pre-designed for the rate of clearance optimal for their function and turnover in the body. Protein engineering has led to significant advances in improving some of these parameters and the trend would predict that additional advances will be made in the near future (reviewed in [109]). Protein modifications can be engineered such that stability and efficacy can be improved in ways that cannot be achieved solely, if at all, with formulation alterations. While it is clear that more research is needed in the relatively new area of engineered antibody fragments for clinical use [109], they have great potential for diagnostic and therapeutic application.

In general, successful antibody formulation designs are simply the best compromise of minimizing several competing degradation pathways. One approach is to find a pH where the minimal amount of degradation events such as deamidation and oxidation will occur. Another is to identify a buffer system that can stabilize the formulation at that pH without damaging the antibody. One common tactic taken to reduce the rate of many of these chemical degradation events is to prepare lyophilized or freeze-dried antibody preparation but even these can be compromised by aggregation issues due to thermodynamic stress that occurs during freezing, drying and reconstitution. A number of protective excipients, mostly carbohydrates, can reduce the propensity of antibodies to aggregate during these stressful events. Another issue is to produce a formulation that is compatible with delivery — neither hypotonic — nor hypertonic solutions are well-tolerated upon injection. Added to this is the potential use of a surface active agent to help with liquid stability such as a non-ionic detergent at a low concentration [58] as well as the possible addition of preservatives required for multi-use systems [130].

The future of antibody based drugs is likely to include more complex systems than those currently approved for use in man. Possibly the most critical aspect of generating a pharmaceutically successful formulation for antibody based drugs is an understanding the critical aspects of stability for the protein or the materials associated with the protein. It has been known for some time that even a single amino acid change in the Fv region of an antibody can result in dramatic differences in antigen-binding specificity [31]. Also, alterations at specific amino acids in the Fc domain can dramatically change functional aspects of an antibody. Thus, these kinds of changes must be considered more important in the search for a successful formulation than those changes that do not appear to affect function. In the case of antibody or antibody-like agents being used to delivery a payload such as a toxin or chemotherapeutic, the stability of the material to be delivered and its continued association with the delivery antibody is crucial.

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