

P-15727

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

DIRECCIÓN DE NUEVAS CREACIONES

E. S. D.

Re: Expediente No. **NC2018/0008754**Solicitud de patente a nombre de **AMGEN RESEARCH (MUNICH) GMBH y AMGEN INC.**

Yo, CAROLINA DAZA MONTALVO identificada como aparece al pie de mi firma, domiciliada en la Carrera 11 No. 86-53, obrando como apoderada de las sociedades AMGEN RESEARCH (MUNICH) GMBH y AMGEN INC., domiciliadas en Alemania y Estados Unidos de América, respectivamente, solicitantes de la patente de la referencia, en respuesta al oficio No. **200254** notificado electrónicamente el 27 de abril de 2020, relacionado con los requerimientos de fondo de la solicitud, atentamente me permito presentar la siguiente información:

1. Modificación al Título de la Solicitud

El examinador ha indicado que el título no se relaciona con el objeto de la solicitud contenido en la descripción y las reivindicaciones, y ha sugerido modificar el título a: "*COMPOSICIÓN FARMACÉUTICA QUE COMPRENDE UN ANTICUERPO BIESPECÍFICO ANTI-CD33/CD3, β -DEXTRINA Y UN BUFFER*". En respuesta a ello, los solicitantes discrepan respetuosamente del título sugerido, ya que los anticuerpos biespecíficos además de los que se dirigen a CD33 y CD3 se reivindican y ejemplifican en la solicitud tal como se presentó (ver, por ejemplo, la reivindicación 12 vigente). Adicionalmente, el término " *β -dextrina*" está incompleto y debería ser " *β -ciclodextrina*". Por lo tanto, se solicita modificar el título para recitar: "*COMPOSICIÓN FARMACÉUTICA QUE COMPRENDE UN ANTICUERPO BISPECIFICO, β -CICLODEXTRINA Y UN BUFFER*".

2. Modificación a las Reivindicaciones

Con fundamento en la facultad que confiere el artículo 34 de la Decisión 486 al solicitante para modificar la solicitud de patente en cualquier momento del trámite, presento junto con este memorial nuevo capítulo reivindicatorio, el cual consta de catorce (14) reivindicaciones y reemplaza completamente el presentado anteriormente. Específicamente, se realizaron las siguientes modificaciones:

- 2.1** No se incluyen las antiguas reivindicaciones 2 y 15.
- 2.2** Se modificó la antigua reivindicación 1 con el propósito de incluir la materia reclamada en las antiguas reivindicaciones 2 y 15. Adicionalmente, las modificaciones realizadas se encuentran soportadas en la descripción inicialmente presentada.
- 2.3** Las reivindicaciones y sus dependencias han sido reenumeradas, en vista de las modificaciones mencionadas.

En cuanto a estas modificaciones, me permito decir que las mismas no amplían de ninguna manera la materia objeto de la presente solicitud y se realizaron con el ánimo de superar las objeciones de claridad y nivel inventivo planteadas por el examinador en su concepto técnico.

3. Pago de tasas

En relación con el número de reivindicaciones, atentamente nos permitimos decir que no se adjunta tasa por concepto de reivindicaciones adicionales, toda vez que el nuevo capítulo reivindicatorio tiene un menor número de reivindicaciones que el anteriormente presentado, y en esencia es el mismo objeto, pero definido con mayor claridad.

Por otra parte, con respecto al pago de la tasa por concepto de un nuevo examen de patentabilidad, el procedimiento descrito en el numeral 1.2.2.5.1 del Capítulo Primero del Título X de la Circular Única establece que *"Cuando el solicitante en su respuesta al examen de patentabilidad modifique: i.) el capítulo reivindicatorio; ii) la descripción de la invención a proteger, iii) o presente un nuevo capítulo reivindicatorio, la Dirección de Nuevas Creaciones, si así lo considera necesario, podrá realizar hasta dos nuevos exámenes. En todo caso, el número de requerimientos por no patentabilidad estará supeditado a que el término del estudio de patentabilidad no sea superior a 18*

meses, contados desde la publicación de la solicitud de patente para patentes de invención.” (el énfasis es nuestro).

Al respecto, se hace notar que la presente solicitud fue publicada en la Gaceta de la Propiedad Industrial el 31 de agosto de 2018, por lo que el término de 18 meses para la realización de los hasta dos nuevos exámenes de patentabilidad expiró el 31 de marzo de 2020. Sin embargo, la presente acción oficial fue notificada el 27 de abril de 2020, por lo que el plazo de 18 meses se cumplió antes de que fuera notificada la presente acción oficial, con lo cual no se les permitió a los solicitantes acceder a los hasta dos nuevos exámenes de patentabilidad.

Teniendo en cuenta que tales exámenes corresponden a aspectos sustanciales en el marco del artículo 45 de la Decisión citada en precedencia, con los cuales se esperan realizar los análisis y discusiones de fondo que le permitan a su Despacho poder tomar una decisión en los términos del artículo 48 de la norma comunitaria, y que dentro de nuestro ordenamiento jurídico existe el principio de prevalencia del derecho sustancial sobre los aspectos procedimentales en atención a lo dispuesto por el artículo 228 de la Constitución Política de Colombia de 1991, el cual se debe observar dentro del presente trámite, se solicita respetuosamente al examinador determinar si considera necesaria la realización de un nuevo examen de patentabilidad, y en dado caso, notificarnos para proceder a realizar el pago correspondiente.

4. Claridad y concisión de las reivindicaciones

Los solicitantes manifiestan que mediante la presentación del nuevo capítulo reivindicatorio quedan subsanadas la totalidad de las objeciones, de modo tal que las nuevas reivindicaciones proporcionan claridad y concisión a la invención descrita, siguiendo las recomendaciones realizadas por el examinador en su concepto técnico.

- 4.1** El examinado ha indicado que “[l]as reivindicaciones 1 y 12 no son concisas porque mencionan un anticuerpo biespecífico y lo caracterizan por los antígenos a los que se unen y de esta manera puede incluir un sinnúmero de anticuerpos que no presentarían sustento en la descripción”. En dicho respecto, se informa que la reivindicación 1 se modificó con el propósito incluir las características estructurales de las secuencias de aminoácidos (tanto las CDR como las secuencias de aminoácidos completas del dominio de unión o el constructo de anticuerpo biespecífico completo, respectivamente, cuando estén disponibles),

de acuerdo con las antiguas reivindicaciones 1, 2 y 15, así como características descritas en la descripción inicialmente presentada. Específicamente:

El primer dominio de unión se ha especificado estructuralmente como que comprende CDR seleccionadas del grupo que consiste en SEQ ID Nos 91 a 96, SEQ ID Nos 101 a 106, SEQ ID Nos 111 a 116, SEQ ID Nos 120 a 125, SEQ ID Nos 129 a 134, SEQ ID Nos 138 a 143, SEQ ID Nos 147 a 152, SEQ ID Nos 156 a 161, SEQ ID Nos 165 a 170, o en donde la secuencia de aminoácidos del primer dominio de unión del constructo biespecífico de cadena simple es seleccionado del grupo que consiste en 183, 185 y 187. Dicha modificación se encuentra soportada en la descripción tal como se presentó originalmente (ver la tabla en las páginas 67 a 71 que describen las CDR de los respectivos dominios de unión a CD33) y en la antigua reivindicación 13 que especifica otros primeros dominios de unión completos.

El segundo dominio de unión se ha especificado estructuralmente como que comprende CDR seleccionadas del grupo que consiste en SEQ ID Nos 1 a 6, SEQ ID Nos 10 a 15, SEQ ID Nos 19 a 24, SEQ ID Nos 28 a 33, SEQ ID Nos 37 a 42, SEQ ID Nos 46 a 51, SEQ ID Nos 55 a 60, SEQ ID Nos 64 a 69, SEQ ID Nos 73 a 79, SEQ ID Nos 82 a 87, o en donde la secuencia de aminoácidos del segundo dominio de unión del constructo biespecífico de cadena simple es 179, o en donde el constructo de anticuerpo biespecífico de cadena simple (completo) tiene la secuencia de aminoácidos SEQ ID NO 174. Soporte para las CDR se puede encontrar en la descripción tal como se presentó originalmente (ver tabla en las páginas 64 a 67 que describe las CDR de los respectivos dominios de unión a CD33), la SEQ ID NO 179 se describe en la antigua reivindicación 13 y la SEQ ID NO 174 en la antigua reivindicación 15.

Adicionalmente, se definió la β -ciclodextrina indicando que ésta es seleccionada del grupo que consiste en metil- β -ciclodextrina, hidroxietil- β -ciclodextrina, etil- β -ciclodextrina, butil- β -ciclodextrina, succinil-(2-hidroxiopropil)- β -ciclodextrina, heptakis(2,3,6-tri-O-metil)- β -ciclodextrina, heptakis(2,3,6-tri-O-benzoil)- β -ciclodextrina, sal de sodio de fosfato de β -ciclodextrina, sal de sodio de sulfato de β -ciclodextrina, triacetil- β -ciclodextrina, sal de heptasodio de heptakis(6-O-sulfo)- β -ciclodextrina, sal de sodio de carboximetil- β -ciclodextrina, sal de sodio de sulfobutileter- β -ciclodextrina, 6-O-p-toluenesulfonil- β -ciclodextrina, de acuerdo con la antigua reivindicación 2.

4.2 El examinado ha señalado que *“la expresión “la composición es estable” en la parte caracterizadora y la estabilidad es relativa a las condiciones para determinarla”*. Ante ello, se informa que el término “estable” se especificó con base en la descripción presentada originalmente (ver pág. 8, párrafo [39]): *“Los términos “estabilidad” o “estable” en el contexto de la composición farmacéutica de la invención y el constructo de anticuerpo biespecífico de cadena simple en particular se refiere a la reducción o prevención de la formación de agregados de proteína (HMWS)”*. Además, la reducción de agregados se cuantificó a menos del 10% (p/v) con base en lo divulgado en la página 10, párrafo [47] de la descripción inicialmente presentada.

En consecuencia, nos permitimos indicar que el presente capítulo reivindicatorio, que incluye catorce (14) reivindicaciones, se ajusta a las disposiciones del artículo 30 de la Decisión 486/00.

5. Nivel Inventivo

El examinador establece en su concepto técnico la falta de nivel inventivo de las antiguas reivindicaciones 1 a 16 a la luz de las enseñanzas técnicas descritas en los documentos Aigner *et al.*, 2012 (D1) y WO2015036583 (D2).

En este sentido, me permito decir que el artículo 18 de la Decisión 486 establece que *“Se considerará que una invención tiene nivel inventivo, si para una persona del oficio normalmente versada en la materia técnica correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica”*.

Lo anterior significa que el análisis de nivel inventivo **NO** es una comparación de los objetos de las anterioridades y la solicitud en estudio. El análisis de nivel inventivo **debe demostrar** la obviedad de la invención y establecer cómo ésta no contiene un salto cualitativo, que demuestre su altura inventiva, teniendo en cuenta que se hace **necesario demostrar** que la invención analizada carece de nivel inventivo y hubiese sido obvia para una persona versada en la materia.

Considerando la anterior definición, es deseo de los solicitantes manifestar que la invención reclamada en el nuevo capítulo reivindicatorio es inventiva en relación con el estado de la técnica citado, por las siguientes razones:

Los constructos de anticuerpos biespecíficos a las que se hace referencia en la presente invención se conocen de hecho como tales en la técnica anterior, pero su estabilización particularmente efectiva mediante β -ciclodextrinas específicas como se describe en el presente documento no ha sido enseñada ni sugerida por ninguna de las técnicas anteriores citadas.

D2 divulga que “[e]n ciertas realizaciones, la composición farmacéutica puede contener materiales de formulación para modificar, mantener o preservar, por ejemplo, el pH, osmolaridad, viscosidad, claridad, color, isotonicidad, olor, esterilidad, estabilidad, velocidad de disolución o liberación, adsorción o penetración de la composición. En tales realizaciones, los materiales de formulación adecuados incluyen, pero no se limitan a [...] beta-ciclodextrina o hidroxipropil-beta-ciclodextrina); [...]”. En dicho documento, “beta-ciclodextrina o hidroxipropil-beta-ciclodextrina” son parte de una larga lista de lavado de cientos de excipientes que podrían usarse para una multitud de propósitos, desde la conservación hasta la penetración, como se mencionó anteriormente. En absoluto, D2 proporciona una indicación o motivación específica para elegir β -ciclodextrina o hidroxipropil- β -ciclodextrina (HP- β -CD) con el propósito de inhibir la agregación. Sin embargo, incluso si la persona experta lo hizo, en vista de la enmienda a la nueva reivindicación 1, D2 no podría afectar el nivel inventivo de la presente solicitud. La materia de la nueva reivindicación 1 especifica que la β -ciclodextrina y HP- β -CD no se encuentra entre estas β -ciclodextrinas especificadas. Por lo tanto, la combinación de D1 y D2 no puede enseñar la materia de la nueva reivindicación 1.

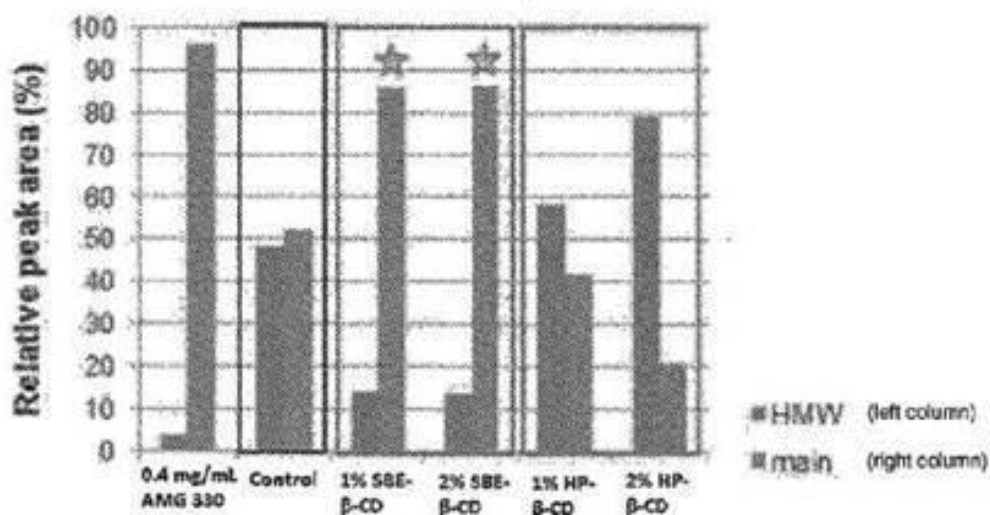
El examinador afirma además que un experto normalmente versado en la materia sabe que las ciclodextrinas ayudan a estabilizar las proteínas y evitan su agregación a través del mecanismo de formación compleja. En dicho respecto, los solicitantes discrepan respetuosamente y afirman que **no todas** las ciclodextrinas pueden ser igualmente adecuadas para cada proteína a estabilizar. Por lo tanto, es difícil para la persona experta hacer predicciones con este fin. Este es aún más el caso cuando una ciclodextrina particular es elogiada como la ciclodextrina de elección para los anticuerpos.

Por ejemplo, Serno *et al.*, 2010 (una copia de dicho documento se adjunta a este memorial, el cual fue citado como documento D10 durante la fase internacional) enseña la inhibición de la agregación inducida por la agitación de un anticuerpo IgG por HP- β -CD. Serno *et al.*, en la pág. 1204, columna derecha, supuestamente enseña que sólo HP- β -CD debe incluirse rutinariamente en estudios de formulación en pruebas durante el desarrollo de formulaciones líquidas de IgG. En contraste, Serno *et al.* aparentemente

enseña que SBE β CD no exhibió una estabilización significativa de IgG (ver Fig. 1) y que las muestras incubadas con MbCD o HP β CD tuvieron una pérdida mucho mayor de monómero y altos niveles de agregados solubles (ver página 1198, columna derecha).

Sin embargo, la materia de la nueva reivindicación 1 está dirigida a una composición farmacéutica que no comprende HP- β -CD porque, en contraste con, por ejemplo, la enseñanza de Serno- se encontró que para los constructos de anticuerpos biespecíficos HP- β -CD no es adecuado debido a la formación de agregados masivos como se muestra en la Figura 14 como se presentó originalmente:

Figure 14



Por el contrario, otras β -ciclodextrinas como SBE- β -CD, las cuales han sido previamente mostradas por Serno *et al.* no ser adecuadas para la estabilización de anticuerpos, sí estabilizan el constructo de anticuerpos biespecíficos ejemplar como lo demuestra la baja presencia de agregados (especies de alto peso molecular).

Debido a que HP-beta – CD no se reivindica específicamente en la nueva reivindicación 1 y D1 y D2 a la luz de Serno *et al.* no enseñan ni sugieren que otras β -ciclodextrinas son efectivas en una composición de constructo de anticuerpos biespecíficos como se reivindica específicamente, no es posible indicar que la presente invención es obvia.

Como la técnica citada combinada no enseña todas las características de la materia reivindicada que conducen al efecto técnico de una estabilidad mejorada para moléculas

específicas como se demuestra en los Ejemplos de la presente solicitud, la materia de las nuevas reivindicaciones 1 a 14 cumple con el requisito de nivel inventivo, tal como se define en el artículo 18 de la Decisión 486 y como se explica detalladamente en el Manual Andino y la Guía para Examinadores de la Superintendencia de Industria y Comercio.

6. Conclusión

Con las modificaciones introducidas y los argumentos presentados puede concluirse que:

- i) La presente solicitud es clara, concisa y está debidamente soportada en la descripción, cumpliendo con los requerimientos del Artículo 30 de la Decisión 486; y
- ii) Las reivindicaciones actuales son patentables sobre D1 y D2, cumpliendo así con los requerimientos de los Artículos 16 y 18 de la Decisión 486.

Habiendo atendido cabalmente las objeciones presentadas por ese despacho, atentamente solicitamos se conceda el privilegio de patente de invención a la solicitud en estudio o si el señor examinador considera que subsisten razones para objetar la presente invención, rogamos expedir otro examen de fondo con los debidos motivos y razones que sustenten su objeción con el fin de que nuestro mandante tenga la posibilidad de exponer sus argumentos con base en la interpretación de los artículos 45 de la Decisión 486 -el cual otorga la facultad al examinador para emitir los exámenes de fondo- y 42 del Código de Procedimiento Administrativo y de lo Contencioso Administrativo que señala que: *"habiéndose dado oportunidad a los interesados para expresar sus opiniones, y con base en las pruebas e informes disponibles, se tomará la decisión, que será motivada. La decisión resolverá todas las peticiones que hayan sido oportunamente planteadas dentro de la actuación por el peticionario y por los terceros reconocidos"*, (el subrayado es nuestro).

En consecuencia, solicitamos a ese Despacho pronunciarse en relación con todos los argumentos presentados en este memorial dando estricta aplicación al principio del debido proceso consagrado en el artículo 29 de la Constitución Nacional.

7. Anexos

- Nuevo capítulo reivindicatorio conformado por catorce (14) reivindicaciones.
- Copia de documento Serno *et al.*, 2010 (Inhibition of Agitation-Induced Aggregation of an IgG-Antibody by Hydroxypropyl- β -Cyclodextrin).

Señora Directora,



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REIVINDICACIONES

1. Una composición farmacéutica que comprende
un constructo de anticuerpo biespecífico de cadena simple, que se une
a un antígeno de superficie de una célula diana mediante un primer dominio de unión que comprende CDR seleccionadas del grupo que consiste en SEQ ID Nos 91 a 96, SEQ ID Nos 101 a 106, SEQ ID Nos 111 a 116, SEQ ID Nos 120 a 125, SEQ ID Nos 129 a 134, SEQ ID Nos 138 a 143, SEQ ID Nos 147 a 152, SEQ ID Nos 156 a 161, SEQ ID Nos 165 a 170, o en donde la secuencia de aminoácidos del primer dominio de unión del constructo biespecífico de cadena simple es seleccionado del grupo que consiste en 183, 185 y 187, y
al antígeno de superficie de Células T CD3 mediante un segundo dominio de unión que comprende CDR seleccionadas del grupo que consiste en SEQ ID Nos 1 a 6, SEQ ID Nos 10 a 15, SEQ ID Nos 19 a 24, SEQ ID Nos 28 a 33, SEQ ID Nos 37 a 42, SEQ ID Nos 46 a 51, SEQ ID Nos 55 a 60, SEQ ID Nos 64 a 69, SEQ ID Nos 73 a 79, SEQ ID Nos 82 a 87, o en donde la secuencia de aminoácidos del segundo dominio de unión del constructo biespecífico de cadena simple es 179, o en donde el constructo de anticuerpo biespecífico de cadena simple tiene la secuencia de aminoácidos SEQ ID NO 174,
una β -ciclodextrina, en donde la β -ciclodextrina es seleccionada del grupo que consiste en metil- β -ciclodextrina, hidroxietil- β -ciclodextrina, etil- β -ciclodextrina, butil- β -ciclodextrina, succinil-(2-hidroxipropil)- β -ciclodextrina, heptakis(2,3,6-tri-O-metil)- β -ciclodextrina, heptakis(2,3,6-tri-O-benzoil)- β -ciclodextrina, sal de sodio de fosfato de β -ciclodextrina, sal de sodio de sulfato de β -ciclodextrina, triacetil- β -ciclodextrina, sal de heptasodio de heptakis(6-O-sulfo)- β -ciclodextrina, sal de sodio de carboximetil- β -ciclodextrina, sal de sodio de sulfobutileter- β -ciclodextrina, 6-O-p-toluenesulfonil- β -ciclodextrina; y
un buffer, en donde la composición reduce a menos del 10% (p/v) o evita la formación de agregados de proteínas (especies de alto peso molecular HMWS).
2. La composición de acuerdo con la reivindicación 1, en donde la β -ciclodextrina está presente en una concentración en el rango de entre 0,1 % a 20 % (p/v), preferiblemente de 0,5 % a 2 % (p/v) y más preferiblemente de 0,8 % a 1,5 % (p/v).

3. La composición de acuerdo con una cualquiera de las reivindicaciones precedentes, en donde la β -ciclodextrina es seleccionada del grupo que consiste en sal de sodio de sulfobutileter- β -ciclodextrina, hidroxipropil- β -ciclodextrina.
4. La composición de acuerdo con una cualquiera de las reivindicaciones precedentes, en donde el constructo de anticuerpo biespecífico de cadena simple está presente en un rango de concentración de 0,1-5 mg/ml, preferiblemente de 0,2-2,5 mg/ml, más preferiblemente de 0,25-1,0 mg/ml.
5. La composición de acuerdo con una cualquiera de las reivindicaciones precedentes, en donde el buffer es seleccionado del grupo que consiste en fosfato de potasio, ácido acético/acetato de sodio, ácido cítrico/citrato de sodio, ácido succínico/succinato de sodio, ácido tartárico/tartrato de sodio, histidina/histidina HCl, glicina, Tris, glutamato, acetato y mezclas de los mismos.
6. La composición de acuerdo con la reivindicación 5, en donde el buffer es seleccionado del grupo que consiste en fosfato de potasio, ácido cítrico/citrato de sodio, ácido succínico, histidina, glutamato, acetato y combinaciones de los mismos.
7. La composición de acuerdo con cualquiera de las reivindicaciones precedentes, en donde el pH de la composición está en el rango de 4-7,5.
8. La composición de acuerdo con una cualquiera de las reivindicaciones precedentes, que además comprende uno o más excipientes seleccionados del grupo que consiste en sacarosa, trehalosa, manitol, sorbitol, arginina, lisina, polisorbato 20, polisorbato 80, poloxámero 188, plurónico y combinaciones de los mismos.
9. La composición de acuerdo con cualquiera de las reivindicaciones precedentes, que comprende uno o más conservantes.
10. La composición de acuerdo con la reivindicación 9, en donde el uno o más conservantes es seleccionado del grupo que consiste en alcohol bencílico,

clorobutanol, fenol, meta-cresol, metilparabeno, fenoxietanol, propilparabeno y tiomerosal.

11. La composición de acuerdo con cualquiera de las reivindicaciones 1 a 9, en donde el primer dominio de unión del constructo de anticuerpo biespecífico de cadena simple se une a CD19, CD33, MSLN, FLT3 o BCMA.
12. La composición de acuerdo con la reivindicación 11, en donde la secuencia de aminoácidos del primer dominio de unión del constructo biespecífico de cadena simple se selecciona del grupo que consiste en SEQ ID 99, 109, 119, 128, 137, 146, 155, 164, 173, 183, 185 y 187 y la secuencia de aminoácidos del segundo dominio de unión del constructo biespecífico de cadena simple se selecciona del grupo que consiste en SEQ ID NO: 9, 18, 27, 36, 45, 54, 63, 72, 81, 179 y 90.
13. La composición de acuerdo con la reivindicación 12, en donde la formulación está libre de conservantes, la secuencia de aminoácidos del constructo de anticuerpo biespecífico de cadena simple es SEQ ID NO 100 o 110 y el constructo está en una concentración de aproximadamente 0,5 mg/ml, la ciclodextrina es sal de sodio de sulfobutileter- β -ciclodextrina en una concentración de aproximadamente 1% (p/v), el buffer es fosfato de potasio en una concentración de aproximadamente 10 mM y la formulación comprende además sacarosa en una concentración de aproximadamente 8% (p/v) y polisorbato 80 en una concentración de aproximadamente 0,01% (p/v) a un pH de aproximadamente 6,0.
14. La composición de acuerdo con la reivindicación 11, donde la ciclodextrina es sal de sodio de sulfobutileter- β -ciclodextrina en una concentración de aproximadamente 1% (p/v), el buffer es fosfato de potasio en una concentración de aproximadamente 10 mM y la formulación comprende además manitol en una concentración de aproximadamente 2%, sacarosa en una concentración de aproximadamente 2% (p/v) y polisorbato 80 en una concentración de aproximadamente 0,01% (p/v) a un pH de aproximadamente 6,0.

BIOTECHNOLOGY

Inhibition of Agitation-Induced Aggregation of an IgG-Antibody by Hydroxypropyl- β -Cyclodextrin

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ABSTRACT: In order to provide an alternative to nonionic surfactants as excipients for protein formulations, cyclodextrin-derivatives (CDs) were examined for their potential to inhibit agitation-induced aggregation of an IgG in aqueous solution. Loss of monomeric protein and protein aggregation were monitored throughout the agitation experiments by size exclusion chromatography. Hydroxypropyl- β -cyclodextrin (HP β CD) completely suppressed IgG-aggregation at a remarkably low concentration (2.5 mM) and in contrast to other CDs it also did not negatively affect IgG-stability during storage in nonagitated solution. Further agitation experiments demonstrated the superiority of HP β CD to other common excipients in protein formulation, such as sugars and sugar alcohols or polysorbate 80 in low concentrations. Spectroscopic (fluorescence spectroscopy and Fourier transform infrared spectroscopy), thermodynamic (microcalorimetry), and physical investigations (surface tension measurements) were carried out to elucidate the mechanism of stabilization of the IgG. In contrast to other studies with HP β CD, protein stabilization could not be attributed to direct interaction between hydrophobic amino acids on the IgG and this excipient. Competition with the protein for the air–water interface appears to be the dominating mechanism of stabilization. © 2009 Wiley-Liss, Inc. and the American Pharmacists Association *J Pharm Sci* 99:1193–1206, 2010

Keywords: calorimetry (DSC); cyclodextrins; excipients; stability; formulation; FTIR; proteins; physicochemical properties; protein aggregation; protein formulation

INTRODUCTION

Monoclonal antibodies (mAbs) have by far become the most important group of protein therapeutics.

More than 300 mAbs are under development and more than 20 are already approved and on the market.^{1,2} However, the application of these oftentimes highly efficient or promising molecules remains problematic due to their tendency to aggregate. Physical stability of the mAbs must be maintained throughout all steps of pharmaceutical processing such as packaging, shipping, and storage as well as during administration to the patient. Aggregation of mAbs

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can be triggered by many stresses, including exposure to elevated temperatures, the presence of impurities,³ freeze-drying, and exposure to interfaces such as the air–water interface.⁴ This article focuses on the effects of the latter stress on mAb formulations: agitation-induced aggregation of a mAb and its prevention by cyclodextrin-derivatives (CDs).

MAbs—like other proteins—are prone to accumulate at interfaces. Most proteins exhibit a remarkable adsorption to hydrophobic surfaces, with the air–water interface not only being among the most hydrophobic ones but also the most frequently encountered, for example, during mechanical agitation and mixing, spray-drying or filtration.⁵

After adsorption proteins rearrange at the surface, a process which may involve partial unfolding of the adsorbed protein segments.⁵ The altered protein structure along with high local concentrations at the interface are thought to promote aggregation.⁴ It is well known that protein aggregation may have serious implications for the safety and efficacy of protein drugs.^{6–10} Hence, for a new protein formulation, surface-induced aggregation during processing and storage must be circumvented.

Agitation-induced aggregation has been reported^{4,11} for a variety of proteins,^{12–17} and it is a serious concern for the formulation of mAbs^{18–20} and also fusion proteins containing parts of immunoglobulins.²¹ Currently, nonionic surfactants are the excipients most commonly used to inhibit surface-induced protein aggregation. For example, polysorbate 80, polysorbate 20, Brij 35 (polyoxyethylene alkyl ether), Pluronic F 68, and Pluronic F 88 (polyoxyethylene polyoxypropylene block polymer) were demonstrated to stabilize rhGH against aggregation during vortexing.^{17,22} MAbs as well were demonstrated to be stabilized by polysorbate against mechanical stress^{18–20} and so were a number of additional proteins.

Unfortunately, nonionic surfactants and most notably the members of the frequently used polysorbate family are associated with a number of disadvantages. The use of polysorbate in protein formulations raises major concerns due to autooxidation of the ethylene oxide subunits, which may be followed by the formation of peroxides that in turn can lead to oxidation of the protein.³ The level of peroxides in formulations of recombinant human granulocyte colony-stimulating factor (rhGCSF) could be correlated to

the extent of protein oxidation.²³ Dual effects of polysorbate 80 on the stability of the model protein IL-2 were reported by Wang et al.²⁴: inhibition of shaking-induced aggregation on the one hand but also increased protein oxidation and aggregation during long-term storage in nonagitated solution. These findings are in agreement with a study on pegylated granulocyte colony-stimulating factor (PEG-GCSF) in which polysorbate 20 suppressed protein aggregation induced by agitation, but during quiescent storage increasing polysorbate concentrations lead to increasing amounts of aggregates.²¹ Increased levels of aggregation after long-term storage despite good stabilization against surface-induced damage were also reported in the case of recombinant hemoglobin.²⁵ In addition, the presence of surfactants in protein solutions may also decrease the protein's native state stability, which can be explained by a preferential binding to the unfolded state.²⁶ Finally, polysorbate 80 is also suspected to form mixed micelles with proteins that simulate a viral structure which has been suggested could possibly trigger immune reactions in patients.⁶

Because of these shortcomings, there is a need for alternatives (either new kinds of surfactants or even a different class of excipients) to the traditional polyoxyethylene-based surfactants. A disadvantage of new surfactants is that they are not used in approved parenteral products. On the other hand, an effective strategy would be to choose excipients that are used in approved products and that may serve the same role as surfactants to inhibit agitation-induced aggregation of therapeutic proteins.

The purpose of the current study was to test the hypothesis that CDs could be used as alternatives to nonionic surfactants to inhibit agitation-induced aggregation of therapeutic antibodies. Cyclodextrins comprise a family of three major cyclic oligosaccharides: α -CDs, β -CDs, and γ -CDs. All CDs have a truncated cone-like structure with a cavity that is lined by a wide and a narrow hydrophilic end consisting of primary and secondary hydroxyl groups. The cavity interior is marked by pairs of glycosidic oxygen atoms lying in a plane, thereby providing a hydrophobic microenvironment in the interior of the CDs.²⁷ These structural properties of CDs are responsible for the aqueous solubility of the CDs on the one hand and for their ability to encapsulate hydrophobic moieties on the other hand.²⁸

Depending on the size of the molecules either the entire guest molecule may be included into the cavity or only certain parts of larger molecules may be incorporated into the cavity. Most commonly the application of CDs in parenteral drug formulation is due to their capability to solubilize drugs for intravenous and intramuscular dosing. There are marketed intravenous formulations available containing hydroxypropyl- β -cyclodextrin (HP β CD), for example, in formulations of the antifungal agent itraconazole.²⁹ In addition water-soluble sulfobutylether- β -cyclodextrin (SBE β CD) appears in approved formulations of voriconazole i.v. and ziprazidone i.m.

To date, products with CDs that have been marketed for parenteral use exclusively contain low-molecular-weight drugs. However, the inhibition of protein aggregation by different CD-derivatives has been demonstrated. But so far no studies have been published examining the potential use of CD-derivatives to inhibit aggregation of mAbs in aqueous solution formulations. The stresses applied in the previous studies on inhibition of protein aggregation by CD-derivatives were varied: chemical denaturation of lysozyme, bFGF, rhGH, or bovine carbonic anhydrase with guanidinium-HCl, or exposure of the same proteins to thermal stress,^{30–32} acid inactivation of rhGH in the presence of salt;³³ and also agitation stress due to vortexing.^{32,34}

However, CDs also often lead to a decrease of the thermal stability of proteins, presumably due to preferential binding to exposed hydrophobic amino acids in the unfolded state, which is associated with a shift of the folding equilibrium towards the unfolded state. For globular proteins this was demonstrated by Cooper.³⁵ They found that the binding of CDs to exposed hydrophobic side chains destabilizes the native folded form of the protein resulting in a decrease of T_m , as observed in DSC studies. This observation was confirmed, for example, recently by Tavornvipas et al.³¹ Thus, when binding of CDs to therapeutic proteins occurs, a balance of stabilizing (in terms of aggregation-inhibition by shielding of hydrophobic interaction) and also destabilizing (in terms of a decreased temperature of unfolding and lower thermal resistance of the protein) effects is expected.

Here, we present studies on the use of CDs in IgG-formulations. The focus will be on the CD-effect on agitation-induced aggregation as well as on possible destabilizing effects at elevated

temperature. CD effects are compared to other common excipients for mAb formulations such as polysorbate and sugars. Furthermore, the underlying mechanisms of mAb stabilization by CDs will be analyzed.

MATERIALS AND METHODS

Materials

An IgG mAb was a gift from Roche Diagnostics GmbH (Penzberg, Germany). It was formulated in a 20 mM histidine (His) buffer at pH 5.8 at a protein concentration of 2.4 mg/mL. Protein solutions were filtered through Acrodisc[®] 0.2 μ m PVDF syringe filter units (Pall GmbH, Dreieich, Germany) prior to usage in formulations. The IgG bulk material consisted of 91.70% monomer and 8.30% soluble aggregates of which 5.77% can presumably be attributed to dimers and the rest to higher order oligomers.

HP β CD, methyl- β -cyclodextrin (M β CD), and hydroxypropyl- γ -cyclodextrin (HP γ CD) were supplied by Wacker Chemie AG (Burghausen, Germany). SBE β CD was a gift by CyDex Inc. (Lenexa, KS). All CDs as well as polysorbate 80 (Tween[®] 80, purchased from Sigma-Aldrich GmbH, Seelze, Germany) were dissolved to yield 100 mM stock solutions in His-buffer that were used for the preparation of the formulations. These stock solutions were filtered through 0.22 μ m cellulose acetate syringe filters (VWR International, Darmstadt, Germany).

Trehalose dihydrate (Ferro Pfanstiehl Laboratories Inc., Waukegan, IL), sucrose (Sigma-Ultra, St. Louis, MO), D-mannitol (Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany) and D-sorbitol (Merck KGaA, Darmstadt, Germany) were prepared as 1 M stock solutions in His buffer.

All formulations prepared for stability testing had a mAb concentration of 1.8 mg/mL. They were prepared by dilution of the protein bulk solution with CD-stock solution and His buffer. All formulations were filtered through 0.2 μ m PVDF syringe filters before the beginning of the incubation period. For stability studies, samples were tested in triplicate and triplicate samples were left unstressed as control samples.

Agitation Studies

Agitation experiments were carried out on a "Thermomixer" or on an "Orbit 300" shaking

device. Polypropylene centrifugal tubes (1.5 mL) were placed vertically onto these devices which were then shaken at a speed of 1,200 or 800 rpm, respectively. Thereby, the air–water interface within in the tubes was greatly increased and constantly renewed with substantial entrainment of air bubbles into the solution. The temperature was 20°C throughout all agitation experiments. The tubes were initially filled with 1 mL of the respective formulation, leaving enough headspace for the formation of a large air–water interface. At certain intervals (depending on the experiment) 100 μ L aliquots of the samples were drawn, centrifuged at 12,100*g* to remove potential precipitates before subjecting the supernatants to further analysis for remaining monomer and soluble aggregates. In addition, tubes were filled with 1.5 mL formulation (leaving no headspace) as a reference in order to evaluate the effect of the absence of an air–water interface.

Incubation Without Agitation at Elevated Temperature

For the evaluation of mAb stability at elevated temperature, 1 mL samples were incubated in polypropylene centrifugal tubes for 8 days at 60°C. Prior to analysis, all samples were first gently homogenized and then centrifuged at 12,100*g*. The supernatants were analyzed for monomer and soluble aggregates by size exclusion chromatography.

Size Exclusion Chromatography

After removing insoluble aggregates by centrifuging, the supernatants were analyzed by size exclusion high performance liquid chromatography (SE-HPLC) on an Ultimate[®] 3000-system (Dionex Softron GmbH, Germering, Germany). A sample of 20 μ L was injected into a Tosoh TSK Gel 3000 SW_{xl}-column. Detection was carried out on a UV–Vis variable wavelength detector at 280 nm. The mobile phase consisted of 250 mM potassium phosphate and 200 mM potassium chloride at pH 7, and a flow rate of 0.5 mL/min was used. Peak areas of monomer, dimer, soluble oligomers, and fragments were integrated and monitored throughout the incubation experiments. The amount of remaining monomer was calculated in relation to the amount of soluble monomer of the respective formulation at $T = 0$ (Figs. 1, 3 and 4). The amount of remaining monomer and soluble aggregates after 72 h (Fig. 2)

was obtained by dividing the respective peak areas of the incubated sample by the total protein peak area of an unincubated control sample ($c = 1.8$ mg/mL) in the same formulation buffer. The amount of insoluble aggregates can be calculated as the difference between total amount of protein at $T = 0$ and the total amount of protein of an incubated sample after centrifugation and removal of insoluble protein aggregates. In Figure 5, the relative amount of soluble aggregates is the fraction of soluble aggregates divided by the total amount of soluble protein at certain time points.

Infrared Spectroscopy

In order to study secondary structural changes within the precipitates of agitated samples, precipitates were collected as described above and analyzed by infrared (IR) spectroscopy. These IR spectra were then compared to those of the native mAb in solution. To overcome problems due to a too low concentration of the mAb bulk material ($c = 2.4$ mg/mL) the native mAb was concentrated to 17.5 mg/mL using Centricon[®] Tubes. For analysis of the precipitates' secondary structure, precipitates of samples containing the mAb without CD were collected after a 24 h and a 1 week incubation period.

IR measurements were performed on a Bomem MB series Fourier transform infrared spectrometer (ABB Bomem, Quebec, Canada). Approximately, 20 μ L of the aqueous precipitate samples were placed into a cell with CaF₂-windows and a 7.5 μ m spacer. Each spectrum was collected as a 256-scan interferogram with a 4/cm resolution in single-beam mode. Spectra were recorded in the presence of buffer only, buffer and CD as well as in the presence of all three; protein, CD, and buffer. Protein spectra were obtained by subtraction procedures and criteria that were established earlier.^{36,37} The spectra were area-normalized and calculation of the second-derivative-spectra was performed using a seven-point Savitsky–Golay derivative function.

Apparent Melting Temperature by Fluorescence Spectroscopy

The effect of CDs on thermal stability of the mAb was monitored using intrinsic steady-state fluorescence spectroscopy. A 0.24 mg/mL solution of the mAb in presence of 25 mM CD in a 1 cm

Quartz cuvette was excited at 280 nm and the shift of the emission wavelength maximum during heating was monitored. Scans were collected at 2.5°C increments as the solution was heated from 10 to 90°C with a 5 min equilibration time after each heating step. The measurements were performed on a PTI QuantaMaster (Photon Technology International, Inc., Birmingham, NJ) fluorescence spectrometer. Slits for both excitation and emission were set at 3 nm.

Microcalorimetry

Thermal stability of the mAb in the formulations was also monitored by high sensitivity differential scanning calorimetry (μ DSC) on a Microcal differential scanning calorimeter (MicroCal Inc., Northampton, MA). The degassed samples and references were loaded into the cells with a Hamilton syringe and subsequently heated from 30 to 100°C with a scan rate of 60°C/h. Reversibility of unfolding was tested by performing two consecutive upscans (immediate cooling of the sample after the first upscan).

The samples consisted of mAb (1.8 mg/mL) in His buffer and either HP β CD, M β CD, or SBE β CD (all CD-derivatives in a concentration of 2.5 mM) or polysorbate 80 (0.04% or 0.004%) or no excipient. The respective reference samples consisted of the buffer only, and the thermograms for the buffer background were subtracted from the mAb-thermograms using Origin 7.0 software. All thermograms were normalized to the concentration of the mAb. Origin 7.0 software was also employed for all data analysis and data deconvolution processes. The melting temperatures (midpoint of unfolding, T_m) of the transitions were determined.

Fluorescence Titration

Concentrated stock solutions of CD-derivatives were titrated into 2 mL of a 0.24 mg/mL solution of the mAb in His 20 mM buffer and changes in intrinsic steady-state fluorescence spectra were monitored. Each titration was carried out three times and after recording the spectra were corrected for dilution. The rest of the experimental conditions were identical to those described above for the assessment of changes in the apparent melting temperature.

Surface Tension Measurements

Surface tension measurements were performed to evaluate the extent of mAb, CD, and polysorbate adsorption to the air–water interface and to analyze possible competition at the interface. Measurements were carried out on a Krüss digital tensiometer K 100 (Krüss GmbH, Hamburg, Germany) using a Wilhelmy plate made of roughened platinum which was heated in a Bunsen burner flame prior to each measurement. Three milliliter of the respective freshly prepared solution were placed in a circular, thermostatted, and thoroughly cleaned dish. Each measurement was performed for 240 s and the last 20 values determined within this period were averaged. These measurements were repeated at least five times and averaged to yield the reported surface tension value.

RESULTS AND DISCUSSION

A variety of methods can be used to investigate the effect of protein exposure to the air–water interface: air bubbling methods,²² vigorous vortexing for seconds or minutes,³² agitating vials either horizontally or vertically with and without headspace, or performing the agitation experiment on shaking devices like a temperature-controlled Thermomixer, as was done in this case. The approach used in the current study was chosen because it led to significant loss of monomer (more than 50%) within 3–5 days.

Agitation Study Comparing CD-Derivatives

In Figure 1, the results of an agitation study comprising mAb-formulations with four different CD-derivatives (HP β CD, M β CD, SBE β CD, and HP γ CD; each in three different concentrations) in comparison to a formulation without any CDs are shown. Loss of monomer was almost exclusively due to the formation of insoluble aggregates that were removed by centrifugation prior to analysis of the supernatants for SE-HPLC. It can be seen that after subjecting the samples to agitation for 72 h the formulation without CDs contains less than 10% of the original amount of soluble monomer. In contrast, all the formulations containing M β CD and the formulations containing HP β CD in a concentration higher than or equal to 2.5 mM were found to completely suppress agitation-induced aggregation. However, formulations

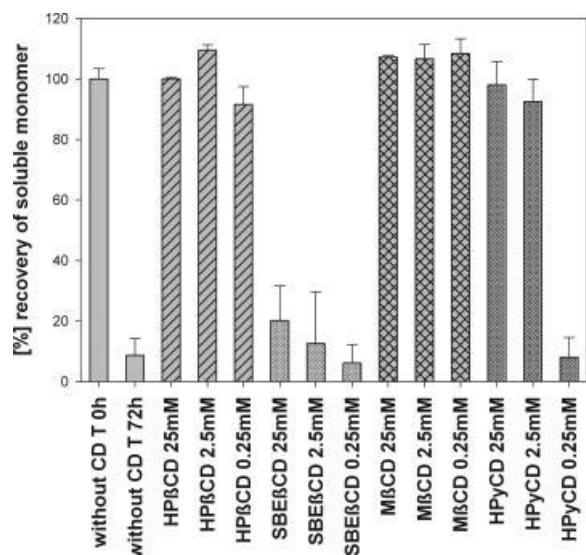


Figure 1. IgG-aggregation after 72 h of agitation at 800 rpm. Remaining monomer followed by size exclusion chromatography. Each value represents the mean of three samples $\pm$ standard deviation.

containing HP γ CD were less effectively protected, and formulations containing the ionic CD-derivative SBE β CD exhibited no significant stabilization compared to the formulation without CD.

Some samples in Figure 1 appear to have a monomer content that exceeds the theoretical maximum of 100% which would be higher than in the sample before incubation. This phenomenon can be explained by the presence of about 8% soluble aggregates in the bulk starting material. These aggregates partially or even completely dissolve into monomer throughout the course of the experiment.

Reference samples with no headspace left in the centrifugal tube were subjected to the agitation experiment as well. These samples did not show any aggregation, supporting the conclusion that aggregation in the presence of headspace is due to the presence of the extensive air–water interface. Furthermore, samples with regular headspace that were not agitated for the duration of the experiment also did not show any aggregation. Soluble aggregates that were present in the starting material did not vanish in the control samples when agitated without headspace or when stored with headspace but without agitation.

To date this is the first published demonstration that HP β CD prevents aggregation of an IgG during agitation. Compared to earlier studies that

investigated the potential of CDs for the stabilization of other proteins, the concentrations found to be sufficient for complete aggregation suppression in the current study were surprisingly low [≤ 2.5 mM, or about 0.35% (w/w)].^{32,33} Furthermore, considering the relatively high concentrations of HP β CD employed in commercially available parenteral formulations of LMW-drugs, mAb formulations with 2.5 mM HP β CD should be safe from a toxicologic point of view.^{38,39}

Incubation at Elevated Temperature

After an incubation period of 8 days at 60°C the amount of monomer in the samples without any CDs decreased to about 80%, which was mainly due to the formation of soluble aggregates (Fig. 2). In samples containing HP β CD at 0.25 and 2.5 mM the level of remaining monomer was slightly higher, for the 25 mM concentration the monomer content remained nearly unchanged compared to the reference. However, samples incubated with M β CD or HP γ CD had much greater loss of monomer and high levels of soluble aggregates. At this point it is unclear why some of the CD-derivatives stabilize the mAb and others do not. Since SBE β CD was the only ionic CD-derivative tested here the detrimental effects on

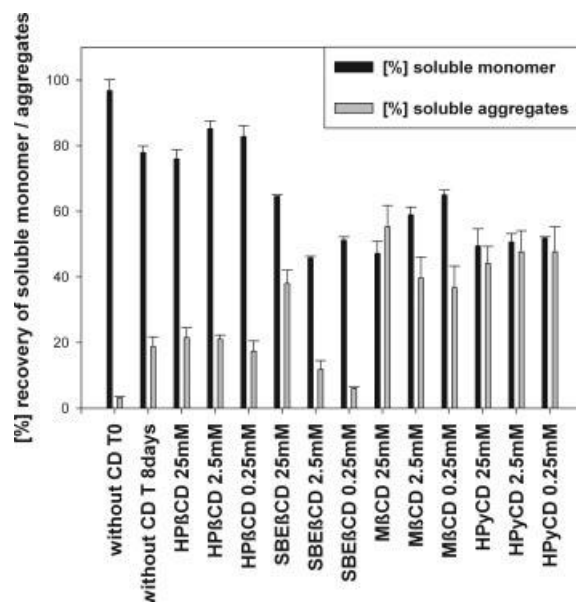


Figure 2. Aggregation of IgG after incubation at 60°C for 8 days. The levels of monomer and soluble aggregates as determined by size exclusion chromatography are displayed. Each value represents the mean of three samples $\pm$ standard deviation.

protein stability could be related to its charge. If competition at the air–water interface plays a role in inhibiting agitation-induced aggregation the comparably low surface activity (data not shown) of SBE β CD could also play a role. HP γ CD could have turned out to be less for effective aggregation–suppression than HP β CD because of its larger cavity diameter that does not allow as good a fit of hydrophobic amino acid residues into its interior as the corresponding β CD-derivative.⁴⁰ SBE β CD-containing samples contained insoluble as well as soluble aggregates.

When taken together the results from the agitation and elevated temperatures experiments, it appears that HP β CD at concentrations of about 2.5 mM in mAb formulations would be effective at inhibiting agitation-induced aggregation while not promoting aggregation at elevated temperatures. In order to get an idea of how the most promising CD-derivative tested here—HP β CD—compares to other common excipients for protein formulation (sugars and polysorbates), further agitation experiments were conducted. It was also intended to gain insight into the CDs' mechanism of action by comparing to other excipients with well-investigated effects on proteins in solution.

Effects of Different Excipient Classes on Agitation-Induced Aggregation

First, the stabilization by HP β CD and M β CD was compared to that afforded by sugars and sugar alcohols (sucrose, trehalose, mannitol, and sorbitol) at concentrations typically used in protein formulations. These excipients are frequently employed in protein formulation and mainly increase protein stability via the preferential exclusion mechanism.⁴¹ The results of the study are summarized in Figure 3. The two formulations containing HP β CD and M β CD completely inhibited aggregation, whereas the reference formulation without any CD or sugar excipients had a monomer content of about 40% after 80 h of agitation. Surprisingly, all the sugar- and sugar alcohol-containing formulations exhibited accelerated aggregation during agitation. After 80 h of agitation, the IgG monomer content decreased to less than 10% in these samples. As a consequence we conclude that CDs—although chemically classified as sugars—act completely differently from these low-molecular-weight sugars. In addition it can be stated that in contrast to the general opinion in protein for-

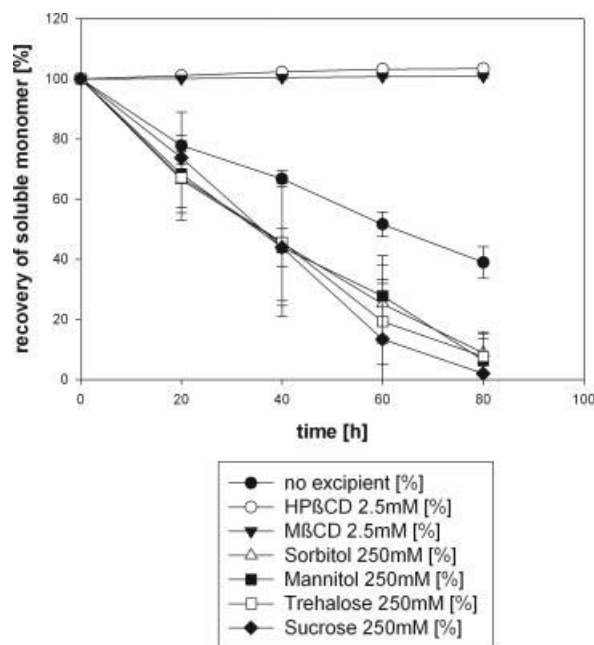


Figure 3. Comparison of the extent of IgG-aggregation after agitation between CD-formulations and low-molecular-weight sugar-formulations. Each point represents the mean of three samples $\pm$ standard deviation.

mulation⁴² sugars may promote IgG-aggregation during agitation. The reasons for this behavior remain speculative and the investigation of the mechanism(s) for this surprising phenomenon exceeded the scope of the current study.

As noted in the Introduction Section there is a need for alternatives to the traditional polyoxyethylene-based surfactants like polysorbate 80 which often is added to protein solutions in order to prevent aggregation due to interfacial stresses. In order to compare the behavior of HP β CD to polysorbate 80 an additional agitation experiment was conducted. It compared a formulation containing polysorbate 80 at a rather low concentration (0.004% = 0.03 mM, molar ratio polysorbate 80:mAb = 2.5:1) close to the CMC of polysorbate 80 (0.012 mM in water⁴³) to a formulation containing polysorbate 80 in a high concentration far above the CMC (0.04% = 0.3 mM, molar ratio 25:1) and to a formulation containing 2.5 mM HP β CD. Polysorbate 80 at 0.3 mM provided the same degree of protection as HP β CD did at 2.5 mM (Fig. 4). However, at the lower polysorbate 80 concentration the formation of soluble aggregates was observed (Fig. 5), and the HP β CD-formulation had a superior resistance to agitation-induced aggregation.

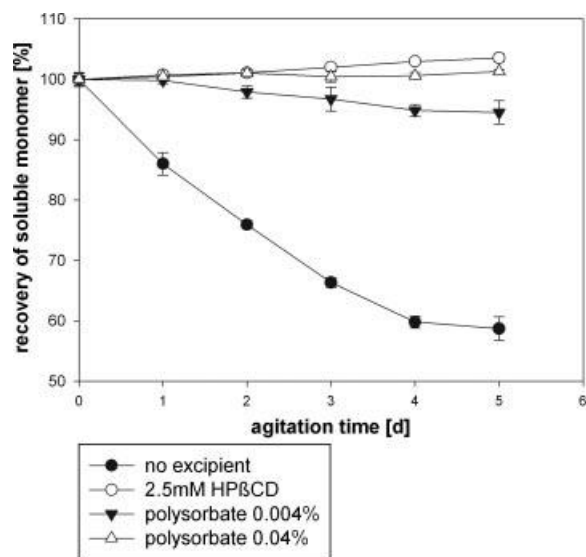


Figure 4. Comparison between formulations containing polysorbate 80 and formulations containing HPβCD during agitation for 5 days. Each point represents the mean of three samples $\pm$ standard deviation.

One can assume that CDs might prevent protein aggregation at the air–water interface in a similar fashion as nonionic surfactants. However, different mechanisms of stabilization, which appear to depend on the protein being studied, of proteins by nonionic surfactants are described. The most obvious mechanism is a

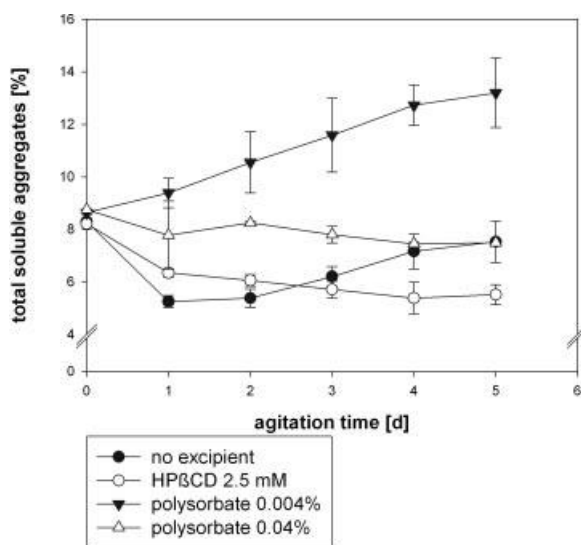


Figure 5. Soluble aggregates in formulations containing either polysorbate 80 or HPβCD during agitation for 5 days. Each point represents the mean of three samples $\pm$ standard deviation.

competition for adsorption at the air–water interface, which likely occurs in all cases even if additional routes of polysorbate-induced protein stabilization are operative. In the case of inhibition of aggregation of recombinant Human Factor XIII during agitation,⁴⁴ a saturation of the protective effect of polysorbate 20 was reached at a surfactant concentration near the critical micelle concentration—regardless of which protein concentration, that is, which surfactant–protein ratio, was employed. In addition no evidence for direct binding to the native or the partially unfolded species could be obtained using spectroscopic methods.⁴⁴

However, in other cases direct binding between the native protein and the nonionic surfactant could be observed and also clearly related to the mechanism of aggregation inhibition.^{13,15} For example, polysorbate 20 and polysorbate 80 stabilize the fusion protein albutropin against agitation-induced aggregation. By spectroscopic and calorimetric means the two surfactants were shown to bind to this fusion protein in a defined and saturable ratio at concentrations below their respective CMCs. These ratios were also applicable for effective stabilization against aggregation at the air–water interface. In addition it could be demonstrated that the presence of polysorbate increases the thermodynamic stability of albutropin, thereby reducing the propensity for denaturation and subsequent aggregation.¹⁵ In another study, weak direct binding of polysorbate to hydrophobic patches on native rhGH was demonstrated suggesting the blocking of aggregation-prone sites on the protein surface.¹³ Finally, nonionic surfactants may also act as “molecular chaperones” assisting refolding of partially denatured protein. For example, by adding nonionic surfactants to denatured carbonic anhydrase II (CAII) refolding of the enzyme could be assisted and aggregation was suppressed.⁴⁵ Similar findings were reported for rhGH where a nonionic surfactant reduced the extent of aggregation during refolding of a molten globule intermediate.¹²

The effects of HPβCD on agitation-induced aggregation described above qualitatively resemble those of polysorbate 80. Because CDs like nonionic surfactants are known to be able to bind to hydrophobic parts of proteins (most often the potency of CD-derivatives to suppress protein aggregation is ascribed to their ability to incorporate hydrophobic protein residues in their interior^{46,47}) and because some CD-derivatives are

also known to be surface-active, the hypothesis for the following mechanistic investigations is that HP β CD functions in manners analogous to nonionic surfactants in mAb-formulations. Our approach is intended to identify or exclude the potential mechanisms by which CDs stabilize the IgG against aggregation at the air–water interface.

Secondary Structure of mAb in Insoluble Aggregates

To gain insight into the potential route of aggregation during agitation, and hence mechanism for inhibition, secondary structural changes in the protein molecules in the precipitate formed were studied. In contrast to other spectroscopic methods, IR spectroscopy is well suited to measure samples in almost any physical state. Therefore, it was decided to compare the secondary structure of the protein in precipitates with the native protein in solution by employing IR spectroscopy.⁴⁸

Because no significant shifts within the characteristic IR bands are apparent when comparing the spectra (Fig. 6) it has to be assumed that protein molecules in the precipitates have a very native-like secondary structure and that they presumably did not expose large hydrophobic regions by unfolding before aggregating. This observation suggests that the prevention of mAb-

aggregation at the air–water interface by CDs does not involve the inhibition of structural alterations on a secondary structural level but it must be due to different mechanisms such as competition at the air–water interface or prevention of partial unfolding on a tertiary structural level.

These findings are in agreement with an earlier study conducted on mAb-precipitates that were generated during agitation.¹⁸ The secondary structure of the precipitates in that study was also analyzed by IR and found to maintain a native-like structure.¹⁸

Also, the IR spectroscopic results are important from a patient safety viewpoint. This is because large aggregates containing an array of nondenatured protein molecules are thought potentially to be the most immunogenic types of aggregates in therapeutic protein products.¹⁰ Therefore, it is particularly important to inhibit the formation of such agitation-induced aggregates in mAb products.

Use of Intrinsic Fluorescence to Detect CD Binding to Protein

Since almost all studies conducted on the matter of protein–CD interaction have identified aromatic amino acid residues as the main site of interaction with CDs, notably with β -CD whose cavity diameter allows a good fit of Phe, Tyr, His, and Trp into the hydrophobic cavity,^{33,40,46,49–51} the binding of nonionic surfactants or CDs to proteins can be monitored by steady-state fluorescence spectroscopy. Changes in the microenvironment of hydrophobic amino acids on the protein are monitored with this method. By analyzing titration curves generated by plotting fluorescence parameters versus amount of compound added, binding affinities can be calculated.^{32,33} Thus, we monitored steady-state fluorescence spectra of the IgG upon the addition of HP β CD or M β CD. When the spectra were corrected for dilution after the titration procedure, they almost perfectly overlaid (Fig. 7). Neither shifts in the maximum emission wavelength nor significant changes in fluorescence emission intensity could be observed. Thus, binding to the IgG could not be detected by this method, and it does not appear that the CDs inhibit aggregation by binding to the native IgG and increasing stability of the native state or by blocking hydrophobic patches on the protein's surface.

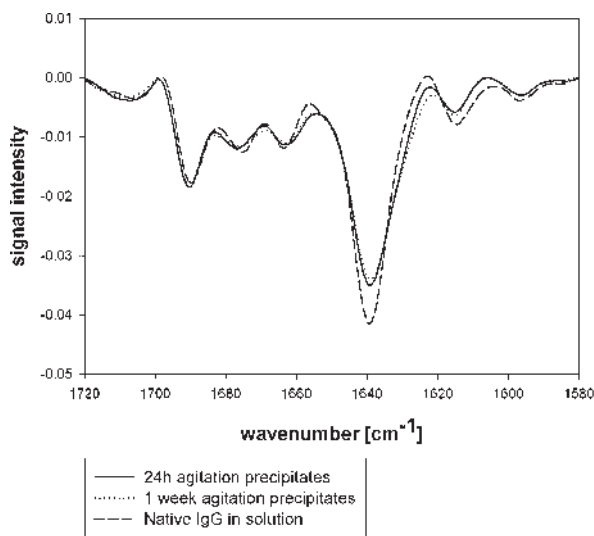


Figure 6. Second-derivative IR-spectra of IgG in precipitates formed after 24 h and 1 week of agitation in comparison to the spectrum for the native protein in solution.

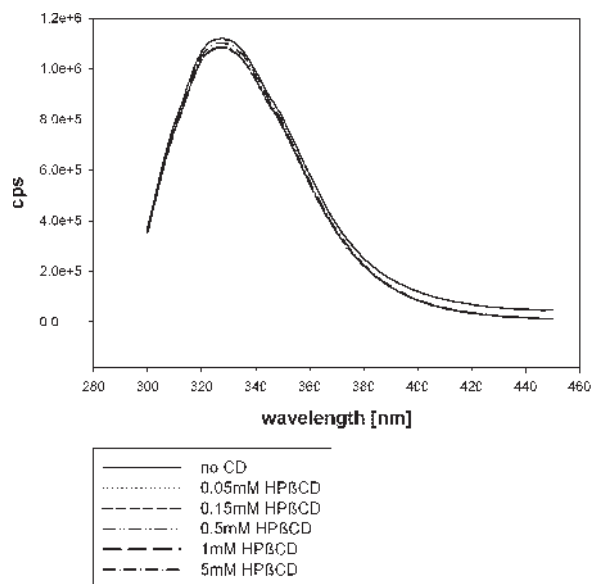


Figure 7. Titration of a 100 mM HP β CD stock-solution into a 0.24 mg/mL solution of the IgG and monitoring of intrinsic steady-state fluorescence.

Effect of CDs on Apparent Melting Temperature of IgG

It is reported that the reduction of agitation-induced aggregation by nonionic surfactants can also be due to an increase in the thermodynamic stability of a protein.⁴⁴ In order to find out whether this behavior might play a role for the stabilization of the IgG by CDs, the apparent

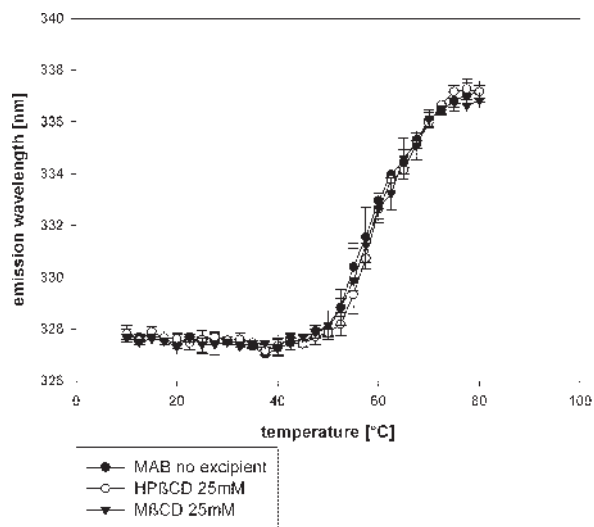


Figure 8. Shift of the IgG maximum emission wavelength during temperature ramping. Comparison between formulations containing HP β CD, M β CD, and the IgG in the presence of buffer only.

Table 1. Experimental Values for Melting Temperatures of Formulations Containing CDs, Tween80, Sugars, and a Reference Formulation

Formulation	Apparent T_m ($^{\circ}\text{C}$)
His 20 mM no excipient	75.64 ± 0.12
HP β CD 2.5 mM	75.73 ± 0.07
M β CD 2.5 mM	75.69 ± 0.15
Tween80 0.004%	75.62 ± 0.16
Tween80 0.04%	75.64 ± 0.08
Sucrose 250 mM	76.54 ± 0.10
Trehalose 250 mM	76.86 ± 0.15

melting temperature of the mAb was measured in presence and absence of CDs.

First, fluorescence spectroscopy was employed to record melting curves. The resulting curves show a good overlay between the two formulations containing 25 mM CD and the formulation containing IgG in buffer alone (Fig. 8). Thus, the apparent melting temperature as determined by this method (monitoring of tertiary structural transitions) remains unaffected even in the presence of high concentrations of HP β CD.

Microcalorimetric measurements were also carried out (Tab. 1). The apparent melting temperature by microcalorimetry for the IgG in buffer alone is nearly identical to those for the protein in samples with CDs. Thus, it appears that CDs have no measurable effect on the thermodynamic stability of the IgG. Together with the findings shown in Figure 8 it can be concluded that a change in thermodynamic stability is not an explanation for the aggregation inhibition of the mAb in the presence of CDs. These results can be interpreted as a further indication that no binding between CDs and the IgG occurs which is in contrast to some previous publications in which T_m is reported to decrease due to a preferential binding of CDs to the partially unfolded state of the protein and a subsequent shift of the folding equilibrium.³⁵

Interestingly, the apparent T_m is increased by about 1°C in the presence of the sugars and sugar alcohols that were used for the agitation studies earlier. However, this increase in thermodynamic stability does not correspond to increased stability against aggregation at the air–water interface. In contrast, agitation-induced aggregation is even more pronounced in the presence of these sugars and sugar alcohols (Fig. 4). Thus, thermodynamic stability of the IgG in the bulk solution probably plays a minor role for the explanation of the aggregation behavior at interfaces.

Competition of IgG and CDs for Adsorption to the Air–Water Interface

Surface tension measurements were carried out to evaluate the behavior of CDs at the air–water interface in absence and presence of the mAb. In addition, a comparison to polysorbate 80 was made. As described in the Materials and Methods section, the surface tension recordings were only conducted for 240 s which is far shorter than the time that is necessary for establishing an equilibrium condition between the mAb and other components at the interface (>12 h, data not shown). However, since it is intended to relate the surface tension results to the results of agitation studies in which constant renewal of the interface takes place, it seems that short measurement times better reflect the situation of the aggregation-experiment than equilibrium data. The 240 s interval was chosen because it offered the shortest period of time in which it was possible to achieve acceptably reproducible results in solutions containing the IgG alone. The surface tension of solutions without mAb containing either polysorbate 80 or HP β CD more quickly reached constant values, and the obtained values at 240 s can be considered close to the equilibrium value.

HP β CD lowers the surface tension and thus it accumulates at the interface (Tab. 2). From the results it can be concluded that saturation of the interface with HP β CD is reached at a concentration of about 2.5 mM. This concentration is the same as the minimum needed for complete protection of the mAb against agitation-induced aggregation.

As expected, the surface tension measurements also show that the mAb accumulates at the air–water interface as well (reflected in the decrease of the surface tension by 11.47 mN/m). Since both

HP β CD and the IgG concentrate at the air–water interface, competition between the two molecules at the interface is likely to occur. This finding is further substantiated by the fact that the surface tension of the solutions containing both the IgG and HP β CD (at ≥ 2.5 mM, i.e., after saturation of the interface) is only slightly lower (≤ 3.83 mN/m) than the surface tension of the solutions containing either one of the single components. If both components concentrated at the interface in the same manner as in absence of the respective other component the decrease of the surface tension would be more pronounced. It can thus be concluded that competition at the air–water interface is likely the dominating reason for the stabilization of the IgG against agitation-induced aggregation by CDs. This conclusion is also in agreement with a study conducted by Tavornvivas et al.³² on agitation-induced aggregation of rhGH. This study identified HP β CD and 2,6-di-*O*-methyl- β -CD to be superior to other CDs for the inhibition of aggregation. This finding was attributed to the relatively high surface activity of those two CD-derivatives, which could result in a competition between the rhGH molecules and the CDs at the air–water interface.

Interestingly, polysorbate 80 even at the lowest concentration tested caused a more pronounced decrease of surface tension than HP β CD did even in its highest concentration. However, as Figures 4 and 5 demonstrated this lower concentration of polysorbate 80 was not sufficient to completely stabilize the IgG against agitation-induced aggregation. Thus, the extent of surface tension decrease alone cannot be used for prediction of the stability of a formulation against aggregation at the air–water interface. The surface tension increment alone might not directly correlate with inhibition of protein adsorption to the air–water interface, especially when taking into account the time-scale (very short) at which the air–water interface is renewed during agitation. Presumably, mass-transfer limitations due to the relatively low polysorbate 80 concentration compared to that of HP β CD could result in slower equilibration kinetics of polysorbate 80 versus HP β CD at newly formed air–water interfaces. A different interpretation could be that other factors play a role in aggregation–inhibition. Perhaps CDs exert further stabilizing effects besides competition at the interface, which seems unlikely taking into account the data from the biophysical investigations presented above. Alternatively, polysorbate 80 might promote protein aggregation

Table 2. Surface Tension of Different mAb-Formulations as Determined by the Wilhelmy-Plate-Method

Formulation	σ (mN/m) Buffer	σ (mN/m) With mAb
His 20 mM no excipient	73.00 $\pm$ 0.510	61.53 $\pm$ 0.19
HP β CD 0.25 mM	66.51 $\pm$ 0.46	62.25 $\pm$ 0.42
HP β CD 2.5 mM	61.74 $\pm$ 0.29	59.15 $\pm$ 0.72
HP β CD 25 mM	59.32 $\pm$ 0.34	57.70 $\pm$ 0.72
Tween 0.04%	37.42 $\pm$ 0.44	38.76 $\pm$ 0.14
Tween 0.004%	43.96 $\pm$ 0.86	43.35 $\pm$ 0.18
Sucrose 250 mM	70.75 $\pm$ 0.26	62.83 $\pm$ 0.36

independent of its effects on the air–water interface.

The overall effects of excipients on protein aggregation during agitation can be related to a theory for how these compounds influence protein interactions with and their stability at the air–water interface. Because sugars like sucrose are excluded from the protein–water interface they can be expected to increase the surface tension σ_{pw} at the protein–water interface.^{52–54} The surface tension at the air–water-interface (σ_{aw}) also increases with increasing concentrations of the low-molecular-weight sugars and sugar alcohols. In contrast, the interfacial tension at the air–protein interface (σ_{ap}) is expected to be independent of the concentration of excipients in the bulk liquid.⁵⁵ As described in more detail by Mahler et al.⁵⁵ when taking into account the relation between the contact angle, the adhesion energy of the protein per unit area of the protein–air interface and the free energy of protein unfolding on the air–water interface ($\Delta G_{N \rightarrow U}$) it can be concluded that the $\Delta G_{N \rightarrow U}$ will decrease with increasing sucrose concentrations. As a consequence the protein, although more stable in the bulk due to preferential exclusion, will become less stable upon adsorption to the air–water interface. On the other hand, this relation means that the excipients that lower the surface tension at both the air–water interface and the protein–water interface (decreasing both σ_{aw} and σ_{pw} and thereby increasing the contact angle) result in less spreading and a more stable protein at the surface.⁵⁵ However, nonionic surfactants and cyclodextrins, such as M β CD (but not HP β CD) in this case, may at the same time accelerate aggregation rates in the bulk.

In general a compromise has to be made in protein formulation: excluded excipients such as sucrose, trehalose, sorbitol, and mannitol which were employed for this work are expected to stabilize the protein in the bulk, but clearly destabilize it at interfaces, whereas surfactants such as polysorbate 80 and some cyclodextrins may destabilize the protein in the bulk. For HP β CD stabilization at the interface could be achieved but thermodynamic stability in the bulk was not affected.

CONCLUSIONS

To our knowledge for the first time it has been demonstrated that HP β CD is well suited to inhibit

aggregation of an IgG monoclonal antibody at the air–water interface. Remarkably low (≥ 2.5 mM) and thus presumably nontoxic concentrations of HP β CD were satisfactory for complete inhibition of IgG-aggregation at the air–water interface. Low-molecular-weight sugars and sugar alcohols (sucrose, trehalose, mannitol, and sorbitol) turned out to even promote agitation-induced aggregation and thus show a completely different behavior than the oligosaccharide HP β CD. In a rather high concentration of 0.04% polysorbate 80 provided the same, complete degree of stabilization. However, when employed in a lower concentration of 0.004% the formation of soluble aggregates was observed.

Spectroscopic investigations and surface tension measurements suggest that competition between the IgG and HP β CD at the air–water interface is the dominating mechanism of aggregation protection during these agitation studies rather than direct binding between the mAb and HP β CD.

Since HP β CD is not associated with a number of disadvantages of polysorbates (formation of micelles, generation of peroxides during storage) it poses a valuable alternative to polysorbates in mAb-formulation. We therefore suggest that they should be routinely included in formulation studies during the development of liquid IgG-formulations. Of course, it has to be acknowledged that long-term studies with a greater number of different mAbs and a more thorough investigation of the effects on chemical stability will be necessary in order to draw general conclusions on the applicability of HP β CD for the formulation of mAbs.

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