Señor
SUPERINTENDENTE DE INDUSTRIA Y COMERCIO
DIVISIÓN DE NUEVAS CREACIONES
E. S. D.

Trámite: 11
Evento: 378
Actuación: 444

Re: PI.-**MITSUBISHI TANABE PHARMA CORPORATION, NATIONAL UNIVERSITY CORPORATION CHIBA UNIVERSITY, OSAKA UNIVERSITY.**-Solicitud de Patente de Invención en Fase Nacional PCT para **"PROTEÍNA DE UNIÓN A RGMA"**.-Clase **C07K 16/28**.-Expediente número **NC2017/0011975.**

1. Me refiero al auto dictado por ese Despacho notificado por fijación en línea del 8 de julio del 2019.

2. Con el fin de responder las objeciones expuestas por la Señora Examinadora en su Concepto Técnico correspondiente al Oficio N° **5851** en relación con la claridad y concisión del Capítulo Reivindicatorio, y el nivel inventivo de la presente invención, me permito dirigir su atención a las siguientes modificaciones y argumentos técnicos por medio de los cuales no sólo se superan completamente dichas objeciones sino que además se establece de manera incontrovertible la patentabilidad de la solicitud en estudio.

3. CAPÍTULO REIVINDICATORIO.

3.1. Modificaciones al Capítulo Reivindicatorio.

En primer lugar, me permito manifestar que es deseo del solicitante presentar un **capítulo reivindicatorio modificado**, que remplaza al actualmente presentado, con el propósito de superar las objeciones presentadas como parte del concepto técnico realizado por ese Despacho.

En conexión con lo anterior, me permito resaltar que dicho capítulo no constituye de ninguna manera una ampliación al alcance de la solicitud, tal como fue originalmente presentada, toda vez que define claramente el objeto de la presente invención de acuerdo con las enseñanzas de la descripción. Las modificaciones realizadas se exponen a continuación:

- Se limitó el alcance de la **reivindicación 1** actualmente presentada, eliminando las referencias a las secuencias SEQ ID Nos:36 a 40 de la reivindicación.

- Se eliminó la expresión "*y un derivado del mismo*" de la **reivindicación 6** actualmente presentada.

- Se reemplazó en la **reivindicación 8** actualmente presentada el término "*recuperar*" por "*recolectar*" a fin de dar más claridad a la materia reclamada y estar más acorde con el contexto del objeto de dicha reivindicación.

De esta forma, **el capítulo reivindicatorio modificado queda constituido por 9 reivindicaciones,** las cuales se refieren de manera más clara y concisa a anticuerpos anti-RGMa, moléculas que codifican para los mismos, y métodos para producirlos, que es objeto de la presente solicitud, y cuenta con pleno sustento en las enseñanzas de la descripción.

3.2. Claridad y Concisión del Capítulo Reivindicatorio.

Ahora bien, con respecto a la claridad y la concisión del capítulo reivindicatorio, la examinadora afirma que *"La reivindicación 6 no es clara, porque la expresión "un derivado del mismo" al hacer referencia a un vector recombinante no define adecuadamente el tipo de vector. Se sugiere eliminar dicha expresión o hacer las aclaraciones pertinentes"*

Con respecto a lo anterior, por favor note que la expresión objetada ha sido eliminada de la reivindicación 6, por lo que esta objeción queda completamente superada.

Así las cosas, me permito señalar que el capítulo reivindicatorio modificado presentado junto con el presente memorial supera completamente las objeciones presentadas por la Señora Examinadora. Asimismo, el capítulo reivindicatorio modificado cuenta con la suficiente claridad, concisión y sustento para definir la materia a reclamar y, por ende, cumple a cabalidad con lo estipulado por el Artículo 30 de la Decisión Andina 486 de 2000.

4. NIVEL INVENTIVO

Finalmente, la examinadora objeta el nivel inventivo de las reivindicaciones 1 a 9 a la luz de las enseñanzas de los siguientes documentos:

- D1: US2010/0028340. "*Antibodies against the RGMa protein and uses thereof*"

- D2: US2009/297527. "*Binding domains of proteins of the repulsive guidance molecule (RGM) Protein family and functional Fragments thereof, and their use*"

En opinión de la examinadora, el documento D1 es el estado de la técnica más cercano a la invención definida en la reivindicación 1 porque revela un anticuerpo anti-RGMa (anticuerpo 5F9) capaz de unirse a RMGa y modular la unión entre RGMa y neogenina. Dicho documento también revela que se pueden obtener diferentes fragmentos de unión o anticuerpos con especificidad de unión por diferentes métodos, por ejemplo, hibridomas, que pueden implicar la modificación de algunos residuos de CDR para mantener la afinidad del anticuerpo.

En este sentido, la examinadora establece que la diferencia entre la invención definida en la reivindicación 1 y los anticuerpos de D1 consiste en las CDR de cadena pesada y cadena ligera, las cuales se unen específicamente al extremo C-terminal del RGMa humano (posiciones de aminoácidos 298 a 377).

Sin embargo, la examinadora señala que el documento D2 revela diferentes fragmentos que se unen a RGMa y neogenina, en donde dichos fragmentos incluyen péptidos que se unen a diferentes regiones de RGMA, incluidas las posiciones entre los aminoácidos 266-355, 316-387, 168-422, entre otros.

En consecuencia, y acorde con lo manifestado por la examinadora, la persona versada en la materia estaría motivada a desarrollar fragmentos de unión capaces de neutralizar la actividad inhibidora del crecimiento neurítico de RGMa sin afectar la unión entre RGMa y neogenina en base a las secuencias CDR de D1, en donde modificaría dichas secuencias de CDR utilizando técnicas bioinformáticas y considerando las enseñanzas del documento D2, con el fin de llegar a las enseñanzas de la presente solicitud.

Con respecto a lo anterior, me permito aclarar que la persona versada en la materia no encontraría suficiente motivación en las enseñanzas de D1 y D2 para llegar al objeto de la presente solicitud, en base a las diferencias técnicas entre la invención y el estado de la técnica, más aún, considerando el sorprendente efecto asociado al anticuerpo reivindicado.

Diferencias entre la invención y los documentos D1 y D2

A este respecto, me permito recordar que la presente invención se refiere al anticuerpo monoclonal anti-RGMa que comprende secuencias específicas de aminoácidos para las CDR, y particularmente, se refiere al anticuerpo 116A3.

Ahora, con respecto a D1, me permito resaltar que los anticuerpos 5F9 y 8D1, revelados por dicho documento, comprenden CDR con secuencias **completamente diferentes** de las CDR descritas en la reivindicación 1. Adicional a lo anterior, el anticuerpo 5F9 reconoce la cadena ligera de RGMa, ubicada entre las posiciones de

aminoácidos 47 y 168, la cual difiere completamente del epítope reconocido por el anticuerpo de la invención, el cual se une entre las posiciones de aminoácidos 298 y 337 de la proteína RGMa.

Por otro lado, y después de analizar el contenido de D2, es posible corroborar que dicho documento enseña diferentes fragmentos peptídicos de RGM que pueden ser epítopes para anticuerpos anti-RGMa, de los cuales el péptido 1 (referido a los aminoácidos 267-285) muestra una actividad inhibidora confiable y reproducible, siendo así la realización preferida de dicho documento. De este modo, atentamente me permito resaltar que dicho péptido **no es el epítope reconocido por el anticuerpo de la invención**.

Además, me permito señalar que el péptido 6-AK (aminoácidos 300-316), mencionado por la examinadora por tener similitudes con la secuencia de aminoácidos del epítope reconocido por el anticuerpo de la invención (aminoácidos 290-350), **es débilmente activo** en comparación con el péptido 1 (D2; Figuras 6A y 6B; Página 24, Párr. [0293]).

Por lo tanto, y contrario a las afirmaciones de la examinadora en su concepto técnico, D2 enseña que los dominios inhibitorios de RGM A están particularmente ubicados entre las posiciones 260-291, más preferiblemente en las posiciones 267-285.

A su vez, y con respecto a las posiciones 290-350, dicho documento aclara que "*sobre la base de la actividad de los dos péptidos 6-Ak y 7-Ak, que mostraron una tendencia a inhibir el crecimiento a la concentración de péptido más alta (Figura 6B), es perfectamente concebible que RGM A tenga más dominios inhibitorios*" (énfasis añadido), lo que significa que la concentración de dichos péptidos debe ser mayor si se quiere lograr un efecto. Por lo tanto, esta afirmación desalentaría la producción de anticuerpos con afinidad a dichos péptidos porque se requiere una alta concentración de dichos péptidos para obtener el efecto deseado, en comparación con los anticuerpos dirigidos al péptido 1, como lo enseña dicho documento.

En consecuencia, en caso de que la persona versada en la materia decidiera combinar las enseñanzas de D1 y D2, habría encontrado la motivación para producir un anticuerpo anti-RGMa con afinidad a un epítope en la posición del péptido 1 en lugar de al péptido 6-AK.

En consecuencia, el anticuerpo monoclonal anti-RGMa de la presente invención, que se une a un epítope particular y tiene secuencias específicas para las CDR, no podría ser obtenido fácilmente incluso por una persona versada en la técnica, basado en las divulgaciones de D1 y D2.

<u>Efecto técnico alcanzado por los anticuerpos de la presente solicitud</u>

Adicional a lo anterior, me permito traer a colación el hecho de que **<u>el anticuerpo de la presente invención exhibe efectos técnicos sorprendentes e inesperados</u>** como se muestra a continuación.

Como se aprecia en el ejemplo 8 de la presente solicitud, el anticuerpo R116A3 se une a la proteína RGMa humana **con una afinidad 32 veces más fuerte** que 5F9 (revelado en D1) y a la proteína RGMa de ratón 44 veces más fuerte que 5F9. Además, y como se mencionó anteriormente, el anticuerpo R116A3 se une al dominio C-terminal de la proteína RGMa humana, a diferencia de 5F9.

Asimismo, me permito señalar que el anticuerpo 116A3 **exhibe una estabilidad térmica significativa** cuando se compara con otros anticuerpos en la técnica anterior, por ejemplo, el anticuerpo 5F9. En este sentido, la figura 3 de la presente solicitud muestra que la temperatura de fusión (Tm, temperatura en la cual se degrada el 50% del anticuerpo) del anticuerpo 116A3 es de aproximadamente 72 °C, mientras que la Tm del anticuerpo 5F9 es de aproximadamente 67 °C.

En consecuencia, y a pesar de que no hay una diferencia significativa en el comportamiento de los péptidos en el rango de 40 a 65 °C, como lo señala la examinadora, el solicitante se refiere particularmente a la estabilidad térmica evaluada en términos de los valores de Tm que, como se señaló anteriormente, <u>son significativamente diferentes</u>.

En este sentido, me permito referirme al artículo de Goldberg *et al*. (adjunto al presente memorial), el cual enseña una correlación entre el valor de Tm y la agregación del anticuerpo, y concluye, con base en la Figura 5 de dicho artículo, que "*La comparación de Tm en cada pH con propensión a la agregación indica que <u>un aumento en Tm conduce a una disminución en tasa de agregación</u>, lo que sugiere el importante papel de la estabilidad conformacional en el mantenimiento de mAbB estables en condiciones de almacenamiento a largo plazo*" (Página 1313, columna izquierda. Énfasis añadido).

Por lo tanto, y considerando que la Tm de 116A3 es mayor que la Tm de 5F9, el anticuerpo de la invención tenderá a agregarse en menor proporción y, por lo tanto, exhibirá mejores propiedades en el almacenamiento a largo plazo.

En base a todo lo anterior, la persona versada en la materia no estaría motivada a modificar el anticuerpo revelado en D1 basándose en las enseñanzas de D2 para llegar a la materia objeto de la presente solicitud con el sorprendente efecto asociado a la misma. Así las cosas, la presente solicitud indudablemente tiene nivel inventivo.

A la luz de todo lo anterior, me permito entonces señalar que el capítulo reivindicatorio modificado es claramente inventivo a la luz del estado de la técnica. Por lo tanto, cumple a cabalidad con los requisitos del Artículo 18 de la Decisión 486 de la Comunidad Andina.

5. CONCLUSIÓN.

Para finalizar, me permito reiterar que el capítulo reivindicatorio modificado presentado junto con este memorial es totalmente claro y conciso al reclamar anticuerpos anti-RGMa, que están plenamente soportados dentro del texto de la descripción, cumpliendo así con lo exigido por la mencionada Decisión Andina 486 de 2000.

Por otro lado, en virtud de los argumentos discutidos con respecto a los documentos citados, se estableció de manera incontrovertible que los anticuerpos anti-RGMa, que son objeto de la solicitud en estudio, son novedosos y de ninguna manera resultarían evidentes para la persona versada en la materia a partir de las enseñanzas de los documentos referenciados, demostrando irrefutablemente la patentabilidad de la presente solicitud.

6. Anexo al presente memorial, me permito presentar:

- Copia de las páginas **56 y 57** que corresponden al **capítulo reivindicatorio modificado**, tal como se discutió en el presente memorial, constituido por **9 reivindicaciones.**

- Anexo: Copia del artículo "*Formulation Development of Therapeutic Monoclonal Antibodies Using High-Throughput Fluorescence and Static Light Scattering Techniques: Role of Conformational and Colloidal Stability*" Journal of Pharmaceutical Sciences, Vol. 100, No. 4, April 2011.

7. En vista de lo anterior, ruego a usted proceder a estudiar nuevamente la solicitud a la luz de las modificaciones y los argumentos presentados y conceder el privilegio de patente solicitado.

Señor Superintendente,

ALICIA LLOREDA RICAURTE

T.P. 53.215

Direcciones para Notificación: **notificacionesip@lloredacamacho.com**
notificacionespatentes@lloredacamacho.com

REIVINDICACIONES

1. Un anticuerpo anti-RGMa aislado, o un fragmento de unión a antígeno del mismo, en donde la secuencia de aminoácidos de cada una de la región determinante de complementariedad de la cadena ligera 1 (LCDR1), la región determinación de la complementariedad de la cadena ligera 2 (LCDR2), la región determinante de la complementariedad de la cadena ligera 3 (LCDR3), la región determinante de la complementariedad de la cadena pesada 1 (HCDR1), la región determinante de la complementariedad de la cadena pesada 2 (HCDR2) y la región determinante de la complementariedad de la cadena pesada 3 (HCDR3) comprenden lo siguiente: LCDR1: RASQDISSYLN (SEQ ID NO: 30), LCDR2: YTSRLHS (SEQ ID NO: 31), LCDR3: QQLNTLP (SEQ ID NO: 32), HCDR1: DAWMD (SEQ ID NO: 33), HCDR2: EIRSKANNHATYYAESVKG (SEQ ID NO: 34) y HCDR3: RDGAY (SEQ ID NO: 35).

2. El anticuerpo anti-RGMa o un fragmento de unión a antígeno del mismo de conformidad con la reivindicación 1, caracterizado además porque la región variable de cadena pesada (VH) comprende lo siguiente:
VH:
EVQLVESGGGLVQPGRSLRLSCTASGFTFSDAWMDWVRQAPGKGLEWVAEIR SKANNHATYYAESVKGRFTISRDDSKSIVYLQMNSLRTEDTALYYCTRRDGAYW GKGTTVTVSS (SEQ ID NO: 41); y
en el que la región variable de cadena ligera (VL) comprende lo siguiente:
VL:
DIQMTQSPSSVSASVGDRVTITCRASQDISSYLNWYQQKPGKAPKLLIYYTSRLH SGVPSRFSGSGSGTDFTLTISSLQPEDFASYFCQQLNTLPWTFGGGTKVEME (SEQ ID NO: 42).

3. El anticuerpo anti-RGMa o un fragmento de unión a antígeno del mismo de conformidad con la reivindicación 1 ó 2, caracterizado además porque el anticuerpo anti-RGMa es un anticuerpo humano, un anticuerpo humanizado o un anticuerpo quimérico.

4. El anticuerpo anti-RGMa o fragmento de unión a antígeno del mismo de conformidad con cualquiera de las reivindicaciones 1 a 3, caracterizado además porque el anticuerpo anti-RGMa comprende regiones constantes de IgG1, IgG2, IgG3 o IgG4 humano.

5. Una molécula de ácido nucleico que codifica para la porción de proteína del anticuerpo anti-RGMa o un fragmento de unión a antígeno de la misma de cualquiera de las reivindicaciones 1 a 4, caracterizada además porque las secuencias de nucleótidos que codifican para las secuencias de aminoácidos VH y VL cada una son una secuencia de nucleótidos que comprende:
VH:
gaagtgcagctggtggaatctggcggcggactggtgcagcctggcagatccctgagactgtcctgtaccgcctcc
ggcttcaccttctccgacgcctggatggattgggtgcgacaggctcctggcaagggcctggaatgggtggccgag
atccggtccaaggccaacaaccacgccacctactacgccgagtctgtgaagggccggttcaccatctcccggga
cgactccaagtccatcgtgtacctgcagatgaactccctgcggaccgaggacaccgccctgtactactgcaccag
aagggacggcgcctactggggcaagggcaccacagtgacagtgtcctcc (SEQ ID NO: 43) y
VL:
gacatccagatgacccagtccccctcctccgtgtctgcttccgtgggcgacagagtgaccatcacctgtcgggcct
cccaggacatctcctcctacctgaactggtatcagcagaagcccggcaaggcccccaagctgctgatctactaca
cctcccggctgcactccggcgtgccctctagattttccggctctggctccggcaccgactttaccctgaccatctcca
gcctgcagcccgaggacttcgcctcctacttctgtcagcagctgaacaccctgccctggacctttggcggaggcac
caaggtggaaatggaa (SEQ ID NO: 44).

6. Un vector recombinante que comprende la molécula de ácido nucleico de la reivindicación 5, caracterizado porque el vector recombinante se selecciona del grupo que consiste en pcDNA 3.1, pConPlus, pcDM8, pcDNA I/Amp, pcDNA 3.1, pREP4, y pDON-AI DNA.

7. Una célula hospedera que contiene el vector recombinante de la reivindicación 6.

8. Un método para producir el anticuerpo anti-RGMa o un fragmento de unión a antígeno del mismo de cualquiera de las reivindicaciones 1 a 4, en donde el método comprende:

(i) un paso de cultivar la célula hospedera de la reivindicación 7;
(ii) un paso de recolectar el sobrenadante del cultivo obtenido en (i); y
(iii) un paso de purificar el anticuerpo a partir del sobrenadante del cultivo obtenido en (ii).

9. Una composición farmacéutica que comprende el anticuerpo anti-RGMa o un fragmento de unión a antígeno del mismo de cualquiera de las reivindicaciones 1 a 4.

Formulation Development of Therapeutic Monoclonal Antibodies Using High-Throughput Fluorescence and Static Light Scattering Techniques: Role of Conformational and Colloidal Stability

DEBORAH S. GOLDBERG†, STEVEN M. BISHOP, AMBARISH U. SHAH, HASIGE A SATHISH

Department of Formulation Sciences, MedImmune, Gaithersburg, Maryland 20874

Received 9 July 2010; revised 13 September 2010; accepted 16 September 2010

Published online 19 October 2010 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.22371

ABSTRACT: In this work, we describe the application of two different high-throughput screening (HTS) techniques that can be used to determine protein stability during early formulation development. Differential scanning fluorescence (DSF) and differential static light scattering (DSLS) are used to determine the conformational and colloidal stability of therapeutic monoclonal antibodies (mAbs) during thermal denaturation in a high-throughput fashion. DSF utilizes SYPRO® Orange, a polarity-sensitive extrinsic fluorescent probe, to monitor protein unfolding. We found that melting temperatures determined by DSF have a linear correlation with melting temperatures of the first domain unfolding determined by differential scanning calorimetry, establishing DSF as a reliable method for measuring thermal stability. The DSLS method employs static light scattering to evaluate protein stability during thermal denaturation in a 384-well format. Overall comparison between mAb aggregation under typical accelerated stress conditions (40°C) and the thermal stability obtained by DSF and DSLS is also presented. Both of these HTS methods are cost effective with high-throughput capability and can be implemented in any laboratory. Combined with other emerging HTS techniques, DSF and DSLS could be powerful tools for mAb formulation optimization. © 2010 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 100:1306–1315, 2011

Keywords: monoclonal antibody; formulation; fluorescence spectroscopy; light scattering; calorimetry (DSC); high-throughput screening stability; conformational stability; colloidal stability; protein aggregation

INTRODUCTION

A primary goal in formulation of therapeutic antibodies is to achieve acceptable protein stability during processing and storage. In general, proteins are only marginally stable in solution and are susceptible to several modes of degradation, including aggregation, fragmentation, and chemical modification. Most of these degradation pathways can be controlled or minimized by optimizing solution conditions such as pH, ionic strength, and added excipients.[1–5] Optimization of each of these parameters is often complicated due to the interactions between them. Traditionally, the optimum conditions are determined by long-term stability studies, which involve incubating proteins in various solution conditions under several stresses. This excipient screening strategy is time consuming and requires large amounts of protein. Therefore, it is of great interest to find high-throughput techniques for formulation screening that would allow for more conditions to be tested in a short amount of time, resulting in optimized stability.[6–8]

Protein aggregation is often a significant degradation pathway for monoclonal antibodies (mAbs).[9] Aggregation may be controlled by conformational and/or colloidal stability, and either could play a role in the rate and extent of aggregation, depending on the solution conditions and surface properties.[10–12] The conformational stability of a mAb, or its ability to maintain its native, folded state, can be impacted by pH, ionic strength, added excipients, and protein concentration.[13] Protein aggregation models suggest that aggregation is initiated when hydrophobic residues from partially unfolded proteins come

Corresponding to: Ambarish U. Shah (E-mail: shaha@medimmune.com); Hasige A. Sathish (E-mail: sathishh@medimmune.com)

†Deborah S. Goldberg's present address is Fischell Department of Bioengineering, University of Maryland, College Park, Maryland.

Journal of Pharmaceutical Sciences, Vol. 100, 1306–1315 (2011)

together and form dimers, which serve as nucleation sites for further aggregation.[9] Therefore, increasing the conformational stability of a protein could shift the equilibrium toward native state and thus lower the extent of protein aggregation.

Conformational stability is traditionally measured by differential scanning calorimetry (DSC), circular dichroism spectroscopy, intrinsic tryptophan fluorescence, and Raman spectroscopy. Unfortunately, all of these techniques have relatively low throughput, and cannot be used for screening large numbers of conditions. Therefore, it is desirable to find a high-throughput method to screen for solution conditions that promote conformational stability.

There have been several reports on the use of fluorescent dyes such as SYPRO® Orange (Invitrogen, Carlsbad, California) and anilinonaphthalene-8-sulfonate as external fluorescent probes to monitor protein unfolding.[14–16] In these reports, hydrophobically sensitive dyes are used to track the thermal unfolding of proteins subjected to increasing temperature. From the thermal unfolding curve, the midpoint of unfolding transition (T_m) can be obtained and used to determine whether the protein is stabilized (increased T_m) or destabilized (decreased T_m) relative to a reference condition. This technique of differential scanning fluorescence (DSF) has been used to investigate ligand binding in globular proteins[17,18] and protein stability in different excipient conditions.[19] More recently, it has been applied to the formulation of mAbs.[20] Importantly, this technique is amenable to a high-throughput setup and can be run in small volumes using a polymerase chain reaction (PCR) instrument as a thermocycler.[12,13]

Light scattering has been used to measure the colloidal stability of protein solutions.[21] Both native and nonnative aggregation is the result of assembly of protein molecules into higher order aggregates, which possess an enhanced ability to scatter incident light. By measuring scattered light, one can detect protein aggregation in different solution conditions. Recently, this technique has been employed during rapid high-throughput identification of small molecules that interact with protein targets.[18,22] This method measures the colloidal stability associated with thermal denaturation in a 384-well plate format, allowing for simultaneous screening of the impact of different buffers, pH conditions, and excipient permutations on protein aggregation.

In this work, we investigated the use of high-throughput DSF and DSLS in determining the conformational and colloidal stability of four mAbs in different solution conditions. We compared the melt temperatures obtained by DSF with melt temperatures determined by DSC, an established method of measuring conformational stability. In particular, we investigated the ability of DSF to predict conformational stability of proteins in the presence of different concentrations and combinations of excipients. We found that for all conditions and mAbs tested, there was a linear correlation between DSF and DSC melt temperatures. We also examined the effect of different excipient conditions on the colloidal stability of mAbs using DSLS. We found that the amount of time lag before the onset of aggregation as measured by DSLS was a reliable means of determining stabilizing and destabilizing excipient effects. Finally, we compared stability under accelerated conditions (40°C, 75% relative humidity or RH) for several mAbs with the DSF and DSLS data to determine correlations between either DSF or DSLS and long-term stability. The findings from this study demonstrate that although mAbs have a high degree of sequence similarity, each mAb has unique physicochemical properties, which determine the relative importance of conformational and colloidal stability in overall protein stability. Therefore, screening excipients/conditions based on their ability to increase both conformational stability and colloidal stability are required to improve overall protein stability. The two techniques evaluated here have the potential to measure conformational and colloidal stability in a high-throughput fashion, allowing for testing of multiple conditions in a short amount of time, hence serving as valuable tools for rational formulation design in a rapid manner.

MATERIALS AND METHODS

Monoclonal Antibodies

mAbs were obtained from bulk drug lots that were manufactured at MedImmune, Gaithersburg, MD, USA. The sample purity (>97%) was analyzed and confirmed by size exclusion chromatography and gel electrophoresis. Protein concentrations were determined by ultraviolet (UV) absorption at 280 nm and 20°C using an Agilent 8453 UV-visible (Vis) spectrophotometer (Agilent Technologies, Palo Alto, California) fitted with a Peltier temperature controller. The mAbs used in the study include immunoglobulin G (IgG)1 (mAbA, mAbB, and mAbC) and IgG2 (mAbD) subtypes.

Reagents

SYPRO® Orange was obtained from Invitrogen. Sodium chloride, glycine, lysine, arginine, sucrose, trehalose, and histidine were obtained from J.T. Baker, Phillipsburg, NJ, USA. All excipients and buffer salts were of analytical grade. High-performance liquid chromatography (HPLC) grade water was used for preparation of all buffers and excipient stocks.

Differential Scanning Fluorescence

Buffer conditions were achieved via extensive dialysis of concentrated protein stocks. The dialyses consisted of a minimum of two buffer exchanges for more than 24 h at a volume ratio of buffer to sample greater than 200:1. The final dialysate was reserved and used for all dilutions, blanks, and controls as needed. Samples in relevant excipient conditions were prepared at a final protein concentration of 0.5 mg/mL by spiking in stock excipients that were prepared in relevant buffer. The buffer conditions that were used for each mAb are described in Table 1. SYPRO® Orange was added immediately before the experiment to achieve a final dilution of 1000× relative to the initial stock concentration. The addition of dye in itself did not induce any changes in the thermal stability (as determined by both the intrinsic fluorescence and DSC). Twenty-five microliters of prepared samples was added in duplicate to white-walled PCR plates. A Chromo4 Real Time PCR Detector (Bio-Rad, Hercules, California) was used as a thermal cycler, and the fluorescence emission was detected using the software's custom dye calibration routine. The PCR plate containing the test samples was subjected to a temperature ramp from 20°C to 90°C in increments of 0.2°C with 10 s pauses after each temperature increment. The total assay time was approximately 3 h. The T_m was calculated by the software using a mathematical second derivative method to calculate the inflection point of the curve. The reported T_m is an average of two measurements.

Differential Scanning Calorimetry

Differential scanning calorimetry studies were performed using a VP capillary DSC system (MicroCal, Piscataway, New Jersey). The test samples were prepared in the buffer alone and in various excipient(s) of interest at a final protein concentration of 0.5 mg/mL. Change in the heat capacity (Cp) was measured as the samples were heated from 20°C to 90°C at a rate of 90°C/h. For each sample, a relevant buffer blank containing the same buffer and excipient concentration was used as a reference. Normalized Cp data were corrected for buffer baseline. Data analysis was preformed using OriginLab software (OriginLab Corporation, Northampton, Massachusetts). Raw data from the DSC run were fit using Origin™ scientific plotting software, which uses the Levenberg–Marquardt nonlinear least square method.

Differential Static Light Scattering

The colloidal stability was measured using a static light scattering plate reader (StarGazer-384; Harbinger Biotechnology and Engineering Corporation, Markham, Ontario, Canada). All samples were prepared at a final protein concentration of 1 mg/mL in the buffer alone and excipient(s) of interest. Twenty-five microliters of prepared samples was added to a clear-bottom 384-well plate (Nunc, Rochester, New York) in triplicate and heated at 70°C using a peltier heating system. Protein aggregation was monitored by measuring the intensity of the scattered light every 30 s with a CCD camera. Changes in light scattering at 600 nm were measured by analyzing the intensities of the image with the image analysis software. The pixel intensities in a preselected region of each well were integrated to generate a value representative of the total amount of scattered light in that region. These total intensities were then plotted against time for each sample well. The amount of time lag before the onset of aggregation was used to screen stabilizing and destabilizing excipient effects.[18,22]

Accelerated Stability Studies

Accelerated stability studies were performed at a protein concentration of 10–50 mg/mL in the buffer alone and with excipient(s) of interest. The formulated proteins were placed in glass vials and stored in a (40°C, 75% RH) controlled chamber for 1 month. Samples were tested at weekly intervals for percent aggregate by high-performance size exclusion chromatography (HPSEC). HPSEC analysis was performed on an Agilent HPLC system with a TSK-Gel G3000 (Agilent

Table 1. Buffer and Excipient Conditions Studied for mAbs in Differential Scanning Fluorescence Experiments

	mAbA	mAbB	MAbC	mAbD
Base buffer	10 mM histidine, pH 6	25 mM sodium citrate + 7% (w/v) sucrose, pH 6.5	10 mM histidine, Ph 6	10 mM histidine, pH 5.5
Conditions	+ 300 mM glycine	25 mM sodium citrate + 7% (w/v) sucrose, pH 5	+ 50 mM sodium chloride	+ 50 mM sodium chloride
	+ 300 mM trehalose	25 mM sodium citrate + 7% (w/v) sucrose, pH 6	+ 300 mM sodium chloride	+ 6% (w/v) sucrose
	+ 150 mM sodium chloride	25 mM sodium citrate + 7% (w/v) sucrose, pH 7	+ 4% (w/v) trehalose	+ 50 mM sodium chloride + 6% (w/v) sucrose
	+ 150 mM lysine hydrochloride + 150 mM arginine hydrochloride		+ 8% (w/v) trehalose	+ 4% (w/v) trehalose + 150 mM arginine hydrochloride

 DOI 10.1002/jps

Technologies). Aggregation rates over time were determined by linear regression.

RESULTS AND DISCUSSION

Correlation Between DSF and DSC

The correlation between DSF and DSC was investigated by comparing mAb thermal unfolding curves obtained by SYPRO® Orange fluorescence and by DSC. It has been demonstrated that SYPRO® Orange, an environmentally sensitive external fluorescent probe, shows a distinct sigmoid-shaped fluorescence response during a protein thermal melt due to its increased quantum yield in hydrophobic environments.[18] When a protein is folded, SYPRO® Orange shows very little fluorescence, but as the protein unfolds and hydrophobic resides are exposed, SYPRO® binds to the hydrophobic residues and shows increased fluorescence until the protein finally aggregates, causing a reduction in the available hydrophobic residues and a decreased fluorescent signal. This fluorescence response can be used to track the unfolding of the protein and compare the effect of different solution conditions on the midpoint of this transition.

Figures 1a and 1b show thermal unfolding of mAbA in the presence of various excipients, as monitored by DSC and DSF, respectively. The DSC profile for mAbA shows two well-separated transitions in all the conditions. For multidomain proteins such as antibodies, multiple DSC transitions usually indicate reduced interdomain interactions. Similar to DSC, DSF was also able to distinguish two separate transitions in the thermal unfolding of mAbA. In each excipient condition tested, the inflection point of the DSF curve corresponds to the peak of the DSC curve, with only a small offset. The small offset was expected due to the fact that DSF monitors exposure of hydrophobic residues, whereas DSC measures the change in excess Cp. Therefore, although the T_m obtained by both these techniques is slightly different, the rank order and degree of stabilization or destabilization of mAbA in the presence of each excipient were comparable.

In comparison to mAbA, where two distinct transitions were seen, the DSC unfolding of mAbB at pH 6, 6.5, and 7 (Fig. 2a) shows up to three overlapping transitions, indicating strong interdomain stability/interactions that are pH dependent. DSF results show similar overlapping transitions and offsets as well, with good general agreement between the two techniques (Fig. 2b). At pH 5, two distinct transitions are evident by both techniques, with good separation of the first transition from the main peak (Figs. 2a and 2b), indicating either reduced interdomain interactions and/or destabilization of first transition. Both techniques show increased overlap at pH 6

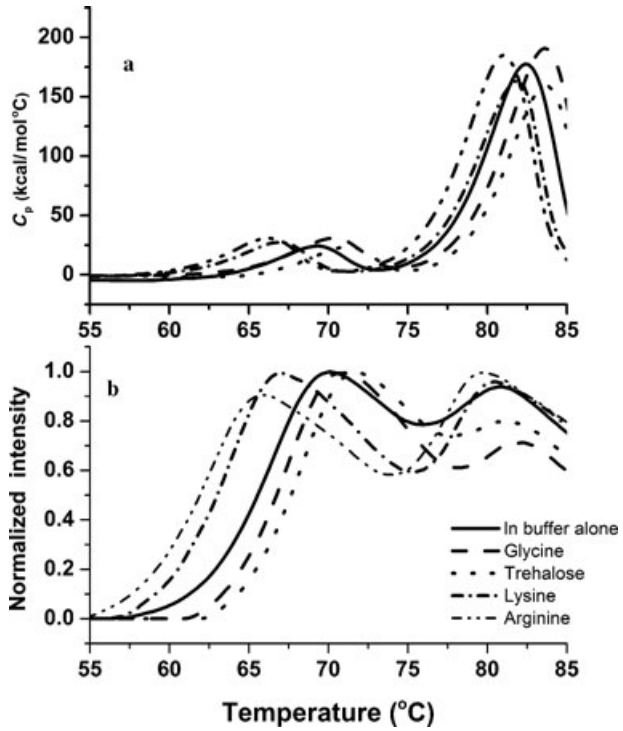

Figure 1. Thermal unfolding of monoclonal antibody A (mAbA) as analyzed by differential scanning calorimetry (DSC) (a) and differential scanning fluorescence (DSF) (b). Both DSC and DSF experiments were run in 10 mM histidine, pH 6, at a protein concentration of 0.5 mg/mL under various solution conditions (see line code in panel b). For both figures, mAbA control in buffer alone is shown in solid black line.

and 7, suggesting increased interdomain interactions. In addition, for the different pH conditions tested, the same rank order of stability was determined by DSF and DSC, as shown by the shift of the thermal transitions.

The similarity of these observations for mAbA and mAbB by both techniques suggests that DSF can be used as a high-throughput technique for measuring not only the thermal stability but also to monitor interdomain interactions in multidomain proteins. Although it appears that DSC can provide additional information if the domain unfolding regions overlap, DSF can still be used as a rapid screening technique for the first domain unfolding event, which is often what leads to protein aggregation.[9]

Table 2 shows a comparison of the first transition melt temperature obtained by DSC and DSF for two additional antibodies (mAbC and mAbD) in the presence of various excipients. In addition, the difference between the T_m of the protein in each condition and protein in buffer alone (ΔT_m) is shown for each excipient tested, with the stabilizing effects indicated by a positive ΔT_m value and destabilizing effects indicated by a negative ΔT_m value. In addition, we

Table 2. Thermal Melting Temperature of mAbC and mAbD as Obtained by DSC and DSF

mAb	Condition	DSF T_m (°C)	DSC T_m (°C)	ΔT_m DSF (°C)	ΔT_m DSC (°C)
mAbC	Base buffer[a]	65.4	68.9	–	–
mAbC	+ 50 mM sodium chloride	64.4	67.8	−1.0	−1.1
mAbC	+ 300 mM sodium chloride	63.4	66.7	−2.0	−2.2
mAbC	+ 4% (w/v) trehalose	66.1	69.7	0.7	0.8
mAbC	+ 8% (w/v) trehalose	66.6	70.2	1.2	1.3
mAbD	Base buffer[b]	63.2	65.5	–	–
mAbD	+ 50 mM sodium chloride	60.3	63.1	−2.9	−2.5
mAbD	+ 6% (w/v) sucrose	64.5	66.4	1.3	0.8
mAbD	+ 50 mM sodium chloride + 6% (w/v) sucrose	61.3	63.9	−1.9	−1.7
mAbD	+ 4% (w/v) trehalose	63.7	66.0	0.5	0.4

Both DSC and DSF experiments were run at a protein concentration of 0.5 mg/mL under various solution conditions. ΔT_m is the difference in the T_m between protein in presence of a particular excipient and the protein in buffer alone without any excipients. Experiment conditions are explained in methods section.

[a]Base buffer of mAbC is 10 mM histidine, pH 6, [b] Base buffer of mAbD is 10 mM histidine, pH 5.5.

DSC, differential scanning calorimetry; DSF, differential scanning fluorescence.

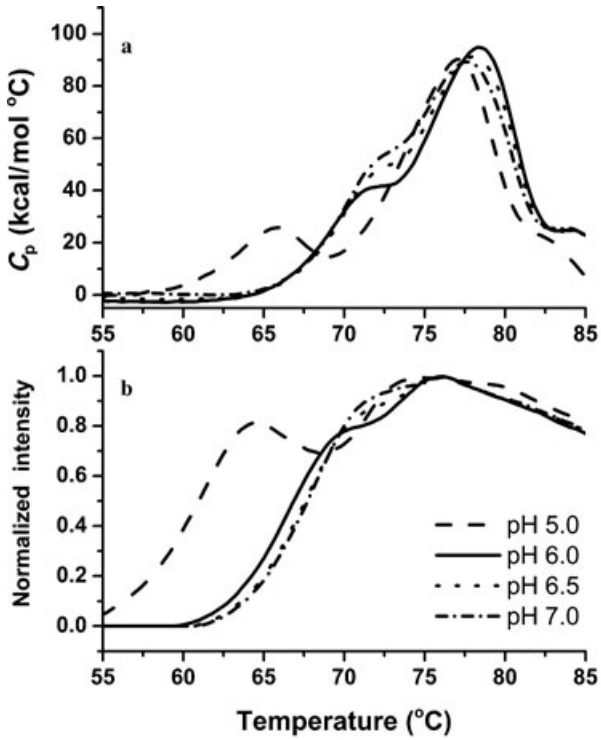

Figure 2. Effect of pH on conformational stability of monoclonal antibody B as seen by (a) differential scanning calorimetry (DSC) and (b) differential scanning fluorescence (DSF). Both DSC and DSF experiments were run in 25 mM sodium citrate buffer with 7% (w/v) sucrose, at a protein concentration of 0.5 mg/mL and under various pH conditions (see line code in panel b).

investigated whether ΔT_m calculated by DSF could distinguish the effects of different concentrations of sugars and salts on conformational stability using mAbC as a case study. The results suggest that it is possible to measure changes in ΔT_m by DSF. For mAbC, 300 mM sodium chloride showed a lower ΔT_m (more destabilizing) than 50 mM sodium chloride, and 8% (w/v) trehalose showed a higher ΔT_m (more stabilizing) than 4% (w/v) trehalose. These results are in agreement with results obtained by DSC. Therefore, DSF can be used to determine whether a given excipient is stabilizing. It can also be used to determine the effect of different concentrations of a given excipient on stability to identify trends and patterns.

In addition to investigating the effect of single excipient concentrations on mAb conformational stability, it is important to understand the impact of excipient combinations on stability. When excipient combinations are used, the stability is difficult to predict because the excipients may not always result in additive effects due to different modes of interaction. For mAbD, we investigated the impact of sugars and salts separately and in combination. Table 2 shows that 50 mM sodium chloride was destabilizing (negative ΔT_m), whereas 6% (w/v) sucrose was stabilizing (positive ΔT_m); however, the combination of 50 mM sodium chloride and 6% (w/v) sucrose was slightly destabilizing (negative ΔT_m). DSF showed good correlation with DSC results, indicating the value of using DSF to study the effect of excipient combinations on the conformational stability of mAbs. Because this technique can be performed in a 96-well plate format, many excipient concentrations can be tested individually and in combination, saving time and protein, and permitting the formulation scientist to more comprehensively explore the formulation space.

Figure 3 shows the overall correlation between T_m values obtained by DSF and DSC for all conditions studied. The linear fit for the curve shows a slope of 1.03 and an intercept of 0.87 with an R^2 of 0.95, establishing a linear correlation between DSC and DSF for all of the antibodies and solution conditions investigated. The intercept of 0.87 indicates a slight offset between the two techniques, with DSF detecting protein unfolding at slightly lower temperatures. This strong correlation between DSF and DSC has significant implications for formulation development

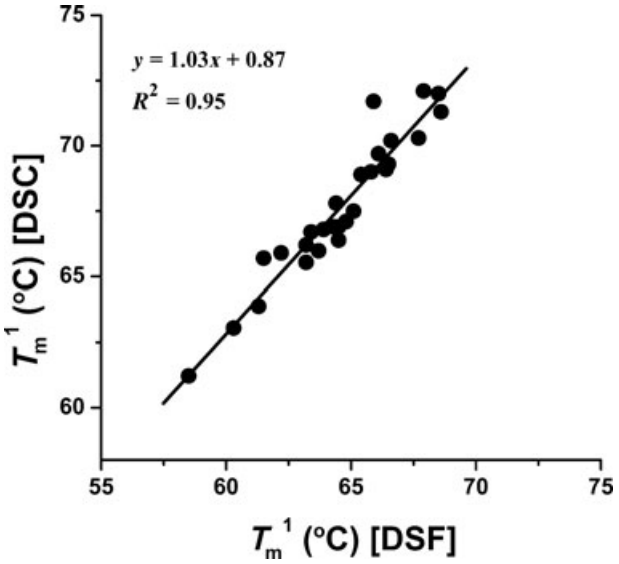

Figure 3. Correlation between the T_m obtained by differential scanning calorimetry (DSC) and differential scanning fluorescence (DSF). A strong linear correlation is seen between the melting temperatures determined by DSF and DSC with $R^2 = 0.95$. An intercept of 0.87 indicates a slight offset between the T_m obtained by two methods, with DSC showing slightly higher melt temperatures.

because DSF can provide similar information to DSC in early formulation screening. Because 96 samples can be analyzed in a single 3 h assay, this technique allows for a more rapid screening of multiple excipients, different concentrations, and varying combinations. The correlation appears to be independent of antibody structure and isotype, as shown in the experiments using a variety of antibody isotypes in various solution conditions.

DSLS Can Monitor Colloidal Stability of a Protein in High-Throughput Fashion

Colloidal stability, or the ability of solution conditions to prevent association of native or partially unfolded molecules, is another important method to prevent protein aggregation. Aggregated protein has an increased ability to scatter incident light because of its larger size. Light scattering techniques have been used to study protein aggregation for many years, and one of the most commonly used methods is UV-Vis absorbance at >350 nm. Unfortunately, most of the commercially available UV-Vis plate readers can give accurate readings only up to 65°C, and the T_m^{-1} of most mAbs is at temperatures higher than 65°C. Because of these limitations, the colloidal stability of mAbs is usually determined in single or multiple cuvette instruments, which operate at higher temperature ranges, making this a low-throughput screening method.

The Stargazer 384 system (Harbinger Biotechnology and Engineering Corporation), which detects static light scattering from samples in a 384-well microplate, was used in our study to monitor colloidal stability of mAbs at elevated temperatures. When proteins are subjected to stress, aggregates form over time and can be detected by increased light scattering. Protein stability in the DSLS system is quantified by the delay time between the onset of the stress (increased temperature) and protein aggregation.

Solution conditions are considered stabilizing when they prolong the time a protein stays folded compared with the time it stays folded in the buffer alone. Conversely, solution conditions that induce aggregation earlier compared with the buffer alone are considered destabilizing. In this study, we choose two mAbs for additional investigation with StarGazer. Figures 4a and 4b show the light scattering traces of mAbC and mAbD, respectively, over time under different solution conditions. The datum shown is an average of three experiments.

For both mAbC and mAbD, sugars are shown to be stabilizing, and higher concentrations show greater

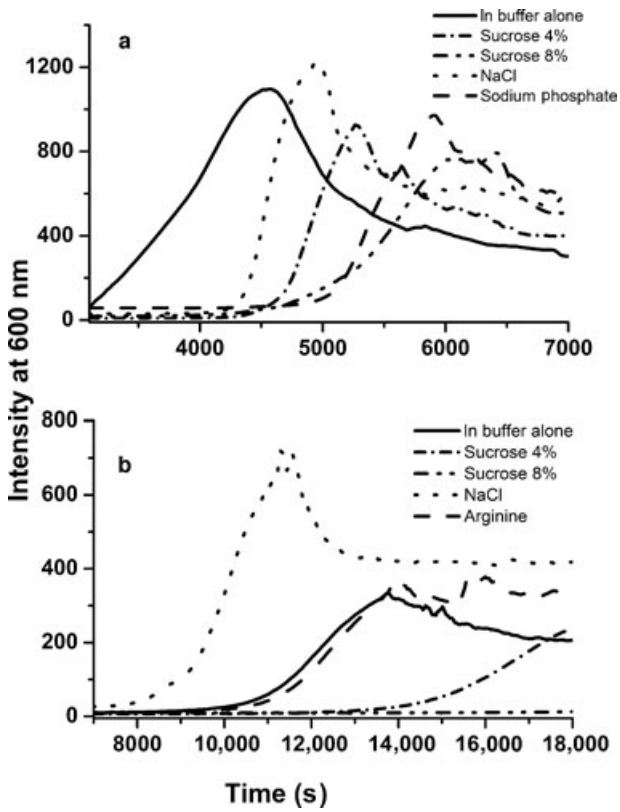

Figure 4. Effect of different excipients on colloidal stability of (a) monoclonal antibody (mAb)C and (b) mAbD as measured by differential static light scattering. Both experiments were run in 10 mM histidine buffer at a protein concentration of 1 mg/mL at 70°C under various solution conditions (see line code in panel a and b).

stabilization, which is consistent with DSF. Interestingly for mAbC, DSLS data showed that sodium chloride was stabilizing, whereas DSF and DSC data showed that sodium chloride was destabilizing. Thus in the case of mAbC, salt seems to have a different effect on conformational and colloidal stability. For mAbD, there is a good correlation between DSLS and DSF for sodium chloride, which acts as a destabilizer. Importantly, these studies showed that distinct differences in aggregation time can be seen between different solution conditions using this technique. Because it was run in a 384-well plate format, it can be a very powerful tool for early formulation screening, especially when a protein is known to be prone to aggregation.

Role of Conformational and Colloidal Stability in Protein Aggregation

For large multidomain proteins such as mAbs, protein aggregation is controlled by conformational and/or colloidal stability.[10,23] Nonnative protein aggregation pathways of several proteins have been well characterized using the well-established Lumry–Eyring framework and are known to occur through the formation of an aggregation-prone intermediate step.[24–27]

$$N \leftrightarrow I \rightarrow A \quad (1)$$

The main goal of a formulation scientist is to stabilize against the last irreversible aggregation step that is kinetically controlled.[10] This can be achieved either directly by stabilizing the N state (conformational stability) or by preventing or minimizing the conversion of partially unfolded intermediates to the A state (colloidal stability). Stabilizers such as sucrose and trehalose are known to minimize aggregation by stabilizing the N state,[28–31] thereby reducing the concentration of reactive or aggregation-prone intermediates (I). In contrast, salts are known to affect the irreversible I to A transition, which is dependent on the colloidal stability.[32] The effect of salt on this transition can be either stabilizing or destabilizing depending on the protein surface charge distribution.[27]

The effect of salt on colloidal stability has been observed for α-amylase.[32] Olsen et al.[32] have postulated that aggregation is decreased in the absence of salt because the electrostatic repulsion between partially unfolded intermediate states prevents interaction. In contrast, in the presence of salt, the charges are screened and repulsion is reduced, allowing the hydrophobic surfaces of partially unfolded I state proteins to interact with each other, increasing aggregation. It is interesting to note the different effects of salt and sugar on conformational and colloidal stability of mAbC and mAbD (Figs. 4a and 4b). For both proteins, sugar increases the conformational stability, whereas salt decreases colloidal stability of mAbD

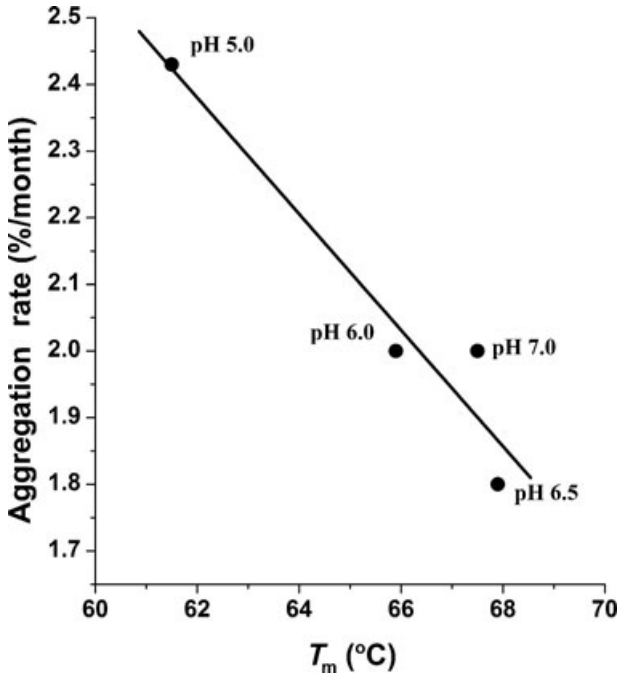

Figure 5. Correlation between conformational stability and aggregation rate at accelerated conditions of monoclonal antibody B at different pH conditions. T_m was determined by differential scanning fluorescence in 25 mM sodium citrate buffer with 7% (w/v) sucrose at a protein concentration of 0.5 mg/mL. Aggregation rates were determined under accelerated condition (40°C, 75% RH) at a protein concentration of 10 mg/mL in the same buffer composition using high-performance size exclusion chromatography.

but increases colloidal stability for mAbC. As mentioned earlier, sugars are known to prevent aggregation by effecting the reversible N to I transition that is generally independent of charge distribution. In contrast, salts can either increase or decrease aggregation propensity by affecting the second irreversible step of the aggregation pathway (I to A) that is dependent on the charge distribution on the surface of partially unfolded intermediate. Therefore, for colloidal stability, the nature of the intermediate state formed is critical, hence we see a different effect of sodium chloride on the stability of mAbC and mAbD (Figs. 4a and 4b). Ultimately our goal is to determine which of the two pathways is more important for the stability of mAbs under long-term storage, as discussed in the following section.

Correlation Among DSF, DSLS, and Real-Time Incubation Studies for Select mAbs

One of the purposes of this study was to determine the feasibility of applying high-throughput techniques to correlate and/or predict real-time stability under storage conditions. To address this, stability determined by DSF and DSLS was compared with mAb

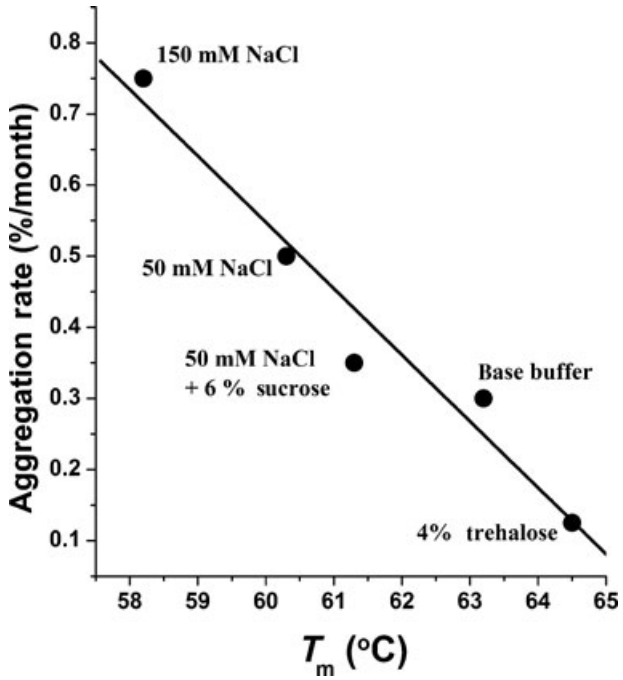

Figure 6. Correlation between conformational stability and aggregation rate at accelerated conditions for monoclonal antibody (mAb)D. T_m of mAbD in the presence of various excipients was determined by differential scanning fluorescence at a protein concentration of 0.5 mg/mL. Aggregation rates in presence of various excipients were determined under accelerated conditions (40°C, 75% relative humidity) at a protein concentration of 10 mg/mL using high-performance size exclusion chromatography.

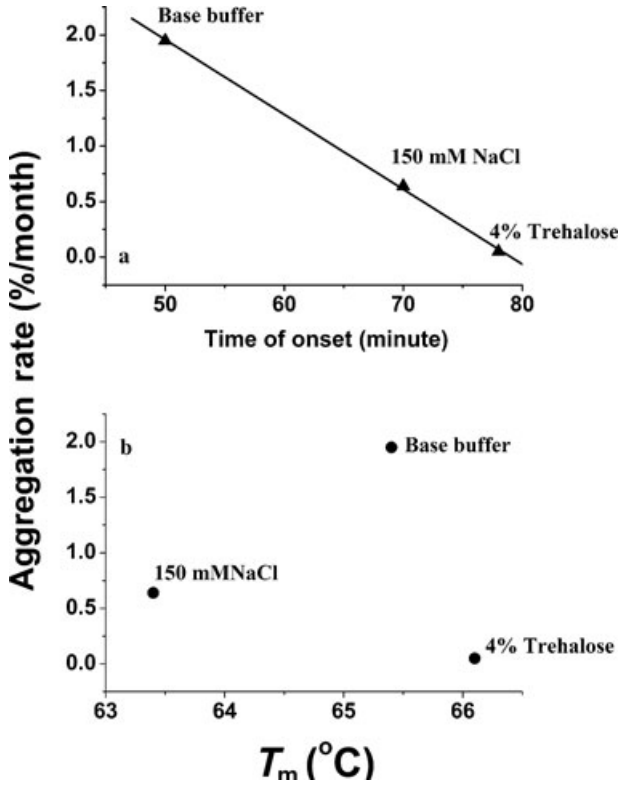

Figure 7. (a) Correlation between colloidal stability and aggregation rate at accelerated conditions for monoclonal antibody (mAb)C. Time of onset of mAbC in presence of various excipients was determined by heating the samples at 70°C and monitoring intensity at 600 nm over time by differential static light scattering at a protein concentration of 1 mg/mL. Aggregation rates in presence of various excipients were determined under accelerated conditions [40°C, 75% relative humidity (RH)] at a protein concentration of 10 mg/mL using high-performance size exclusion chromatography (HPSEC). (b) Comparison of conformational stability and aggregation rate at accelerated conditions for mAbC. T_m of mAbC in the presence of various excipients was determined by differential scanning fluorescence at a protein concentration of 0.5 mg/mL. Aggregation rates in presence of various excipients were determined under accelerated conditions (40°C, 75% RH) at a protein concentration of 10 mg/mL using HPSEC.

degradation (aggregation) under accelerated stress conditions (40°C). These comparisons were performed to determine the extent to which DSF and DSLS stability tests translate to accelerated stability studies, a well-established predictor of long-term formulation stability. T_m, as calculated by DSF, and aggregation rate versus pH are shown for mAbB in Figure 5. Comparison of T_m at each pH with aggregation propensity indicates that an increase in T_m leads to a decrease in aggregation rate, suggesting the important role of conformational stability in keeping mAbB stable under long-term storage conditions. For mAbD, we see a good correlation between DSF and DSLS (Table 2 and Fig. 4b), with sugars stabilizing and salt destabilizing both conformational and colloidal stability. Because the two methods are well correlated, only DSF is compared with aggregation propensity that was obtained under accelerated conditions, as shown in Figure 6. Similar to mAbB, for mAbD, we see a strong correlation between T_m and aggregation rate under accelerated conditions, illustrating that high-throughput screening (HTS) techniques can reliably predict accelerated stability for some mAbs.

For mAbC, we do not see a good correlation between conformational and colloidal stability (Table 2 and Fig. 4a), hence we compared both DSF and DSLS with real-time data for select conditions (Fig. 7). DSF data indicate that salt is destabilizing the native state by decreasing the conformational stability, but the DSLS data show that salt is increasing the colloidal stability by preventing/minimizing the association of intermediate states. This raises the fundamental question of which of these transitions should be stabilized in order to increase the storage stability. Comparison of DSLS (colloidal stability) and DSF (conformation stability) data to aggregation propensity under accelerated conditions for mAbC suggests colloidal stability as a better predictor of long-term stability for

this antibody (Fig. 7). The results from this study underline the importance of monitoring both conformational stability and colloidal stability during excipient screening. In this case, high-throughput DSF and DSLS could both be run to their full capacity with different concentrations and combinations of excipients. The solution conditions with the lowest T_m by DSF and longest delay to aggregation by DSLS would then be chosen for further evaluation using traditional stability studies.

CONCLUSION

We have shown that mAb aggregation is controlled by both conformational and colloidal stability, and depending on protein and solution conditions, either could be rate limiting. We demonstrate the implementation of two HTS techniques (DSF and DSLS) that can monitor conformation and colloidal stability of a protein. These techniques will not only allow us to screen stabilizing excipients but can also enable us to differentiate between different concentrations of excipients as well as the effects of multiple excipients on mAb conformational and colloidal stability. Because of the requirement of less material and shorter analysis time, both these techniques can be used to examine a wider range of pH conditions, excipients, concentrations, and combinations of excipients, compared with traditional formulation techniques, thereby significantly increasing the formulation optimization space. The combination of HTS using DSF and DSLS with the knowledge of the major protein degradation pathways could serve as a valuable tool for optimal formulation design.

ACKNOWLEDGMENTS

Authors are thankful to Hardeep Samra, Shufeng Bai, Neil Mody, Monika Sharma, Anthony Tuesca, William Leach, Brian Lobo, Orit Scharf, and Cynthia Oliver at MedImmune for sharing stability data and/or critical review of the manuscript. Part of this work was funded by MedImmune summer internship program to D.G.

REFERENCES

1. Manning MC, Matsuura JE, Kendrick BS, Meyer JD, Dormish JJ, Vrkljan M, Ruth JR, Carpenter JF, Sheftert E. 1995. Approaches for increasing the solution stability of proteins. Biotechnol Bioeng 48(5):506–512.
2. Wang W. 1999. Instability, stabilization, and formulation of liquid protein pharmaceuticals. Int J Pharm 185(2):129–188.
3. Welfle K, Misselwitz R, Hausdorf G, Höhne W, Welfle H. 1999. Conformation, pH-induced conformational changes, and thermal unfolding of anti-p24 (HIV-1) monoclonal antibody CB4–1 and its Fab and Fc fragments. Biochim Biophys Acta 1431(1):120–131.
4. Fesinmeyer RM, Hogan S, Saluja A, Brych SR, Kras E, Narhi LO, Brems DN, Gokarn YR. 2009. Effect of ions on agitation- and temperature-induced aggregation reactions of antibodies. Pharm Res 26(4):903–913.
5. Volkin DB, Sanyal G, Burke CJ, Middaugh CR. 2002. Preformulation studies as an essential guide to formulation development and manufacture of protein pharmaceuticals. Pharm Biotechnol 14:1–46.
6. Capelle MAH, Gurny R, Arvinte T. 2007. High throughput screening of protein formulation stability: Practical considerations. Eur J Pharm Biopharm 65(2):131–148.
7. Fan H, Ralston J, Dibiase M, Faulkner E, Middaugh CR. 2005. Solution behavior of IFN-beta-1a: An empirical phase diagram based approach. J Pharm Sci 94(9):1893–1911.
8. Ramsey JD, Gill ML, Kamerzell TJ, Price ES, Joshi SB, Bishop SM, Oliver CN, Middaugh CR. 2009. Using empirical phase diagrams to understand the role of intramolecular dynamics in immunoglobulin G stability. J Pharm Sci 98(7):2432–2447.
9. Baynes BM, Trout BL. 2004. Rational design of solution additives for the prevention of protein aggregation. Biophys J 87(3):1631–1639.
10. Chi EY, Krishnan S, Randolph TW, Carpenter JF. 2003. Physical stability of proteins in aqueous solution: Mechanism and driving forces in nonnative protein aggregation. Pharm Res 20(9):1325–1336.
11. Middaugh CR, Edwards KL. 1998. Recent advances in our understanding of protein conformational stability from a pharmaceutical perspective. Expert Opin Investig Drugs 7(9):1493–1500.
12. Grillo AO, Edwards KL, Kashi RS, Shipley KM, Hu L, Besman MJ, Middaugh CR. 2001. Conformational origin of the aggregation of recombinant human factor VIII. Biochemistry 40(2):586–595.
13. Wang W. 2005. Protein aggregation and its inhibition in biopharmaceutics. Int J Pharm 289(1–2):1–30.
14. Bhattacharjee C, Das KP. 2000. Thermal unfolding and refolding of beta-lactoglobulin. An intrinsic andextrinsic fluorescence study. Eur J Biochem 267(13):3957–3964.
15. Hawe A, Sutter M, Jiskoot W. 2008. Extrinsic fluorescent dyes as tools for protein characterization. Pharm Res 25(7):1487–1499.
16. Cummings MD, Farnum MA, Nelen MI. 2006. Universal screening methods and applications of ThermoFluor. J Biomol Screen 11(7):854–863.
17. Crowther GJ, Napuli AJ, Thomas AP, Chung DJ, Kovzun KV, Leibly DJ, Castaneda LJ, Bhandari J, Damman CJ, Hui R, Hol WGJ, Buckner FS, Verlinde CLMJ, Zhang Z, Fan E, Van Voorhis WC. 2009. Buffer optimization of thermal melt assays of plasmodium proteins for detection of small-molecule ligands. J Biomol Screen 14(6):700–707.
18. Vedadi M, Niesen FH, Allali-Hassani A, Fedorov OY, Finerty PJ, Wasney GA, Yeung R, Arrowsmith C, Ball LJ, Berglund H, Hui R, Marsden BD, Nordlund P, Sundstrom M, Weigelt J, Edwards AM. 2006. Chemical screening methods to identify ligands that promote protein stability, protein crystallization, and structure determination. Proc Natl Acad Sci USA 103(43):15835–15840.
19. Ericsson UB, Hallberg BM, Detitta GT, Dekker N, Nordlund P. 2006. Thermofluor-based high-throughput stability optimization of proteins for structural studies. Anal Biochem 357(2):289–298.
20. He F, Hogan S, Latypov RF, Narhi LO, Razinkov VI. 2009. High throughput thermostability screening of monoclonal antibody formulations. J Pharm Sci 9(4):1707–1720.
21. Kurganov BI. 2002. Kinetics of protein aggregation. Quantitative estimation of the chaperone-like activity in test-systems based on suppression of protein aggregation. Biochemistry (Mosc) 67(4):409–422.

22. Senisterra GA, Markin E, Yamazaki K, Hui R, Vedadi M, Awrey DE. 2006. Screening for ligands using a generic and high-throughput light-scattering-based assay. J Biomol Screen 11(8):940–948.
23. Fast JL, Cordes AA, Carpenter JF, Randolph TW. 2009. Physical instability of a therapeutic Fc fusion protein: Domain contributions to conformational and colloidal stability. Biochemistry 48(49):11724–11736.
24. Roberts CJ. 2007. Nonnative protein aggregation kinetics. Biotechnol Bioeng 98(5):927–938.
25. Weiss WFt, Young TM, Roberts CJ. 2009. Principles, approaches, and challenges for predicting protein aggregation rates and shelf life. J Pharm Sci 98(4):1246–1277.
26. Kendrick BS, Cleland JL, Lam X, Nguyen T, Randolph TW, Manning MC, Carpenter JF. 1998. Aggregation of recombinant human interferon gamma: Kinetics and structural transitions. J Pharm Sci 87(9):1069–1076.
27. Lumry R, Eyring H. 1954. Conformation changes of proteins. J Phys Chem 58(2):110–120.
28. Timasheff SN. 2002. Protein hydration, thermodynamic binding, and preferential hydration. Biochemistry 41(46):13473–13482.
29. Timasheff SN. 1995. Solvent stabilization of protein structure. Methods Mol Biol 40:253–269.
30. Kaushik JK, Bhat R. 2003. Why is trehalose an exceptional protein stabilizer? An analysis of the thermal stability of proteins in the presence of the compatible osmolyte trehalose. J Biol Chem 278(29):26458–26465.
31. Sathish HA, Kumar PR, Prakash V. 2007. Mechanism of solvent induced thermal stabilization of papain. Int J Biol Macromol 41(4):383–390.
32. Olsen SN, Andersen KB, Randolph TW, Carpenter JF, Westh P. 2009. Role of electrostatic repulsion on colloidal stability of Bacillus halmapalus alpha-amylase. Biochim Biophys Acta 1794(7):1058–1065.