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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIVISION DE NUEVAS CREACIONES

E. S. D.

Re: Expediente No. **08.019.768 B**
Solicitud de patente a nombre de **IMMUNOGEN, INC.**

Yo, SILVANA MARIA LOPEZ DIAZ identificada como aparece al pie de mi firma, domiciliada en la Carrera 11 No. 86-53, piso 6, obrando como apoderada sustituta de acuerdo con la sustitución adjunta, de la sociedad de **IMMUNOGEN, INC.** domiciliada en Cambridge, Estados Unidos de América y solicitante de la patente de la referencia, dando respuesta al oficio No. **7431** notificado por fijación en lista el 30 de abril de 2013, me permito manifestar:

1. 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 en el cual se realizaron las siguientes modificaciones:

La antigua reivindicación 1 y antigua reivindicación 11 (nueva modificada 6), se han modificado para incorporar la materia objeto de las ex reivindicaciones 20, 27, 28 y 31, a fin de definir adecuadamente los términos "anticuerpo", "reactivo de reticulación bifuncional" y "maitansinoide".

En vista de estas enmiendas, se han cancelado las antiguas reivindicaciones 18-20, 22-26 y 29-31.

La reivindicación 1 también se ha modificado para especificar que la primera mezcla se somete a filtración de flujo tangencial en el paso (b). En vista de esta enmienda, se han cancelado las ex-reivindicaciones 2-6.

Además de lo anterior, se ha modificado la etapa (d) de la reivindicación 1 en aras de eliminar la palabra "una" antes "filtración de flujo tangencial".

La ex reivindicación 11 (nueva modificada 6) también se ha modificado para especificar que la segunda mezcla se somete a filtración de flujo tangencial o cromatografía de adsorción en el paso (c). Además, dicha reivindicación se ha modificado para incorporar la materia objeto de la antigua reivindicación 12.

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En vista de estas enmiendas, el señor examinador podrá corroborar que la antigua cláusula 12 ha sido cancelada.

Se ha eliminado el término "aproximadamente" de la Reivindicación 1 y de las antiguas reivindicaciones 7, 8, 11, 14 y 15 (modificadas 2, 3, 6, 8 y 9) han sido modificadas para eliminar el término "aproximadamente" y las restantes reivindicaciones se han reenumerado y modificado para reflejar las dependencias correspondientes, como se muestra en el nuevo capítulo reivindicatorio adjunto.

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 novedad planteadas por el examinador en su concepto técnico. Se presenta tasa por 6 reivindicaciones adicionales.

2. Presentación de la solicitud faccionaria

El señor examinador indica en el párrafo anterior al acápite 4, que "...*las etapas del proceso de preparación del conjugado anticuerpo -maitansinoide ya habían sido reivindicadas en la solicitud parenteral...*" Sobre este particular nos permitimos decir que debido a que la solicitud principal ha sido finalmente rechazada no tendría por qué haber el rechazo de la materia reclamada en la presente invención dado que no existe doble patentamiento, toda vez que como se indicó anteriormente, las solicitudes de la cual la presente invención se derivó se encuentran rechazadas.

3. Claridad de la solicitud

Respecto a este punto, el solicitante manifiesta que mediante las modificaciones realizadas en el 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.

No obstante, nos permitimos manifestar lo siguiente:

El examinador rechaza las reivindicaciones 1, 11, 18, 19, y 22-25 por una falta de claridad. En particular, el examinador sostiene que el alcance de los términos "anticuerpo", "reactivo de reticulación bifuncional", "enlazadores", "maitansinoide", "solución", "anticuerpo monoclonal", "anticuerpo quimérico", y "anticuerpo humanizado"; es demasiado amplio.

Sobre este particular nos permitimos decir que las reivindicaciones 18, 19 y 22 a 25 han sido canceladas. En consecuencia, los rechazos relacionados con claridad

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se abordarán en relación con la reivindicación 1, y la antigua reivindicación 11 (nueva cláusula modificada 6).

En aras de facilitar la consecución de la presente invención, y no en la aquiescencia del rechazo, la reivindicación 1 y la antigua reivindicación 11 (reivindicación 6 modificada) se han modificado para especificar que el anticuerpo es huN901, huMy9 - 6, HUB4, huC242, trastuzumab, bivatuzumab, sibrotuzumab, CNTO95, huDS6 o rituximab, según la antigua reivindicación 20, que no fue incluida en las objeciones del señor examinador respecto a la claridad.

Además, según la reivindicación 1, y la nueva cláusula 6 se han modificado para especificar que el reactivo de reticulación bifuncional es N-succinimidil 4-(2-piridilditio)butanoato (SPDB), N-succinimidil 4-(2-piridilditio)pentanoato (SPP) o N-succinimidil 4-(maleimidometil)ciclohexanocarboxilato (CCPA), de acuerdo con la primera reivindicación 31, en el que la reivindicación no se incluyó en los rechazos de claridad.

De otra parte, la reivindicación 1 modificada y la nueva reivindicación 6 se han modificado para especificar que el maitansinoide es N2'-desacetil-N2'-(3-mercapto-1-oxopropil)-maitansina (DM1) o N2'-desacetil-N2'-(4-metil-4-mercapto-1-oxopentil)-maitansina (DM4), como el expuesto en las reivindicaciones anteriores 27 y 28 (reivindicaciones modificadas 13 y 14), las cuales se afirma que no se incluyeron en los rechazos de claridad por parte del señor examinador.

Con respecto al término "solución" indicado en la etapa de reacción de conjugación (etapa (c) de la reivindicación 1 y la etapa (b) de la reivindicación 6 modificada), es importante señalar que una persona versada en la materia técnica correspondiente entenderá con facilidad el alcance de la expresión "solución" en vista de la descripción proporcionada en la memoria descriptiva y el estado de la técnica en el momento de la presentación de la solicitud en estudio.

En relación con lo anterior, amablemente dirigimos la atención del señor examinador al párrafo 0016 de la especificación (páginas 11 y 12) que describe soluciones adecuadas que se pueden utilizar para la reacción de conjugación. También dirigimos su atención a las soluciones ejemplares específicas que se describen en detalle en los ejemplos, esto es, en los párrafos 0069 (Ejemplo 1), 0.080 (Ejemplo 3), 0088 (Ejemplo 4), 0096 (Ejemplo 5), 0101 (Ejemplo 6), y 0116 (Ejemplo 7).

Además, tal como se describe en el párrafo 0003 de la memoria descriptiva, los procesos para conjugar anticuerpos a agentes citotóxicos que contienen sulfhidrilo, tales como maitansinoides se conocen en la técnica en el momento de presentación de la solicitud en estudio, de tal manera que, una persona con

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experiencia ordinaria en la técnica sería fácilmente informado de soluciones adecuadas que se podrían utilizar para la reacción de conjugación. En este sentido, respetuosamente solicitamos que sean retiradas estas objeciones.

4. Del Nivel Inventivo

El examinador es de la opinión que las antiguas reivindicaciones carecerían de una actividad inventiva a la vista de la solicitud de patente internacional WO 03/057163 A2 (Mazzola et al.) (D1) y la solicitud de patente internacional WO 02/098897 A3 (Bander et al.) (D2).

Sobre este particular, es deseo del solicitante informarle al señor examinador que durante la tramitación de la correspondiente solicitud de patente de EE.UU., que publicó como la patente US.7,811,572, el solicitante presentó argumentos coherentes con los argumentos que se exponen a continuación junto con una Declaración conforme al punto 37 CFR § 1.132, la declaración pertenece al señor Godfrey Amphlett fechada del 20 de julio de 2009 (en adelante "Declaración Amphlett"), que describe los resultados sorprendentes e inesperados de los procesos citados en las reivindicaciones modificadas.

Adicionalmente, es necesario señalar que los argumentos que se exponen a continuación también son compatibles con los argumentos que se presentaron recientemente en la solicitud de Costa Rica correspondiente: solicitud de patente de Costa Rica N ° 9742.

Es de anotar que las reivindicaciones pendientes de la presente solicitud, en su versión modificada con esta respuesta, se dirigen a dos formas de realización de un proceso para la preparación de un conjugado anticuerpo-maitansinoide, que se analizan a continuación por separado.

En efecto, la reivindicación 1 modificada y sus reivindicaciones dependientes, se encuentran dirigidas al procedimiento que comprende las etapas (a) - (d), en el que la etapa (d) es una etapa de purificación que comprende someter una mezcla de filtración de flujo tangencial (TFF) para purificar un conjugado anticuerpo-maitansinoide. Como se discute en detalle a continuación, los presentes inventores descubrieron inesperadamente que la TFF se puede usar para purificar directamente un conjugado anticuerpo-maitansinoide, lo que elimina la necesidad de cromatografía de intercambio de iones enseñada en D1 o la cromatografía no adsorbtiva enseñada en D2. Como resultado de ello, el presente procedimiento es más rápido, más eficiente en costos, y tiene un rendimiento más alto que los procesos que se enseñan en D1 y D2.

En este orden de ideas es importante hacer énfasis sobre el hecho que las referencias citadas no describen o sugieren que la TFF pueda ser empleada

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para purificar un conjugado anticuerpo-maitansinoide con alguna expectativa de éxito.

En efecto, D1 describe un procedimiento para conjugar un maitansinoide a un anticuerpo por proceso llamado de "dos pasos", en el que la primera etapa implica hacer reaccionar un enlazante con el anticuerpo para formar un anticuerpo modificado, y el segundo paso implica la conjugación del anticuerpo modificado con maitansinoide (por favor véase la página 2, línea 22 a 35 de D1).

Así las cosas, después de la primera etapa, D1 enseña la eliminación del enlazante sin reaccionar del anticuerpo modificado por filtración de flujo tangencial. Luego de la segunda etapa, D1 enseña la purificación del conjugado de anticuerpo-maitansinoide por cromatografía de intercambio iónico (véase, por ejemplo, la página 2, líneas 22 a 35).

Es así que al mirar en detalle la etapa de purificar el conjugado de anticuerpo-maitansinoide, la clara enseñanza de D1 sólo es para llevar a cabo esta purificación por cromatografía de intercambio iónico. En este sentido, los medios de purificación basados en dos de intercambio iónico adecuados se describen: (i) en SP-Sepharose (página 2, línea 34; página 13, Ejemplo 7, y la reivindicación 8 de D1), y (ii) la hidroxipatita cerámica (página 5, líneas 16 a 17, página 7, líneas 28 a 30, y la reivindicación 7 de D1).

En este orden de ideas, es claro que significativamente, los autores-inventores de D1 sabían de la filtración de flujo tangencial (TFF), pero no deliberadamente eligieron para enseñar el uso de TFF en la etapa de purificar el conjugado de anticuerpo - maitansinoide.

Como prueba de que los autores/inventores de D1 sabían de TFF, observamos que la TFF se conoce sobre: (1) páginas 4 y 6 de la D1 en el contexto de la eliminación de ligante sin reaccionar del anticuerpo modificado, y (2) en la página 8 de D1 en relación con lo que podría considerarse como un paso "acabado", por lo que el conjugado de anticuerpo-maitansinoide- ya purificado se sometió a filtración de flujo tangencial para filtro de diálisis del conjugado en el tampón de formulación a granel y para ajustar la concentración.

Por lo tanto, es más que claro que la divulgación de D1 representa una decisión por los autores/inventores de no enseñar o incluso sugerir el uso de la TFF en relación con la purificación del conjugado de anticuerpo-maitansinoide.

Similarmente a D1, D2 también se refiere a un procedimiento para conjugar un maitansinoide a un anticuerpo por un proceso llamado de "dos pasos". El primer paso implica la reacción de un enlazante con el anticuerpo, y el segundo paso implica la conjugación del anticuerpo modificado con el maitansinoide (véase por

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favor el párrafo 00443, páginas 160-161, los pasos (2) y (4) de D2, respectivamente.

Después de la primera etapa, D2 enseña la eliminación de enlazador sin reaccionar del anticuerpo modificado por cromatografía no adsorbtiva (véase el párrafo 00443, página 161, la etapa (3), de D2). Después de la segunda etapa, D2 enseña la purificación de la modificado conjugado anticuerpo-maitansinoide por cromatografía no adsorbtiva (véase el párrafo 00443, página 161, paso (5)).

En ese orden de ideas, al mirar en detalle la etapa de purificación de la modificación conjugado anticuerpo-maitansinoide, la enseñanza clara y única de D2 es llevar a cabo esta purificación por cromatografía no adsorbtiva. En este sentido, dos métodos de cromatografía no adsorbtiva adecuados se describen: (i) cromatografía de filtración en gel, y (ii) una columna Sephadex G-25TM (véase el párrafo 00443, página 161, los pasos (3) y (5), de D2).

Al igual que en D1, los inventores/autores de D2 también tenían claro conocimiento de la TFF. En particular, D2 enseña que la TFF se utiliza para concentrar el anticuerpo antes de la reacción de un enlazador con el anticuerpo (véase el párrafo 00 443, página 160, paso (1)). D2 también enseña el uso de TFF en relación con lo que podría considerarse como un paso "de finalización o de acabado " mediante el cual el conjugado ya purificado se sometió a filtración de flujo tangencial para filtro de diálisis del conjugado en el tampón de formulación a granel y para ajustar la concentración (véase el párrafo 00443 , páginas 161-162, paso (6)).

Por lo tanto, la divulgación de D2 también representa una decisión clara por los autores / inventores de D2 de no enseñar o incluso sugerir el uso de TFF en relación a la purificación del conjugado. Un experto en la técnica no optaría por utilizar la TFF para purificar el conjugado de anticuerpo-maitansinoide porque la membrana de la TFF tiene una tendencia de obstrucción debido a su pequeño tamaño de poro.

Se entenderá a partir de la discusión anterior que la diferencia entre los procesos que se enseñan en D1 y D2 y el proceso descrito en la reivindicación 1 modificada de la presente solicitud, radica en que la reivindicación 1 requiere de la filtración TFF para purificar el conjugado de anticuerpo-maitansinoide. Esta modificación puede ser vista como una mejora de los métodos de D1 y D2 debido a que la presente invención, como se define en la reivindicación 1 modificada, combina eficazmente la etapa de purificación con el paso de "acabado", simplificando de este modo, el método de la técnica anterior y la mejora de la eficiencia, como se discute en más detalle a continuación.

En efecto, la pregunta, por tanto, que responder es: si hubiera sido evidente para

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una persona medianamente versada en la materia técnica correspondiente apartarse de la enseñanza clara e inequívoca de D1 (para utilizar la cromatografía de intercambio iónico para purificar el conjugado) y D2 (para utilizar la cromatografía no adsorbente para purificar el conjugado) y en lugar de utilizar la TFF como el expuesto en la reivindicación 1 modificada.

La respuesta a todas luces es que **NO** hubiera sido evidente para apartarse de las enseñanzas claras e inequívocas de D1 y D2 y en lugar de utilizar la TFF para purificar el conjugado anticuerpo-maitansinoide de acuerdo con la reivindicación 1 modificada.

La TFF es una técnica de ultrafiltración que utiliza una membrana que tiene un tamaño de poro muy pequeño, y por lo tanto particularmente sensible. Como tal, antes de la fecha de prioridad de la presente solicitud, los expertos en este arte habrían entendido que la TFF tendría que hacerse con una solución transparente cuando se aplicara al proceso de ultrafiltración a fin de que se trabajara de manera eficaz. Por el contrario, se hubiera podido esperar una solución turbia (con componentes disueltos sólo en parte) obstruyera la membrana haciendo que el proceso de ultrafiltración fuera inoperable.

Un experto normal en la técnica habría apreciado que la solución que contiene el conjugado de anticuerpo-maitansinoide junto con maitansinoide sin reaccionar (DM1), DMA, y los agregados que se describen en D1 y D2 habría estado presentes en una solución turbia, debido a la baja solubilidad de maitansinoide sin reaccionar, que habría comenzado a salir de la solución. Por lo tanto, el experto en la materia habría esperado que el proceso de TFF sólo habría hecho este problema empeorara (por eliminación del disolvente), con maitansinoide precipitado obstruyendo el sistema TFF y haciendo que el sistema TFF fuera inoperable como un método de purificación (véase el párrafo 9 de la Declaración Amphlett adjunta a la presente).

De hecho, es importante hacer énfasis sobre el hecho que es bien sabido en la técnica antes de la fecha de prioridad de la solicitud, el ensuciamiento de la membrana, que era un problema importante asociado con el uso de la TFF. Véase, por ejemplo, Huisman et al., Journal of Membrane Science, 179:79-90 (2000)(adjunto a la presente), que describe que el principal problema restringiendo la aplicación práctica de la ultrafiltración, es el ensuciamiento de la membrana y el flujo dependiente del tiempo que resulta y el comportamiento de rechazo (véase la página 79). Huisman et al. también describe que el flujo observado y comportamiento de retención en la ultrafiltración se pueden relacionar con muchos mecanismos de ensuciamiento, tales como interacciones hidrofóbicas, interacciones electrostáticas, y propiedades de la proteína tales como el tamaño y el comportamiento de agregación (véanse las páginas 80-81).

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Por otra parte, antes de la presente invención, no se esperaba que la TFF podría ser utilizada como un sustituto de la cromatografía de intercambio iónico en la etapa (d) para lograr el mismo resultado o uno mejor, debido a las diferentes propiedades de cada una de estas técnicas de purificación. Por ejemplo, Kalyanpur, Biotecnología Molecular, 22:87-98 (2002) (adjunto a la presente), afirma en la página 91 que:

"La Filtración de flujo tangencial con membranas de ultrafiltración se utiliza para concentrar las proteínas y otras moléculas grandes en corrientes acuosas, con la eliminación simultánea de las especies de peso molecular más bajos, sales, y agua. Sin embargo, el método adolece de su incapacidad para fraccionar mezclas de moléculas que tienen pesos moleculares similares. Las purificaciones cromatográficas diferencian moléculas en base a sus propiedades químicas y no simplemente de su tamaño, y estas técnicas contribuyen en gran medida en una gran manera a la fabricación de productos farmacéuticos de alta pureza en la industria biofarmacéutica."

En efecto, aunque D1 describe que la TFF "se utiliza [en el paso (b)] para eliminar el ligante sin reaccionar y etanol a partir del anticuerpo enlazador-modificado" (página 4, líneas 19 21), **no hay nada en D1** que sugeriría el uso de TFF para purificar el conjugado de anticuerpo-maitansinoide en el paso (d). Más bien, de acuerdo con el estado de la técnica antes de la fecha de prioridad de la aplicación, D1 hace hincapié en la importancia de la utilización de cromatografía de intercambio iónico en la etapa (d) para purificar el conjugado de anticuerpo-maitansinoide" del que no ha reaccionado DM1, DMA, y los agregados " (página 5, líneas 16 18).

En vista de lo anterior, se podría considerar que existía **un prejuicio técnico contra el uso de TFF para purificar el conjugado de anticuerpo - maitansinoide** en D1 y D2. Como resultado de este prejuicio técnico, los autores/inventores de D1 y D2, al parecer tomaron una decisión consciente de **no** enseñar o sugerir el uso de la TFF en relación con la purificación del conjugado anticuerpo-maitansinoide.

Como se mencionó anteriormente, D1 enseña sólo el uso de la cromatografía de intercambio iónico para purificar el conjugado anticuerpo-maitansinoide, a pesar de que los inventores/autores tengan un conocimiento claro de la TFF. **En vista de los conocimientos generales comunes de los expertos según lo expuesto anteriormente, y teniendo en cuenta el hecho de que los expertos no habrían ido en contra de un prejuicio establecido**, el solicitante se permite manifestar que **NO** hubiera sido evidente apartarse de la enseñanza clara de D1 y D2 (para usar cromatografía no adsorbtiva o cromatografía de intercambio iónico para purificar el conjugado) y en su lugar, utilizar la TFF como lo expuesto en la reivindicación 1 modificada.

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En resumen, la abrumadora comprensión de los expertos en este arte fue que la solución turbia del conjugado anticuerpo-maitansinoide junto con maitansinoide sin reaccionar no se podría poner a través de un sistema TFF con eficacia con el fin de purificar el conjugado de anticuerpo - maitansinoide. El proceso reivindicado tendría necesariamente un mayor rendimiento y una mejor eficiencia debido a que el procedimiento reivindicado combina las etapas de purificación y de "acabado" en un solo paso.

La presente invención tal como se define por la reivindicación 1 modificada fue posible solamente debido a que los presentes inventores decidieron ir en contra de la opinión generalizada de que la TFF no se podría utilizar para purificar eficazmente un conjugado anticuerpo-maitansinoide. Los resultados obtenidos por los inventores eran totalmente inesperados en la medida en que no sólo el proceso de trabajo, sino el proceso funcionaba tan bien como procesos en los que la etapas de purificación y "de acabado" se realizaron por separado, utilizando diferentes técnicas, como se evidencia por los resultados que se muestran en el Ejemplo 7 de la presente solicitud: Descubrimiento inesperado de los presentes inventores que la TFF se puede utilizar directamente para purificar el conjugado anticuerpo-maitansinoide eliminando la necesidad de la cromatografía de intercambio iónico enseñado por D1 o la etapa de cromatografía de no adsorbtiva enseñada por D2 .

Por lo tanto, el proceso descrito en la reivindicación 1 modificada se puede realizar en menos tiempo que los procedimientos descritos en D1 y D2, y cuesta menos que los procedimientos descritos en dichas anterioridades debido a que el conjugado de anticuerpo-maitansinoide se somete a TFF, en lugar cromatografía de intercambio de iones seguida de TFF, según lo enseñado por D1, o a cromatografía no adsorbtiva seguida de TFF, según lo enseñado por D2.

Es bien sabido en la técnica que cada uno de los pasos de purificación resulta en una reducción del rendimiento. Por ejemplo, D2 da a conocer que el rendimiento de conjugado obtenido de la etapa de cromatografía no adsorbtiva está entre 60 % y 65 %, y que el rendimiento de conjugado obtenido de la etapa de la TFF posterior está entre 92% y 100% (véase el párrafo 00443, páginas 161-162, los pasos (6) y (7)). Dado que el proceso descrito en la reivindicación 1 modificada elimina la necesidad de la etapa de cromatografía de intercambio iónico enseñado por D1 y la etapa de cromatografía no adsorbtiva enseñado por D2, el rendimiento del conjugado anticuerpo-maitansinoide producido por el proceso descrito en la reivindicación 1 modificada necesariamente es mayor que el rendimiento del conjugado producido por los procedimientos descritos en D1 y D2.

Además de las mejoras en el rendimiento, la eliminación de la etapa de purificación cromatográfica conduce a otras ventajas. Se evita el tiempo

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significativo necesario para crear y dirigir la cromatografía, al igual que los costos asociados (por ejemplo, mano de obra y materiales). Por lo tanto la eficiencia operativa se ha mejorado y el costo de fabricación se reduce.

En vista de lo anterior, se destaca un efecto técnico inesperado encontrado por parte de los presentes inventores en relación con que la TFF se puede utilizar directamente para purificar el conjugado de anticuerpo-maitansinoide, como se expone en la reivindicación 1 modificada, y que resulta en un proceso que es (1) más rápido que los procedimientos descritos en D1 y D2 , (2) cuesta menos de los procedimientos descritos en D1 y D2 , y (3) produce un mayor rendimiento del conjugado de los procedimientos descritos en D1 y D2 , todos los cuales son muy importantes ventajas comerciales para la producción de grandes lotes de conjugados anticuerpo-maitansinoide .

Ahora en cuanto a la reivindicación 11 modificada y sus dependientes, es necesario señalar que en la realización definida por la reivindicación independiente 11, el procedimiento comprende las etapas (a) - (c), en el que la mezcla no se somete a purificación entre las etapas (a) y (b), y en el que la etapa (c) es una etapa de purificación que comprende someter la mezcla a filtración de flujo tangencial, precipitación selectiva, filtración de adsorción, cromatografía de adsorción, o una combinación de los mismos.

Como se discute en detalle a continuación, los presentes inventores encontraron inesperadamente que la omisión de la etapa de purificación después de la primera etapa en el proceso de "dos etapas" enseñado en D1 y D2 **NO** afecta a la calidad del conjugado final anticuerpo-maitansinoide. Además, por la omisión de una etapa de purificación, el procedimiento reivindicado tiene un rendimiento significativamente mayor en comparación con un proceso que comprende dos etapas de purificación, similar a los procesos que se enseñan en D1 y D2 (véase el Ejemplo 6 de la presente solicitud, 91 % frente a 79 %, respectivamente).

Aunado a lo anterior y como se discutió más arriba, cada uno de D1 y D2 enseña un proceso llamado de "dos etapas " en el que se utiliza una etapa de purificación después de la primera etapa para eliminar el enlazante sin reaccionar del anticuerpo modificado. En particular, D1 enseña la eliminación de enlazante sin reaccionar del anticuerpo modificado por TFF (véase, por ejemplo, página 4, líneas 19-21; página 5, líneas 3-6, y página 6, líneas 34-36), y D2 enseña la eliminación de enlazante sin reaccionar del anticuerpo modificado por cromatografía no adsorbtiva (véase el párrafo 00 443, página 161, la etapa (3), de D2).

La diferencia entre los procesos que se enseñan en D1 y D2 y el proceso de acuerdo con la reivindicación 11 modificada de la presente solicitud, radica principalmente en que la reivindicación 11 requiere que el anticuerpo modificado

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no se someta a purificación después de la primera etapa. Esta modificación puede ser vista como una mejora a los procesos de D1 y D2 ya que el anticuerpo modificado puede ser conjugado directamente a maitansinoide sin la necesidad de una etapa de purificación, simplificando de este modo el método y la mejora de la eficiencia, como se discute en más detalle a continuación.

En efecto, la pregunta, por tanto, que responder es: si hubiera sido evidente para apartarse de la enseñanza clara e inequívoca de D1 (para usar la TFF para purificar el anticuerpo modificado) y D2 (para utilizar la cromatografía no adsorbtiva para purificar el anticuerpo modificado) y en su lugar, eliminar las etapas de purificación antes mencionadas del todo como lo expuesto en la reivindicación 11 modificada.

Sobre este particular, el solicitante se permite decir que no hubiera sido evidente eliminar las etapas de purificación impartidas con claridad y sin ambigüedades por D1 (utilizar TFF para purificar el anticuerpo modificado) y D2 (utilizar la cromatografía no adsorbtiva para purificar el anticuerpo modificado).

Antes de la presente invención, se entendía por los expertos en esta técnica que para la eliminación de los componentes sin reaccionar, disolventes, y productos de reacción de la reacción de modificación, tales como la eliminación de la enlazante sin reaccionar y etanol del anticuerpo modificado, tal como se describe en D1 y D2, era necesario un proceso eficiente que produjera un conjugado anticuerpo-maitansinoide de buena pureza (véase el párrafo 8 de la Declaración Amphlett adjunta a la presente).

La presente invención tal como se define en la reivindicación 11 modificada sólo fue posible debido a que los presentes inventores decidieron ir en contra de esta opinión generalizada y omitir el uso de una etapa de purificación entre la etapa de modificación y la etapa de conjugación, como el expuesto en la reivindicación 11 modificada.

Los resultados obtenidos por los presentes inventores fueron totalmente inesperados en la medida en que no sólo el proceso de trabajo, sino el proceso funcionaba tan bien como los procesos que comprenden una etapa de purificación entre la etapa de modificación y la etapa de conjugación, como se evidencia por los resultados mostrados en el Ejemplo 6 de la presente solicitud.

Más específicamente, los inventores encontraron de manera inesperada que la omisión de la etapa de purificación entre la etapa de modificación y la etapa de conjugación, como el expuesto en la reivindicación 11 modificada **augmenta de manera significativa el rendimiento del conjugado anticuerpo-maitansinoide, sin afectar a la calidad del conjugado.** Como se muestra en el Ejemplo 6 de la presente solicitud, el conjugado anticuerpo-maitansinoide

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producido por un proceso que comprende una sola etapa de purificación, como se expone en la reivindicación 11 modificada, dado el 91% del conjugado, en comparación con un rendimiento de sólo el 79 % cuando el conjugado fue producido por un proceso que comprende dos etapas de purificación, similares a los procesos enseñados por D1 y D2 (véase la Tabla 7 de la memoria descriptiva).

Los datos presentados en el Ejemplo 6 de acuerdo con la presente invención, también demuestran que la calidad del conjugado anticuerpo-maitansinoide producido por un proceso que comprende una sola etapa de purificación, como se expone en la reivindicación 11 modificada, fue similar a la calidad del conjugado producido por un proceso que comprende dos etapas de purificación, similar a los procesos enseñados por D1 y D2 (véase la Tabla 7). Por lo tanto, los datos presentados en el Ejemplo 6 demuestran que el proceso de acuerdo con la reivindicación 11, en el que el anticuerpo modificado no se sometió a purificación, produce un aumento del rendimiento de conjugado de anticuerpo - maitansinoide que es de la misma calidad que un conjugado anticuerpo - maitansinoide preparado con tal purificación del anticuerpo modificado, al tiempo que reduce el tiempo total y el coste requeridos para preparar el conjugado.

Además de las mejoras en el rendimiento, la eliminación de la etapa de purificación para el anticuerpo modificado conduce a otras ventajas. Se evita el tiempo significativo necesario para configurar y ejecutar la etapa de purificación, al igual que los costos asociados (por ejemplo, mano de obra y materiales). Por lo tanto la eficiencia operativa se ha mejorado y el costo de fabricación se reduce.

En este sentido, nuevamente y de manera respetuosa hacemos énfasis sobre el hecho que cada uno de D1 y D2 enseña un proceso llamado de "dos etapas " en el que se utiliza una etapa de purificación después de la primera etapa para eliminar el enlazante sin reaccionar del anticuerpo modificado. En particular, D1 enseña la eliminación de enlazante sin reaccionar del anticuerpo modificado por TFF (véase, por ejemplo, página 4, líneas 19-21; página 5, líneas 3-6, y página 6, líneas 34-36), y D2 enseña la eliminación de enlazante sin reaccionar del anticuerpo modificado por cromatografía no adsorbtiva (véase el párrafo 00 443, página 161, la etapa (3), de D2).

En este sentido, respetuosamente nos permitimos decir que consideramos que el examinador ha hecho un análisis no permitido para la evaluación del nivel inventivo.

En cuanto a las características técnicas antes mencionadas, nos permitimos hacer hincapié en lo argumentado en la interpretación prejudicial 81-IP-2006 del Tribunal que ha establecido lo siguiente: *"Ante ello, es preciso definir lo que se entiende*

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por "obvio" y por "evidente". Lo obvio es aquello que salta a la vista de manera clara y directa; lo que se encuentra o pone delante de los ojos o que no tiene dificultad. Mientras que lo evidente es algo tan cierto y claro que nadie puede dudar racionalmente de ello; se presenta de manera tan perceptible y manifiesta que muestra certeza y claridad¹. Como se podrá ver, la diferencia radica en lo "tan", de forma que algo que resulte obvio no es necesariamente evidente; empero lo que es evidente, es también obvio." (El subrayado es nuestro)

Es claro que la presente invención no salta a la vista de una persona versada en la técnica cuando se cuenta con la información de D1 y D2. Por consiguiente, nos permitimos ratificar el hecho que la invención es inventiva sobre las enseñanzas de la técnica anterior.

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-, 35 y 59 del Código 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 al menos en forma sumaria si afecta a particulares" y "(..) deberá proferirse la decisión definitiva. Esta se motivará en sus aspectos de hecho y de derecho, y en los de conveniencia si es el caso", respectivamente (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.

Señor Superintendente,


SILVANA MARIA LÓPEZ DÍAZ

C.C. 22468787 de Barranquilla

T.P 147463 del CSJ

notificaciones@clarkemodet.com.co

¹ Diccionario de la Lengua Española. Vigésima Primera Edición. Madrid – España. 1992. Definiciones de evidente, evidencia y obvio. Pp. 655 y 1036.

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ANEXOS

Copia en inglés y español de la declaración de Godfrey Amphlett.
Huisman et al., Journal of Membrane Science, 179:79-90 (2000)
Kalyanpur, Biotecnología Molecular, 22:87-98 (2002)
Nuevo pliego reivindicatorio

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Patent Application No. 11/503,781

Confirmation No. 2131

Applicant: Dai et al.

Filed: August 14, 2006

TC/AU: 1647

Examiner: Ian D. Dang

Docket No.: 253788

Customer No.: 23460

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. § 1.132 OF GODFREY AMPHLETT

I, Godfrey Amphlett, do hereby declare as follows:

1. I received a Doctor of Philosophy degree in Biochemistry from Birmingham University in the United Kingdom. Following my graduate work, I completed my postdoctoral training at the University of Notre Dame in Notre Dame, IN.

2. I have been employed at ImmunoGen, Inc., which is the assignee of the present application, from 2001 to the present. Since 2005, I have been Executive Director of Process and Product Development at ImmunoGen, Inc. Prior to my current position, I served as Senior Director of Process Development and Manufacturing from 2001 to 2005. Prior to joining ImmunoGen, Inc. in 2001, I was employed at Genetics Institute in Cambridge, MA.

3. I am one of the co-inventors of the subject matter disclosed and claimed in the present application.

4. I have reviewed International Patent Application Publication WO 03/057163 (Mazzola et al.) (hereinafter "the Mazzola publication").

5. The Mazzola publication discloses a method for conjugating a maytansinoid to an antibody comprising the steps of (a) reacting a disulfide-containing linker with the antibody at about pH 5.0 to about pH 8.0 to form a modified antibody; (b) removing unreacted linker from the modified antibody by tangential flow filtration; (c) conjugating the modified antibody with the maytansinoid at about pH 6.0 to about pH 6.5 in a solvent comprising dimethylacetamide or acetonitrile; and (d) purifying the modified antibody-maytansinoid conjugate by ion exchange chromatography (see, e.g., the Mazzola publication at page 2, lines 22-34). No alternative purification steps are disclosed in the Mazzola publication for either step (b) or step (d).

6. Tangential flow filtration is a membrane based method that uses pressure and tangential fluid flow to drive the separation of components in a fluid. The membrane separates the components based on size. In contrast, ion exchange chromatography is a process that allows the separation of ions and polar molecules based on the charge properties of the molecules. Ion exchange chromatography does not take particle size into account.

7. The Mazzola publication teaches the use of tangential flow filtration to remove the "unreacted linker and ethanol" from the modified antibody prior to conjugation with the maytansinoid (see page 4, lines 19-21), and the use of ion exchange chromatography (e.g., S-Sepharose or ceramic hydroxyapatite column) to purify the antibody-DM1 conjugate from "unreacted DM1, DMA and aggregates" (see page 5, lines 16-18). In view of the significant differences between tangential flow filtration and ion exchange chromatography, an ordinary researcher as of the priority date of the present application in 2005 would not have considered exchanging the two techniques within the method disclosed in the Mazzola publication, i.e., to use ion exchange chromatography to remove the "unreacted linker and ethanol" from the modified antibody prior to conjugation with the maytansinoid, and to use tangential flow filtration to purify the antibody-DM1 conjugate from "unreacted DM1, DMA and aggregates." Indeed, an ordinary researcher at the time would have understood that tangential flow filtration, which depends on size differences for separation, is not suitable for removing similarly sized

aggregates from the antibody-DM1 conjugates, as is taught to be necessary by the Mazzola publication.

8. Prior to the present invention, it was reasonable to assume that the removal of unreacted components, solvent, and reaction products from the modification reaction, such as removing the unreacted linker and ethanol from the modified antibody, as disclosed in the Mazzola publication, was necessary for an efficient process that yielded a cell-binding agent-cytotoxic agent conjugate of good purity. However, my co-inventors and I unexpectedly discovered that omitting purification of the cell-binding agents having linkers bound thereto, e.g., omitting tangential flow filtration, selective precipitation, adsorptive filtration, or adsorptive chromatography purification between the modification step and the conjugation step, results in a purified cell-binding agent-cytotoxic agent conjugate of at least the same quality and quantity as a conjugate prepared with purification of the cell-binding agents having linkers bound thereto, while reducing the overall time required to prepare the conjugate.

9. To my knowledge, there was no information on the efficiency of tangential flow filtration membranes for use in separating unreacted DM1 and DM4 related species, as well as solvents, from antibody-maytansinoid conjugates as of the priority date of the present application in 2005. As indicated above, researchers at the time believed that tangential flow filtration would not be an effective process for the purification of the antibody-maytansinoid conjugates. In particular, DM1 and DM4 related species have very low solubility in fully aqueous solutions and are maintained in solution during the conjugation reaction by the presence of an organic solvent (e.g., around 5% dimethylacetamide). Once tangential flow filtration of a conjugation reaction mixture is initiated, the organic solvent is expected to be efficiently and rapidly removed. It was thought that the rapid removal of the solvent would cause the DM1 or DM4 related species to become insoluble, leading to precipitation of DM1 and DM4 related species, which would clog the tangential flow filtration system and render it inoperable. Additionally, it was thought that the hydrophobic DM1 and DM4 related species would interact non-covalently with the conjugate molecules, as well as with the tangential flow filtration membranes and system components (e.g., tubing and valves), resulting in clogging and poor performance of the filtration system, and making cleaning and reuse of the filtration system difficult. However, my

co-inventors and I unexpectedly discovered that tangential flow filtration can be used to provide cell-binding agent-cytotoxic agent conjugates of at least similar quantity and purity as cell-binding agent-cytotoxic agent conjugates purified with ion exchange chromatography.

10. I hereby declare that all statements made herein of my own knowledge are true, that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 20th July 2009

Godfrey Amphlett
Godfrey Amphlett

EN LA OFICINA DE MARCAS Y PATENTES DE LOS Estados Unidos

Solicitud de Patente No. 11/503,781

Confirmación No. 2131

Solicitante: Dai et al.

Presentada: 14 de agosto, 2006

TC/AU: 1647

Examinador: Ian D. Dang

Expediente No.: 253788

Cliente No.: 23460

Comisionado para Patentes

P.O. Box 1450

Alexandria. VA 22313-1450

DECLARACIÓN DE CONFORMIDAD CON 37 C.F.R. § 1.132 DE GODFREY**AMPHLETT**

Yo, Godfrey Amphlett, por la presente declaro lo siguiente:

1. Recibí el grado de Doctor en Bioquímica de la Birmingham University en el Reino Unido. Después de mi trabajo de grado, completé mi formación postdoctoral en la University of Notre Dame en Notre Dame, IN.

2. He sido empleado en InmunoGen, Inc., que es el cesionario de la presente solicitud, desde el 2001 hasta la actualidad. Desde el 2005, he sido Director Ejecutivo de Desarrollo de Proceso y Producto en InmunoGen, Inc. Antes de mi cargo actual, me desempeñé como Director Senior de Desarrollo de Procesos y Fabricación desde el 2001 hasta el 2005. Antes de trabajar en InmunoGen, Inc. en 2001, trabajé en Genetics Institute en Cambridge, MA.

3. Soy uno de los co-inventores de la materia objeto descrita y reivindicada en la presente solicitud.

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4. He revisado la Publicación de la Solicitud de Patente Internacional WO 03/057163 (Mazzola et al.) (en los sucesivo "la publicación Mazzola").

5. La publicación Mazzola describe un método para conjugar un maitansinoide con un anticuerpo que comprende las etapas de (a) hacer reaccionar un ligador que contiene disulfuro con el anticuerpo en aproximadamente pH 5.0 a aproximadamente pH 8.0 para formar un anticuerpo modificado; (b) retirar el ligador que no ha reaccionado del anticuerpo modificado mediante filtración de flujo tangencial; (c) conjugar el anticuerpo modificado con el maitansinoide en aproximadamente pH 6.0 a aproximadamente pH 6.5 en un disolvente que comprende dimetilacetamida o acetonitrilo; y (d) purificar el conjugado de anticuerpo-maitansinoide modificado mediante cromatografía de intercambio iónico (ver, por ejemplo, la publicación Mazzola en la página 2, líneas 22-34). No se describen etapas de purificación alternativas en la publicación Mazzola para ya sea la etapa (b) o la etapa (d).

6. La filtración de flujo tangencial es un método basado en membrana que utiliza presión y flujo de fluido tangencial para conducir la separación de los componentes en un fluido. La membrana separa los componentes con base en el tamaño. En contraste, la cromatografía de intercambio iónico es un proceso que permite la separación de los iones y las moléculas polares con base en las propiedades de carga de las moléculas. La cromatografía de intercambio iónico no tiene en cuenta el tamaño de partícula.

7. La publicación Mazzola enseña el uso de filtración de

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flujo tangencial para retirar el "ligador que no ha reaccionado y el etanol" del anticuerpo modificado antes de conjugación con el maitansinoide (ver página 4, líneas 19-21), y el uso de cromatografía de intercambio iónico (por ejemplo, columna de hidroxapatita cerámica o S-Sefarosa) para purificar el anticuerpo-DM1 conjugado a partir de "DM1 que no ha reaccionado, DMA y agregados" (ver página 5, líneas 16-18). En vista de las diferencias significativas entre la filtración de flujo tangencial y la cromatografía de intercambio iónico, un investigador medianamente versado desde la fecha de la prioridad de la presente solicitud en el 2005 no habría considerado intercambiar las dos técnicas dentro del método descrito en la publicación Mazzola, es decir, utilizar la cromatografía de intercambio iónico para retirar el "ligador que no ha reaccionado y el etanol" a partir del anticuerpo modificado antes de conjugación con el maitansinoide, y utilizar la filtración de flujo tangencial para purificar el anticuerpo-DM1 conjugado a partir del "DM1 que no ha reaccionado, el DMA y los agregados." En efecto, un investigador medianamente versado con el tiempo habría entendido que la filtración de flujo tangencial, que depende de las diferencias de tamaño para la separación, no es adecuada para retirar de forma similar los agregados dimensionados de los conjugados de anticuerpos-DM1, como se enseña que es necesario por la publicación Mazzola.

8. Antes de la presente invención, era razonable asumir que el retiro de los componentes que no reaccionan, el disolvente, y los productos de reacción de la reacción de modificación, tal como el retiro del ligador que no ha reaccionado y el etanol del anticuerpo modificado, como se discute en la publicación Mazzola, era necesaria para un proceso

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eficiente que produce un conjugado de agente de unión celular - agente citotóxico de buena pureza. Sin embargo, mis co-inventores y yo descubrimos de forma inesperada que omitir la purificación de los agentes de unión celular que tienen ligadores unidos a estos, por ejemplo, omitir la filtración de flujo tangencial, precipitación selectiva, filtración de adsorción, o purificación con cromatografía de adsorción entre la etapa de modificación y la etapa de conjugación, resulta en un conjugado de agente de unión celular-agente citotóxico purificado de por lo menos la misma calidad y cantidad que un conjugado preparado con la purificación de los agentes de unión celular que tienen ligadores unidos a estos, mientras que se reduce el tiempo general requerido para preparar el conjugado.

9. A mi entender, no existe información sobre la eficiencia de las membranas de filtración de flujo tangencial para uso en separar especies relacionadas DM1 y DM4 que no ha reaccionado, así como también, los disolventes, a partir de los conjugados de anticuerpo-maitansinoide desde la fecha de prioridad de la presente solicitud en el 2005. Como se indicó anteriormente, los investigadores en su momento consideraron que la filtración de flujo tangencial no sería un proceso efectivo para la purificación de los conjugados de anticuerpo-maitansinoide. En particular, las especies relacionadas DM1 y DM4 tienen muy baja solubilidad en soluciones completamente acuosas y se mantienen en solución durante la reacción de conjugación mediante la presencia de un disolvente orgánico (por ejemplo, alrededor de 5% de dimetilacetamida). Una vez se inicia la filtración de flujo tangencial de una mezcla de reacción de conjugación, se espera que el disolvente orgánico sea retirado rápida y eficientemente. Se enseñó que el retiro rápido del

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disolvente provocaría que las especies relacionadas DM1 o DM4 se vuelvan insolubles, lo que obstruiría el sistema de filtración de flujo tangencial y lo haría inoperable. Adicionalmente, se enseña que las especies relacionadas DM1 y DM4 hidrófobas interactuarían de forma no covalente con las moléculas de conjugado, así como también con las membranas de filtración de flujo tangencial y los componentes del sistema (por ejemplo, tuberías y válvulas), que resultan en la obstrucción y pobre desempeño del sistema de filtración, y haría difícil la limpieza y reutilización del sistema de filtración. Sin embargo, mis co-inventores y yo descubrimos de forma inesperada que se puede utilizar la filtración de flujo tangencial para proporcionar los conjugados de agente de unión celular-agente citotóxico de por lo menos una cantidad y pureza similar como los conjugados de agente de unión celular -agente citotóxico purificados con cromatografía de intercambio iónico.

10. Por la presente declaro que todas las declaraciones hechas aquí de mi propio conocimiento son verdaderas, que todas las declaraciones hechas sobre la información y creencia se consideran que son verdaderas, y que estas declaraciones se hacen con el conocimiento que las declaraciones falsas intencionales y similares así hechas se castigan con multa o prisión, o ambos, de conformidad con el Artículo 1001 del Título 18 del Código de los Estados Unidos, y que dichas declaraciones falsas intencionales pueden perjudicar la validez de la solicitud o cualquier patente emitida a la misma.

Fecha: 20 de julio 2009

Aparece firma

Godfrey Amphlett

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The effect of protein–protein and protein–membrane interactions on membrane fouling in ultrafiltration

Ingmar H. Huisman*, Pedro Prádanos, Antonio Hernández

Dpto. Termodinámica y Física Aplicada, Fac. Ciencias, Universidad de Valladolid C/Real de Burgos s/n, Valladolid, Spain

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Abstract

It was studied how protein–protein and protein–membrane interactions influence the filtration performance during the ultrafiltration of protein solutions over polymeric membranes. This was done by measuring flux, streaming potential, and protein transmission during filtration of bovine serum albumin (BSA) solutions at various pH values using various membranes with different cut-off values.

It was found that protein–membrane interactions influence the fouling behaviour in the initial stages of filtration. In the high-fouling regime (later stages of filtration), protein–protein interactions dictate the overall performance.

AFM images of the membrane surfaces taken after the filtration experiments showed that the membranes were totally covered by a protein fouling layer. The structure of this fouling layer depended strongly on pH. In particular, very open structures with high permeabilities were found at low pH (below the iso-electric point of the protein). These induced high values of flux and protein transmission. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Ultrafiltration; Protein; Physicochemical interactions; Protein–membrane interactions; Fouling

1. Introduction

Ultrafiltration is a powerful technique that can be used to concentrate or fractionate protein solutions. It is gentler towards the delicate proteins than separation processes based on phase changes such as evaporation, and more economical than precise separation processes such as gel chromatography. The main problem restricting its practical application is membrane *fouling* and the resulting time-dependent flux and rejection behaviour. The main factors that influence fouling are the *hydrodynamics* of the process and the *physicochemical properties* of membrane

and feed solution. The current paper will focus on the latter of these factors.

The influence of solution pH on fouling has been studied by many authors [1–11,22,25]. One key result, obtained by several authors, is that severe fouling is observed at a pH equal to the iso-electric point of the protein [1–6]. Fluxes show a minimum at this pH value and increase both for lower and for higher pH values. Several explanations have been proposed for this phenomenon.

Some authors argue that proteins are strongly hydrophobic at their iso-electric point and that their adsorption on membranes may therefore be increased due to *hydrophobic interaction* [3,4,6]. The well-known fact that hydrophobic membranes foul more rapidly than hydrophilic membranes is in accordance with this explanation [9]. Other authors point

* Corresponding author. Present address: TNO Voeding, AMKM, P.O. Box 360, 3700 AJ Zeist, The Netherlands.
E-mail address: huisman@bigfoot.com (I.H. Huisman).

out that proteins in solution may *aggregate* more readily at their iso-electric point, because of reduced electrostatic repulsion, and these aggregates may cause the fouling [3,7]. This explanation is supported by the finding that prefiltering of the feed solution (i.e. removing possibly available aggregates) reduces the extent of fouling [7]. Others claim that the permeability of the fouling layer is lower at the iso-electric point, as the proteins have *small sizes* and thus form a densely packed layer [1,2]. This may explain the low fluxes observed at the iso-electric point.

Other results indicate that, during the very first seconds of a filtration experiment, the membrane is covered by a protein monolayer and thus acquires the protein electrostatic charge. Adsorption of more protein is therefore hindered by *electrostatic repulsion* at all pH values except for the iso-electric point [8]. If this assumption is true, it can be shown by applying a simple force balance that the flux is expected to increase linearly with the square of the zeta potentials of the proteins. Palacek and Zydney [18] demonstrated that the flux during the ultrafiltration of protein solutions indeed increased linearly with the square of the zeta potential of the proteins, in accordance with theory. Recently, it was shown that more advanced modelling, describing in detail the diffusion, convection, and physicochemical interactions between the particles in solution, resulted in the same conclusion: the steady-state ultrafiltration flux increases with the square of the zeta potential of the particles [19].

Another result, found by many authors, is that the protein transmission shows its highest value at pH equal to the iso-electric point of the protein [4,6,10,11]. Some authors believe that this is a charge effect: protein retention is thought to be caused in part by *electrostatic repulsion* and this part is absent at the iso-electric point, thus causing low retention, i.e. high protein transmission [6]. Other authors point out that proteins at their iso-electric point have *smaller sizes* than at other values of pH, and that they therefore pass more easily through the membrane [11]. Finally, the observed protein transmission is known to increase with increasing *concentration polarisation*, as the protein concentration at the membrane surface increases. Concentration polarisation is more severe at the iso-electric point [3] and transmissions are thus expected to show a corresponding maximum. From calculations by mass transfer theories, it was

found that 'real' (intrinsic) transmission values were always very close to 0, whereas observed transmission showed much higher values, and depended on solution pH [4,10].

In order to gather support for the theoretical explanations given above, characterisation experiments of membranes and proteins have been performed by many authors. Electrical properties of membranes can be obtained from streaming potential measurements [4,6]. Hydrophobicity of membranes can be obtained from contact angle measurements [12,23]. Information on the properties of the fouling layer can be obtained by performing streaming potential or atomic force microscopy (AFM) measurements on the fouled membrane [24]. It has been reported that the membrane during filtration assumes the electrostatic properties of the protein, i.e. the iso-electric point determined through the pores of the fouled membrane equalled that of the protein [4,6]. This may indicate that the pore surfaces of the fouled membrane are totally covered by proteins or that a thick layer of proteins covers the outer membrane surface and dominates the streaming potential behaviour. Koehler et al. [5] measured protein–protein and protein–membrane interaction forces by a surface forces apparatus. Correlating obtained data with ultrafiltration flux measurements, they showed that both protein–protein and protein–membrane interaction forces are of importance for the fouling behaviour.

In general, protein aggregation and protein adsorption on surfaces is found to show a maximum at the iso-electric point. However, some studies point out that relatively high levels of denaturation, and consequently aggregation, are found in acidic solutions with pH values below the iso-electric point [15,16]. At such low pH values, the protein is more 'bulky' with parts of the polymer chain 'sticking out' [16,17]. By measuring the forces between protein layers and polymer films, it was shown that, below the iso-electric point, the tertiary structure of the proteins was disturbed upon interaction with the polymer films [17]. It thus seems to be of interest to compare membrane fouling results obtained at the iso-electric point with those obtained both above and below the iso-electric point.

It may be clear from the above that the observed flux and retention behaviour in ultrafiltration can be related to many fouling mechanisms (hydrophobic inter-

actions, electrostatic interactions) and protein properties (size, aggregation behaviour). Under different experimental conditions, the main fouling mechanism or the most important protein property may differ. The aim of the current study is to investigate how the dominant mechanism of fouling depends on the experimental conditions. In order to do this, we combined existing experimental methods such as measurement of flux, retention, and streaming potential and AFM measurements, and performed experiments under different experimental conditions, i.e. both with highly retentive and with non- or almost non-retentive membranes and both in the high-fouling and the low-fouling regime.

2. Materials and methods

2.1. Measurement equipment

The membrane fouling experiments were carried out in a flat-sheet ultrafiltration module, equipped with two Ag/AgCl electrodes, which could measure the streaming potential developed across the pores of the membrane. The module (with a single channel of transversal dimensions of 60 mm × 0.5 mm) and the experimental rig of which it was a part, are described in detail in another publication [13].

Protein concentrations in feed and permeate were obtained from absorbance measurements at 270 nm (specific absorption wavelength for bovine serum albumin (BSA)) using a UV–VIS spectrophotometer (Shimadzu UV 160A). Protein transmission values, Tr , were calculated from measured concentrations, using the definition

$$Tr = \frac{C_{perm}}{C_{feed}} \quad (1)$$

where C_{perm} is the BSA concentration in the permeate and C_{feed} the BSA concentration in the feed solution.

Note that transmission is related to the retention, Ret , through

$$Tr = 1 - Ret \quad (2)$$

AFM images were obtained using a Nanoscope IIIa Atomic Force Microscope (Digital Instruments) in Tapping Mode®.

The zeta potential of BSA was calculated from electrophoretic mobilities measured by using a Zetamaster® from Malvern Instruments Ltd. In order to control the pH, a buffer of $H_3PO_4:NaH_2PO_4:Na_2HPO_4:Na_3PO_4$ was used.

2.2. Membranes and chemicals

Polysulphone ultrafiltration membranes were used, with nominal cut-off values of 30, 100, and 300 kDa (M30, M100, M300, Millipore, USA). The characterisation of these membranes has been described in other publications [13,14]. Results of this characterisation (pore size values and iso-electric points) are given in Table 1. Membranes were freed from preservatives by treatment in an ultrasonic bath three times for 3 min with 300 ml of bidistilled, degasified ultrafiltrated and de-ionised (MilliQ treated) water with resistivities over 18 MΩ cm.

All electrolyte and protein solutions used were based on the same high-quality water. The salts used, KCl, HCl and KOH, were of pro-analysis quality. The protein used was BSA purchased from Sigma (A-7638, purity > 99%). BSA has a molecular weight of 67 000 Da and an iso-electric point at pH about 4.9 [20].

2.3. Methods

Ultrafiltration experiments were performed with feed solutions containing 0.5 g/l BSA at a concentra-

Table 1
Data of the membranes used

Membrane	Cut-off (kDa)	Pore radius, nominal (nm)	Pore radius, AFM [14] (nm)	Pore radius, SEM [21] (nm)	Iso-electric point [13]
M30	30	3.78	7	2.4	<3.2
M100	100	7	16	4.6	<3.2
M300	300	15	28	8.8	5

tion of added KCl of 10^{-3} mol/l and at various pH values. pH was regulated by adding KOH or HCl from 0.1 mol/l stock solutions. The pH control changes the ionic strength from 10^{-3} to 2×10^{-3} mol/l which is low enough to avoid significant changes linked to the ionic strength. During each experiment, cross-flow velocity and transmembrane pressure were kept constant and a decrease in flux (because of membrane fouling) was observed. Flux, transmembrane pressure, cross-flow velocity, and potential difference were measured continuously. The protein concentrations in the feed and the permeate and the pH of the feed solution were determined whenever about 100 g of permeate had been collected (what happens after around 100 s) for a total volume of 2 l for the experimental rig. After measuring the protein concentration, the permeate was returned to the feed tank (total recycle mode).

The operating temperature was 30°C. The cross-flow velocity was 1.6 m/s ($Re = 1600$) and the transmembrane pressure was 70 kPa for all experiments, except for those using the M300 membranes, which were performed at 1.5 m/s ($Re = 1500$) and 60 kPa. Each experiment lasted for about 5 h.

The streaming potential is usually obtained from the slope of the graph of potential difference versus transmembrane pressure. As in the current fouling experiments, the pressure was kept at a constant value, such graphs could not be made. Instead, we measured the potential difference across the membrane at zero transmembrane pressure *before* and *after* the experiment. The difference between these values was found to be rather small. It was then assumed that the potential difference at zero membrane pressure (the *asymmetry* of the electrodes, A) changed linearly with time during the experiment: $A(t) = A(0) + at$. The streaming potential, SP, could then be calculated from the measured potential difference $V(t)$ and transmembrane pressure, TMP, by Eq. (3):

$$SP = \frac{V(t) - A(t)}{TMP} \quad (3)$$

The results obtained for the streaming potential according to Eq. (3) proved to be highly reproducible. This confirms that the slope of the linear dependency of potential on applied pressure is accurately enough evaluated by this method. The dependency must be linear as the electrical kinetics is known to be much faster than the fouling one.

3. Results and discussion

3.1. Fouling experiments

Time dependence of flux, streaming potential, and protein transmission at neutral pH are presented in Figs. 1 and 2 for the more retentive M30 membrane and the almost totally non-retentive M300 membrane, respectively. For both membranes, two regimes can be seen: one early regime where transmissions are high and the flux decline is fast, and one later regime where transmissions are much lower and the flux decline slower. For M30, the initial regime can only be noted for the very initial time. In contrast, for the M300 membrane at neutral pH (at least), this early regime extends to larger times. The dominant fouling mechanism in the first regime may be protein adsorption or (less probable) pore plugging, leading to fast flux decline, yet leaving transmissions high. The dominant mechanism in the second regime may be cake layer build-up, leading to slower flux decline and lower values of transmission. A final step, where transmission is almost constant, is reached for both membranes. This final constant transmission is lower and appears later for M30. This behaviour should be due to the appearance of a stationary cake layer whose structure and/or thickness changes only very slowly. AFM images of the membrane surfaces (discussed later in more detail) show that the membranes were covered with thick layers of proteins after the fouling experiments, thus supporting the present mechanistic model.

To investigate this two-step fouling mechanism in more detail, the values of flux, streaming potential, and protein transmission were collected during (or directly after) the first step (at $t = 100$ s) and during (or after) the final step (at $t = 10\,000$ s). Although the length of the two regimes, as determined from graphs in Figs. 2 and 3, seemed to depend on the pH and membrane cut-off, it was found to be more instructive to use the same time intervals (100 and 10 000 s) for all experiments. Around these times, there are very low changes in transmission, thus assuring that the membranes are in similar stages of fouling. Values collected at 100 s are referred to as *initial* values (i.e. related to the initial flux decline), and values collected at 10 000 s are referred to as *final* values.

Initial and final fluxes are presented in Fig. 3 versus the pH of the feed solution. It is clearly seen that

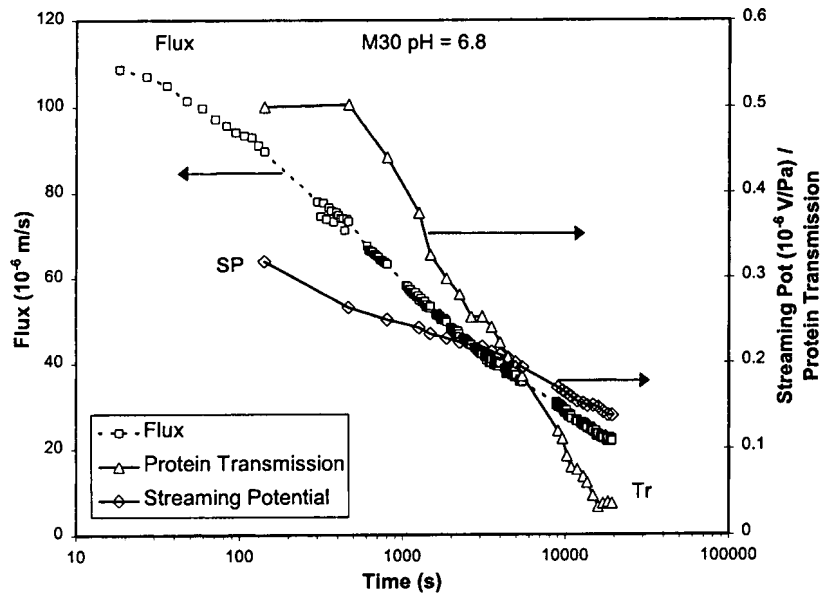


Fig. 1. Permeate flux, streaming potential, and protein transmission vs. time during the ultrafiltration of 0.5 g/dm³ BSA solutions using a 30 kDa membrane at neutral pH.

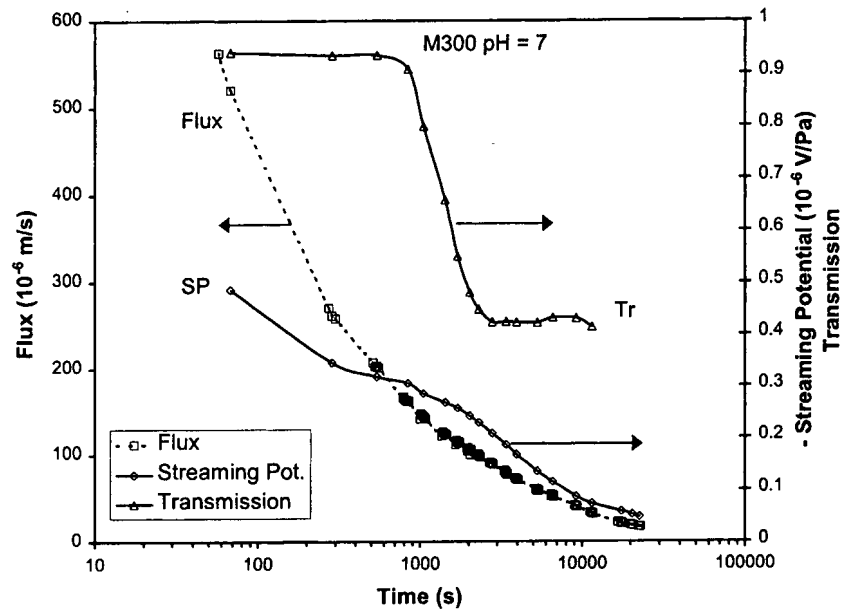


Fig. 2. Permeate flux, streaming potential, and protein transmission vs. time during the ultrafiltration of 0.5 g/dm³ BSA solutions using a 300 kDa membrane at neutral pH.

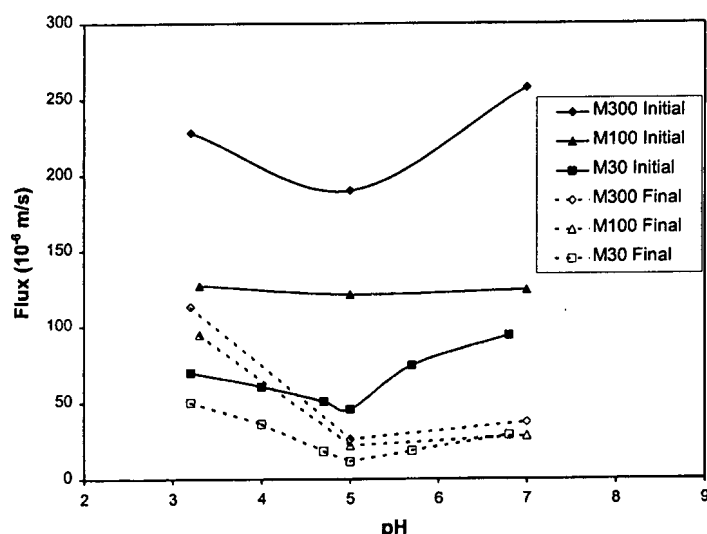


Fig. 3. Initial flux (after 100 s of fouling) and final flux (after 10 000 s of fouling) during the ultrafiltration of 0.5 g/dm³ BSA solutions vs. pH of the feed solution. Results are shown for membranes with cut-off values of 300, 100, and 30 kDa.

both the initial flux and the final flux show a minimum at pH = 5, i.e. both the initial and the final flux decrease are greatest at the iso-electric point of the protein. Note that the fluxes are not symmetrical around the iso-electric point. The initial fluxes show greater values for pH above 5 than for pH below 5. The final fluxes show lower values for pH above 5 than for pH below 5. It will be shown later that this is related to the type of interaction that dominates the fouling at the different stages of filtration.

Initial and final averaged streaming potentials are presented in Fig. 4 versus pH of the feed solution for the M30 membrane. The streaming potential of the clean membrane, before filtration, is also shown. The interpretation of the data presented here is rather difficult, owing to the complexity of the system. The streaming potential during ultrafiltration depends on the pore size, which will be affected by the properties of the protein layer formed on the membrane. Moreover, the streaming potential measured over the membrane and fouling layer will result in some average value reflecting both membrane and fouling layer properties. Finally, protein may adsorb on the electrodes, thus affecting the observed potential difference.

A general observation made from Fig. 4 is that, during fouling, the streaming potential changes slowly

from the pattern characteristic to the clean membrane (iso-electric point < 3) to a pattern characteristic to the protein (iso-electric point = 5). As the fouling at pH = 5 is more severe than at other pH values, this transition is faster at pH = 5, leading to the observed local maximum in streaming potential. One data point deviates from this general behaviour: at pH = 3.2, the streaming potential increases rapidly to strongly negative values upon introduction of the protein solution in the filtration circuit. During filtration, however, the streaming potential decreases slowly, and after some time, changes sign and assumes positive values, i.e. it behaves as would be expected from a negative membrane slowly being covered by positively charged protein. Given that this, as the other data points in Fig. 4, is fairly reproducible, the possibility of an artefact should be rejected. In any case, the reason for the strong increase in streaming potential at the very start of the experiment is not known.

3.2. Protein transmission

It is clear that protein transmission is caused by several effects that occur on the surface and inside the pores simultaneously with different intensities. All

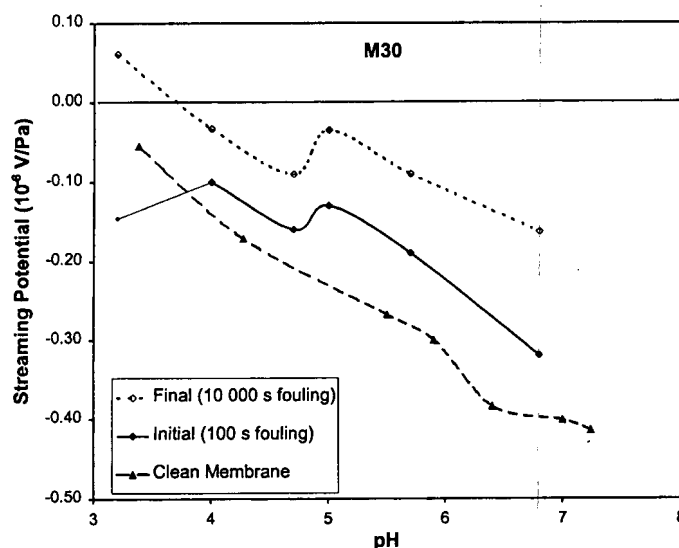


Fig. 4. Initial streaming potential (after 100 s of fouling) and final streaming potential (after 10 000 s of fouling) during the ultrafiltration of 0.5 g/dm³ BSA solutions using a 30 kDa membrane vs. pH of the feed solution. The streaming potential of the clean membrane, before filtration, is also shown.

these effects are related to pH, for example

1. the size of protein molecules and their aggregates;
2. the protein–membrane interaction;
3. the properties of the cake layer of proteins or aggregates.

Fig. 5 shows the initial and final protein transmission for the three different membranes versus pH. For the less retentive membranes M100 and M300, the initial transmission seems to be based on a size effect. Initial transmission is highest at the iso-electric point (pH = 5), where the protein assumes its minimum size. Transmission is lower at pH = 7, where the protein is larger, and it is again lower at pH = 3, where the polymer chain may be partly unfolded and the protein will have its maximum extension [16,17]. Note that the effect of pH is much more important for the M100 membrane, which has a pore size similar to the size of the protein molecule, than for the M300 membrane, which has a pore size that is larger than the size of the protein molecule.

The initial transmission of the M30 membrane follows the same general trend for low and high values of pH as the M100 and M300 membranes. However, at the iso-electric point, the M30 transmission shows a minimum. This minimum must be caused by the high

extent of fouling that occurs at the iso-electric point. Of course, initial fouling affects the transmission of the more retentive M30 membrane much more than that of the less retentive membranes.

The *final* protein transmission for all three membranes follows a general trend opposite of that of the *initial* transmission: protein transmission *decreases* with increasing pH, the transmission at pH = 3 is always higher than that at pH = 7. This trend is explained by the AFM images of the membrane surfaces taken after fouling. As an example, the images of the M300 membrane are shown in Fig. 6. The AFM pictures showed that all membranes were completely covered by protein layers after the fouling experiment. This along with the almost constant transmission in the final steps of fouling (see Figs. 1 and 2) allows the assumption that final protein transmission should be governed mainly by the properties of this fouling layer. As seen in Fig. 6, at pH = 3, the bulky proteins form a very open structure with large pores. At pH = 7, the layer seems to be constituted of small particles that form a structure that is less open. At the iso-electric point (pH = 5), the membrane seems to be covered by a dense layer, that shows strong irregularities in its thickness (as

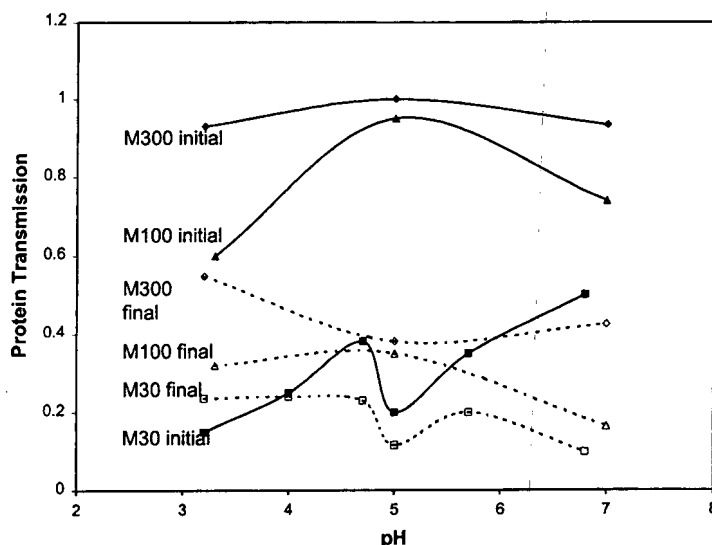


Fig. 5. Initial protein transmission (after 100 s of fouling) and final protein transmission (after 10 000 s of fouling) during the ultrafiltration of 0.5 g/dm³ BSA solutions vs. pH of the feed solution. Results are shown for membranes with cut-off values of 300, 100, and 30 kDa.

indicated by the roughness values), but few 'real pores'.

Comparing the images in Fig. 6 with the final fluxes for the M300 membrane in Fig. 3 and with the final protein transmissions for the M300 membrane in Fig. 5, a clear correlation is observed. At pH = 3, the fouling layer has an open structure: final flux and final transmission are therefore high. At pH = 7, the fouling layer has a less open structure: final flux and final transmission are therefore lower. At pH = 5, the fouling layer is very dense: final flux and final transmission are at a minimum.

Images of the M30 and M100 membranes were also taken (but are not shown). The trends observed were very similar to those shown in Fig. 6, although differences in fouling layer structures for these membranes were less pronounced than for the M300 membrane. Note that the final transmission of the M100 membrane shows a local maximum at the iso-electric point, in contrast to the results obtained for the other two membranes and the explanation given above. As has been mentioned before, the influence of protein size is very important when using the M100 membrane, as its pore size is very similar to the size of the protein. Final transmission is therefore highest at the iso-electric point, where the protein has its smallest extension.

Note from Fig. 5 that the protein transmission of the M30 membrane *increases* with time at pH = 3 (the final transmission is higher than the initial transmission). Both the streaming potential measurements, presented in Fig. 4, and the flux measurements, presented in Fig. 3, show that substantial fouling occurs between the initial and the final measurements. However, the fouling layer, formed on top of the M30 membrane at pH = 3, contains larger pores than the membrane itself and therefore does not hinder the passage of proteins. Although this explains why the transmission does not decrease, it does *not* explain why the transmission *increases*. This could be due to the effect of a high initial adhesion (or adsorption) that leads to a low transitory transmission. This effect is, of course, only relevant for highly retentive membranes as M30.

3.3. Correlation with zeta potentials

As stated in Section 1, the observed fouling behaviour may be a result of at least two different interactions: *protein-membrane* interactions, or *protein-protein* interactions. These interactions can be of two types: electrostatic or hydrophobic. A measure for the strength of the electrostatic interaction between two surfaces is the product of the zeta potentials of the

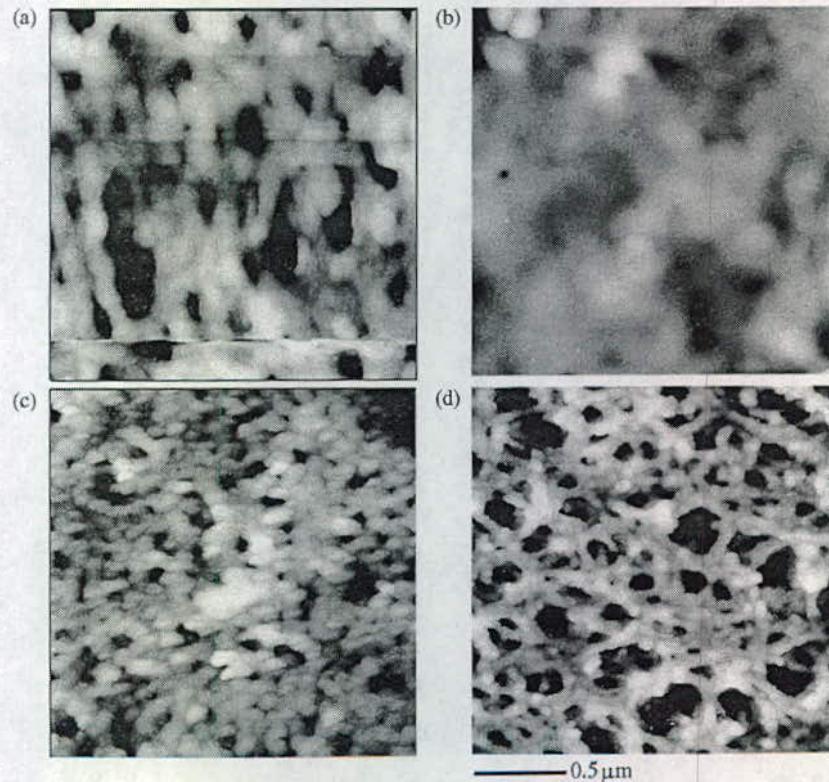


Fig. 6. AFM images of the M300 membrane taken before fouling experiments (d) and after fouling for 20 000 s with a 0.5 mol/dm³ BSA solution at (a) pH = 3, (b) pH = 5, and (c) pH = 7: scan size — 1.72 μm × 1.72 μm; z-range — 150 nm; average roughness obtained from 15 μm × 15 μm — (a) 37.9 nm, (b) 53.4 nm, (c) 29.9 nm and (d) 15.6 nm.

surfaces. The electrostatic interaction between protein and membrane is thus characterised by $\zeta_p \cdot \zeta_m$ and the electrostatic interaction between two protein molecules is characterised by $\zeta_p \cdot \zeta_p$. ζ_p denotes the zeta potential of the protein and ζ_m denotes the zeta potential of the membrane. Positive values of $\zeta_p \cdot \zeta_m$ and $\zeta_p \cdot \zeta_p$ represent *repulsion*. Fluxes are therefore expected to increase (linearly) with increasing values of $\zeta_p \cdot \zeta_m$ and/or $\zeta_p \cdot \zeta_p$.

The hydrophobicity of a surface decreases when more charged groups are present, i.e. the hydrophobicity decreases with increasing absolute value of the zeta potential. This is true if the zeta potential is accepted as an accurate measure of the net charge *at all points* on the corresponding surfaces in what concerns the colloidal behaviour of both membrane and protein. Given the specific characteristics of the membrane–protein

used, we can accept that positive and negative zones do not exist leading to a zeta potential accurately representing their colloidal interactions at all points. The hydrophobic attraction between protein and membrane will therefore decrease with increasing value of $|\zeta_p \cdot \zeta_m|$ and the hydrophobic attraction between two protein molecules will increase with decreasing value of $|\zeta_p \cdot \zeta_p|$. Therefore, if hydrophobic interactions are important, the intensity of fouling is expected to *decrease* with increasing values of $|\zeta_p \cdot \zeta_m|$ or $|\zeta_p \cdot \zeta_p|$ and the *flux* is expected to *increase* accordingly.

In Fig. 7, the initial and the final flux are plotted versus $\zeta_p \cdot \zeta_p$. The zeta potentials of BSA protein (ζ_p) at the various values of pH were obtained from measurements performed earlier in our laboratory [20]. The zeta potentials of the clean membrane (ζ_m) were obtained from streaming potential measurements

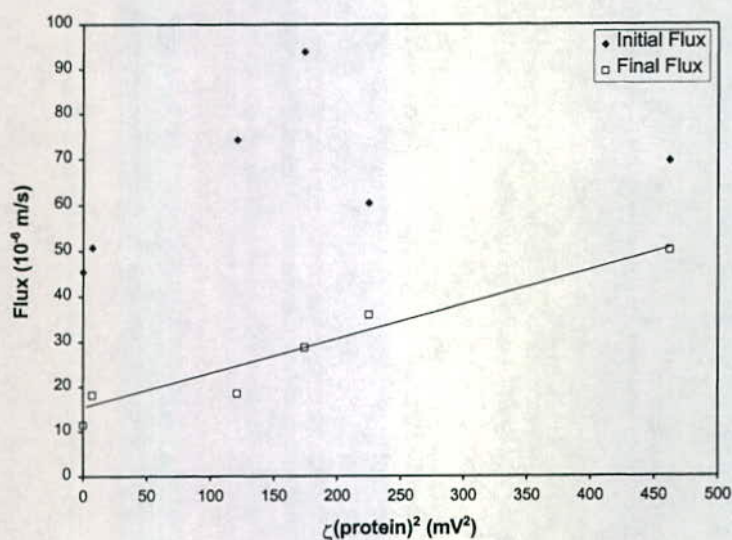


Fig. 7. Initial flux (after 100 s of fouling) and final flux (after 10 000 s of fouling) while filtering a 0.5 mol/dm^3 BSA solution over an M30 membrane vs. the square of the zeta potential of the protein. The solid line is a guide for the eye.

presented elsewhere [13]. Zeta potentials were calculated using the simplified Smoluchowski equation, as more detailed methods need exact information on the pore-size distribution and need complex calculation procedures [13].

It is clearly seen in Fig. 7 that the *final* flux shows a linear increase with $\zeta_p \cdot \zeta_p$, indicating that protein–protein interaction is the main parameter that determines the flux in the later stages of filtration (high-fouling regime). These findings are in agreement with theoretical models and experimental data presented by others [18,19]. The increase in flux with increasing value of $\zeta_p \cdot \zeta_p$ may be caused by the increase in electrostatic repulsion between proteins, or by the decrease in hydrophobic attraction between proteins with increasing value of $\zeta_p \cdot \zeta_p$. Based on the data and the simple modelling presented in this paper, it is not possible to decide which of these causes is the dominant mechanism. The *initial* flux does not show any scaling with $\zeta_p \cdot \zeta_p$, thus indicating that protein–protein interactions are of no importance for the initial fouling of the membrane.

The importance of electrostatic and hydrophobic protein–membrane interactions was investigated by plotting the initial and the final flux versus $|\zeta_p \cdot \zeta_m|$ in Fig. 8. It is clearly seen that the initial flux scales

with $|\zeta_p \cdot \zeta_m|$. Data points for which $\zeta_p \cdot \zeta_m < 0$ and points for which $\zeta_p \cdot \zeta_m > 0$ lie on the same line, thus indicating that electrostatic interactions are of no importance, and that the initial flux decline is governed by hydrophobic attraction between protein and membrane.

The final flux does not scale with $|\zeta_p \cdot \zeta_m|$. Moreover, the data points for which $\zeta_p \cdot \zeta_m < 0$ show higher fluxes than the points for which $\zeta_p \cdot \zeta_m > 0$; in other words: electrostatic attraction between membrane and protein coincides with high fluxes and electrostatic repulsion with low fluxes. The final flux values thus cannot be explained either by considering *hydrophobic* protein–membrane interactions, or by considering *electrostatic* protein–membrane interactions. The explanation must be that the final flux depends *only* on protein–protein interactions, as shown in Fig. 7.

Attempt was made to draw graphs like Figs. 7 and 8 for the M100 and the M300 membrane. The scaling of fluxes with the products of zeta potentials existed but was less clear for these membranes. One of the causes is that the M300 membrane exhibits an iso-electric point close to that of the BSA protein, so that it is difficult to distinguish between protein and protein and protein and membrane. Another cause seems to be that geometric (size) factors seem to play an increasing role

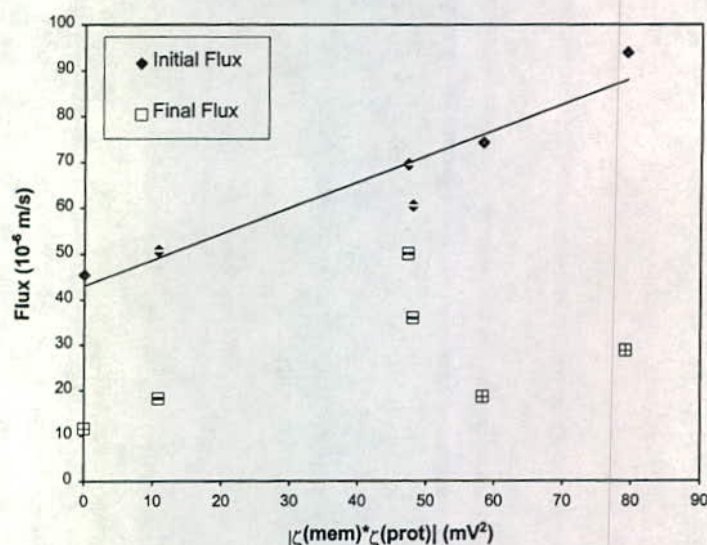


Fig. 8. Initial flux (after 100 s of fouling) and final flux (after 10 000 s of fouling) while filtering a 0.5 mol/dm^3 BSA solution over an M30 membrane vs. the absolute value of the product of the zeta potential of the protein and the zeta potential of the clean membrane: data points for which $\zeta_p \cdot \zeta_m < 0$ are marked by — (horizontal bar), and data points for which $\zeta_p \cdot \zeta_m > 0$ are marked by + (plus). The solid line is a guide for the eye.

for these less retentive membranes. Although these geometric factors depend as well on pH and as on the protein zeta potential, the relation is more indirect. Therefore, the simple scaling, as demonstrated in Figs. 7 and 8 for the M30 membrane, may not be accurate.

4. Conclusions

The factors that influence the fouling of ultrafiltration membranes in protein filtration depend strongly on the filtration conditions. For the more retentive M30 membrane, it was found that, in the low-fouling regime (initial stages of filtration), hydrophobic protein–membrane interactions determine the fouling behaviour. In the high-fouling regime (later stages of filtration), protein–protein interactions dictate the overall performance. For the less retentive M100 and M300 membranes, correlations with protein–protein or protein–membrane interactions were not so strong. The structure of the fouling layer seemed to have a more important influence on the final fouling.

The streaming potential, measured across the membranes during a fouling experiment, changed slowly from a profile characteristic for the membrane to a profile characteristic for the protein, indicating that the membrane was covered with protein.

The protein transmission during a fouling experiment changed slowly from a value related to the ratio protein size/pore size, to a value related to the structure of the fouling layer.

AFM images of the membrane surfaces taken after the filtration experiments showed that the membranes were totally covered by a protein fouling layer. The structure of this fouling layer depended strongly on pH. In particular, very open structures with high permeabilities were found at low pH (below the iso-electric point of the protein). These induced high values of flux and protein transmission.

Acknowledgements

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Downstream Processing in the Biotechnology Industry

Manohar Kalyanpur

Abstract

The biotechnology industry today employs recombinant bacteria, mammalian cells, and transgenic animals for the production of high-value therapeutic proteins. This article reviews the techniques employed in this industry for the recovery of these products. The methods reviewed extend from the centrifugation and membrane filtration for the initial clarification of crude culture media to the final purification of the products by a variety of membrane-based and chromatographic methods. The subject of process validation including validation of the removal of bacterial and viral contaminants from the final products is also discussed with special reference to the latest regulatory guidelines.

Index Entries : bacteria; mammalian cell cultures; centrifuges; homogenizers; tangential flow filtration; ultrafiltration; expanded bed adsorption; chromatography methods; vaccines; bacterial and viral clearance; endotoxin removal; process validation.

1. Introduction

As the 21st century begins, we look with awe at the accomplishments of new technologies in the last century, especially during the last 20 years. Here, molecular biology has made truly significant contributions toward the development of new biopharmaceuticals. We have in our hands today the entire human genome and this is indeed a major milestone in biological sciences. This information will provide clues for researchers to develop new drugs that will cure deficiencies and not simply treat their symptoms, as the older pharmaceuticals did. The biotechnology industry will strive harder than ever before to develop new biotherapeutics, and among these new drugs, proteins and glycoproteins will make up the major part of the targeted drugs. Since the first monoclonal antibody was licensed for sale in 1986, eight more antibody products have already reached the market, while several more are in different stages of development in companies around the world.

The science of biotechnology covers the exploitation of microorganisms and mammalian and insect cell cultures, which are a major source of

high-value medicinal products. In recent years geneticists have succeeded in breeding transgenic sheep, goats, and cattle (1,2) and methods have been developed to get these animals to express the desired drug products in their milk. The scientists insert specific DNA sequences for a therapeutic protein into the genetic material of an animal embryo. The transgenic offspring of this animal then produces the protein of interest in its milk from where the product is isolated and purified. The technique offers a commercially viable alternative to the classical methods of manufacturing recombinant proteins of therapeutic value to treat chronic diseases such as rheumatoid arthritis, cardiovascular problems, numerous cancers, and certain autoimmune diseases. The method has the added advantage that it eliminates the need for expensive bioreactors and other capital equipment required in the traditional methods of manufacturing biotechnology products. Another interesting and even more recent development is the success of some companies who have developed processes for the large-scale production of recombinant proteins in genetically transformed plants (3) such as transgenic corn. This method is

* Author to whom all correspondence and reprint requests should be addressed: Bioseparations and Pharmaceutical Validation, 10 Rue Odilon Redon, 78370 Plaisir, France.

expected to give an economic advantage for drug manufacture.

Most of the biotechnology proteins are present in complex mixtures of products and this makes the task of purifying these molecules very difficult. If these were nonprotein molecules, like antibiotics, for example, one could use solvent extraction to isolate the compounds from solutions in which they are present, making the task of purification much easier. This is quite a challenge to the biochemists, chemical engineers, and other personnel in the downstream processing departments of the industry (4). They use several diverse purification methods in the research laboratory at the bench scale to achieve a high level of purity, and these methods are eventually scaled up to the pilot and production levels. The techniques are used in a complementary fashion to develop cost effective methods that help the companies to produce drugs of the level of purity demanded by the regulatory authorities. The industry today manufactures on a commercial scale compounds that would otherwise have been difficult, if not impossible, to produce in large enough quantities to meet the needs of the sick population. This article attempts to give the reader an overview of the techniques available for downstream purification of biotechnology products.

2. Production Methods in the Biotechnology Industry

As mentioned earlier, the industry manufactures drug products by different methods and their downstream processing varies not only from product to product but also varies depending upon the expression system used for the product manufacture. Each process therefore needs to be fine tuned depending on the particular method of drug manufacture, the process stream from which it is recovered, and the chemical properties of the product.

2.1. Products Made by Recombinant Bacterial Fermentation

The first step in these processes is the separation of the biomass from its surrounding broth. The protein is expressed within the bacterial cell

as a soluble protein but it is quite often present in the form of an insoluble refractive mass referred to as "inclusion bodies." The recovery of the entire biomass including the cells is performed by either preparative centrifugation or by means of tangential flow filtration systems using microporous membranes of appropriate pore diameters. The different filter manufacturers such as Millipore Corporation (Bedford, MA) (5), Pall Corporation (Port Washington, N.Y), and other companies offer membranes with pore diameters of 0.22, 0.45, and 0.65 μm , and the process development scientists must select the membranes suited to their specific needs of biomass concentration. The particulates from the process fluids can get into the membrane pores and cause a significant drop in filtration rates. However, the phenomenon can be controlled by fine tuning the process conditions to obtain the optimum feed and permeate flow rates and transmembrane pressures.

During cell harvesting, either intermittent or continuous cell washing (also referred to as diafiltration) can be performed by adding a suitable solution or a buffer to the cell concentrate, which also helps to maintain the desired pH or ionic strength of the cell suspension and prevents cell lysis. The main advantage of diafiltration is that it helps to wash away the soluble impurities from the process stream. This step is usually started when the cell concentration reaches a point where rapid flux decay is observed.

2.2. Cell Lysis and Clarification of the Lysate

When host cells such as *Escherichia coli* express the desired protein in high concentration, it is converted into micron size *inclusion bodies* within the cytoplasm of the bacterial cells. These bodies usually contain other proteins from the cells as well as nucleic acids and portions of the cell envelope. Valax and Georgion (6) report that these contaminants adhere to the surface of the inclusion bodies. They are released from the cytoplasm in subsequent processing steps. The formation of these bodies is a specific process that, in fact, helps to ease the downstream purification of the protein of interest, since it exists in a relatively

pure form separate from a great majority of the cellular contaminants.

The inclusion bodies are released from within the bacterial cells during mechanical disruption of the cells using homogenizers of different types. Homogenizers such as those manufactured by Niro-Soavi (Parma, Italy), APV-Rennie (Copenhagen, Denmark), and APV-Gaullin (Wilmington, MA) are the principal types in use. Most homogenizers include double-seal designs in order to prevent accidental product loss and can be steam sterilized. They also have a simplified design to facilitate cleaning and sanitization of the system after use. Middleberg (7) provides a comprehensive review of this aspect of downstream processing.

Homogenization of bacterial cells can be made more efficient by chemical treatment of the cells prior to homogenization. The strategy is to weaken the bacterial cell walls by attacking the structural elements that give strength to the cell walls. Vogels and Kula (8) report that the use of the lytic enzyme *cellosyl* helps to increase cell disruption from about 50% to almost 100%. A mixture of ethylenediaminetetraacetic acid (EDTA) and lysozyme also improves the efficiency of cell breakage (9). Cell walls of the yeasts *Saccharomyces cerevisiae* and *Candida utilis* can be weakened by pretreatment with zymolase preparation (10,11) available from Seikagaku America of Rockville, MD. However, the use of pretreatment enzymes can add to the overall cost of processing. It is therefore important to calculate the contribution of the cost increase to the improvement of yield. Quite often product yield can be improved by repeated homogenization passes to obtain a more complete cell disruption. But this has a hidden drawback. The overall rise in temperature during repeated homogenization can denature the protein, thereby lowering the final product yield.

2.3 Lysate Clarification by Centrifugation

The cell lysate is usually clarified by either centrifugation or by tangential flow filtration. The lysate contains the cell debris, soluble proteins from host cells, and the inclusion bodies with the

protein of interest within them. Continuous centrifugation separates the dense inclusion bodies from the rest of the contaminants. The method is described in detail by Middleberg (12). Both small and large production size centrifuges are available from two principal suppliers: the German manufacturer, Westfalia Separator AG, and Alfa Laval Separation AB of Sweden. The centrifuges are operated to obtain a good separation of the inclusion bodies. Next the bodies are washed and dissolved in a strong denaturing agent such as urea or guanidine sulfate, followed by protein refolding. The final purification is accomplished by any number of filtration and/or chromatography steps to recover the protein of interest.

2.4. Membrane Filtration of Cell Lysate

In recent years there has been a lot of interest in the use of membrane filtration in the pharmaceutical and food industries, and this has been extended with much success in the biotechnology industry. Membrane filters are useful in both clarifying and sterilizing gases and aqueous solutions introduced into bioreactors and during downstream recovery and purification of products from a variety of process streams (13). The technologist can today choose from among membranes made from ceramic or organic polymeric materials, and the membranes can be cast with different pore geometries and sizes. The ultimate selection of the membrane for a particular application depends on its durability, cost, and suitability for the intended separation. Also membranes are packed in different filter configurations (14) such as the depth filters, which are operated in the normal flow filtration (NFF) mode, and the hollow fiber, spiral wrap, flat plate, and frame and tubular modules, all of which are operated in the cross flow or tangential flow filtration mode (TFF). The TFF mode is the most commonly used method in downstream processing applications such as clarification of process streams using the microporous membranes and in the separation of biomolecules of different sizes from each other using the ultrafiltration membranes. The topic of tangential flow filtration has been discussed in depth by Michaels

and his numerous coworkers from Millipore Corporation (15).

In the filtration of cell lysate for clarification, the suspended material is retained at the membrane surface and the clear solution with the soluble components ends up in the permeate. The microporous membranes with smaller pore diameters such as 0.2 μm perform better than those with larger pores, since the large pores are more readily plugged by the cell debris. Sometimes ultrafiltration membranes with even smaller pores perform better than the 0.2 μm microporous membranes where the debris is prevented from getting into the pores. This helps to avoid a rapid flow decay. However, the flow rates through the UF membranes are, in general, lower than those obtained with microporous membranes. If the protein of interest is in the soluble fraction it passes through the membrane in the permeate fraction and the solid impurities including the cell debris are retained upstream of the membrane filter.

If the product is present as inclusion bodies, it is in the retentate fraction from the step preceding. It is solubilized by the addition of urea or guanidine sulfate and then separated from the rest of the contaminating proteins using ultrafiltration membranes with an appropriate cut-off. There is often the risk that the membranes might retain some large aggregates of the product of interest and care must be taken to avoid this in order to prevent product loss. The product yields can be improved by washing the retentate fraction with a buffer or another suitable solvent. The combined pool of the permeate and washings contains the protein of interest, which is then sent to the subsequent steps of purification where either membrane or chromatography methods are employed. If at this point the process stream is too dilute, it is concentrated to the desired level, which is adequately performed by ultrafiltration, but any other method that is better suited may also be used.

2.5. Harvesting Mammalian Cell Cultures

Mammalian cells are grown by several methods and in small and large batches for the production of diagnostic and therapeutic proteins. The cells are

typically grown in roller bottles for small-volume applications as in research and product development stages and in bioreactors of varying sizes for production purposes. Whatever the batch size, the first step toward product isolation is the separation of the producing cells, cell debris, and other particulate impurities from the process stream to obtain the clear extracellular fluid for product isolation. Either TFF or NFF methods are commonly employed for the clarification process, and the choice of the method depends mainly on the size of a batch and the frequency of the operation (5).

With small volume batches and infrequent processing, the NFF method is often the preferred method. The filters used here cost much less than the TFF modules and do not require the capital investment in expensive hardware. Moreover, since these are of the disposable type, there is no need to perform the routine time consuming cleaning operation nor does one need to validate the filter cleaning and reuse procedure. On the other hand, TFF modules are expensive but are robust enough to permit multiple use to justify their cost. These are cleaned and sanitized thoroughly after each use, but the procedure must be validated to satisfy regulatory requirements. Overall, the TFF method is preferred for large and frequent production cycles because the costs of the membrane modules and the hardware are spread over production of much larger quantities of the drug product.

The protein of interest in fermentations with mammalian cell cultures are in the extracellular fraction. If the cells are lysed during the separation phase, the intracellular proteins spill out of the cells and contaminate the extracellular fluid. The fragile mammalian cells therefore need careful handling if membrane filtration is employed. An elevated transmembrane pressure and high filtration rate can rupture the fragile cells. An excellent filtration system for this application was developed by Millipore Corporation in the 1980's. The system consists of a microporous membrane, usually with 0.45 μm pore diameters and a recirculating feed pump as in the conventional TFF systems. But a second pump on the permeate side replaces the customary valve used for restricting

the permeate flow. The second pump permits accurate control of the permeate flux and helps to maintain low transmembrane pressures to avoid damage to the fragile cells. The method permits recovery of the desired product of good purity in high yields (15). Here, too, diafiltration with an appropriate buffer improves product yields.

2.6 Concentration of Viruses for Vaccine Production

The first step in the manufacture of viral vaccines and antigens is the concentration of the viruses or portions of the viral coat. This is best performed by ultrafiltration with membranes having a cut-off of 100 kDa but other cut-offs such as 30 kDa, or even 10 kDa may be suitable in specific cases. This is why membrane selection is best done on a case by case basis. The selected membrane must retain the virus and the viral antigens while permitting the passage into the permeate of water, salts, and other impurities with molecular weights smaller than the cut-off of the selected membrane. The use of diafiltration during concentration helps to wash away contaminants of lower molecular weights into the permeate with simultaneous purification. Ultrafiltration membranes in the TFF mode are employed in the manufacture of several vaccines including those used against influenza, Epstein-Barr syndrome, and measles (16–18).

3. Further Purification of Biotechnology Products

Once the desired protein is obtained in a particulate-free process stream, the task of product purification begins and continues until the desired level of purity is reached. The yield in each step is critical because even a small product loss in each purification step adds up to significant losses over the entire downstream process, especially if several purification steps are required to reach a high level of purity. The techniques employed usually fall into two major groups: membrane-based methods such as ultrafiltration and nanofiltration (reverse osmosis) and a variety of chromatography procedures.

3.1 Ultrafiltration

This method is commonly employed to concentrate proteins of different molecular weights while removing smaller molecules such as salts, sugars, and sometimes small or large peptides from the product. The concentration of viruses and viral antigens in the manufacture of vaccines is a typical example. A very wide range of molecular weight cut-offs is available from several suppliers. Prior knowledge of the molecular weight of the protein of interest helps in choosing the membrane best suited for the process. Here again diafiltration is employed to wash off the impurities. In short, the ultrafiltration step helps to enrich the retentate in the higher-molecular-weight species while the permeate contains all the smaller molecules and the solvent.

3.2. High Resolution Tangential Flow Filtration

Tangential flow filtration with ultrafiltration membranes is used to concentrate proteins and other large molecules in aqueous streams, with the simultaneous removal of the lower-molecular-weight species, salts, and water. However, the method suffers from its inability to fractionate mixtures of molecules that have similar molecular weights. The chromatographic purifications differentiate molecules based on their chemical properties and not simply on their size, and these techniques greatly contribute in a large way to the manufacture of high-purity drug products in the biopharmaceutical industry.

The ultrafiltration membranes normally separate molecules, which differ in their molecular diameters by a factor of between 5X and 10X. But this factor of separating efficiency can be further improved by employing a method referred to as high-resolution tangential flow filtration (HRTF) (19). The method aims at separating, in a single-stage process, two compounds that differ in molecular diameter by a factor of about 3X–5X. Diafiltration helps better removal of the smaller molecule in the permeate and increases its yield while at the same time it improves the purity of the larger molecule held back in the retentate fraction.

The HRTF system includes two pumps on either side of the membrane module, the conventional feed pump and a permeate side pump, which is made to operate as a metering pump. The system is not designed to maximize volumetric flux but to maximize solute mass flux by increasing the passage of the solute through the membrane. The system performs best with low-binding membranes to reduce product loss by absorption to the membrane. The value of the HRTF system is demonstrated in the separation of IgG and IgM from albumin in the plasma fractionation industry. The system is also used to remove polyethylene glycols from proteins and serum or allantoic proteins from viruses.

3.3. Nanofiltration or Reverse Osmosis

The reverse osmosis membranes can retain low-molecular-weight compounds such as salts, sugars, and small peptides. These membranes were originally developed for desalination of seawater to make potable water, but they have found a small niche of applications in the biopharmaceutical industry. They are often used for the concentration of antibiotics, peptides, and other molecules with molecular weights between 100 and 3000 Daltons. The newer membranes of this class also permit the desalting of peptide solutions by allowing passage of salts from a solution while retaining the peptides (15).

3.3. Chromatographic Purification

As mentioned in the Introduction, therapeutic proteins in the biotechnology industry are isolated from complex process streams like cell culture supernatants, bacterial fermentation broths, and animal or plant extracts. The products must be very pure to meet the requirements imposed by regulatory agencies. The possibility of the products being contaminated with potential disease-causing viruses have led the regulators to issue strict guidelines for their removal during downstream purification. This has pushed the drug manufacturers to develop very efficient purification techniques. The industry can today access several chromatography methods and media specially developed for the purpose. These are described in the following sections.

3.4. Expanded Bed Adsorption Chromatography

This relatively recent technique is used for whole broth processing and permits the isolation of therapeutic proteins from crude cell culture broths or natural extracts. (20–23). This unique chromatographic method can often combine into one single operation several steps such as centrifugation, filtration, concentration, and purification. The versatile procedure is economical and time saving and can give high yields of the desired protein.

In traditional chromatography the adsorbent columns are tightly packed. In contrast, expanded bed adsorption permits expansion of the adsorbent due to the upward flow of column fluid and crude raw materials such as fermentation broths pass freely through the column without clogging, which is very often observed in packed bed columns. The protein of interest is adsorbed directly on the fluidized column adsorbent, which is stabilized toward uncontrolled turbulence and back mixing with the optimal design of the column and the high density of the adsorbent media. The technique does not suffer from the problems of back pressure and compression of the adsorbent bed when the system is in operation. The method can be scaled up to high volume processes for the manufacture of biomolecules. For a complete discussion of expanded bed adsorption chromatography and the FastMabs system developed at Upfront Chromatography of Denmark for the purification of antibodies, refer to an excellent review by Lihme et al. (24).

3.5. Ion Exchange Chromatography

The technique is relatively inexpensive and is widely used in the purification of biomolecules. The method relies on the use of weak binding and elution conditions, which help to retain the biological activity of the compounds being purified. The method has a high resolving power and capacity and can be used for the purification of almost any charged molecule that is soluble in an aqueous system.

Ion exchange chromatography purifies compounds by ionic interaction between molecules of different charge based on either pH or ionic

strength. The chemists responsible for methods development can choose between the “weak” ion exchangers such as diethylaminoethyl (DEAE) or carboxymethyl (CM) or the “strong” ion exchangers such as the quarternary aminoethyl (QAE) or quarternary ammonium (Q). The term weak or strong refers to the effect of pH on the functional group of the ion exchanger and not to the strength of binding. The choice of the ion-exchange matrix, whether anion or cation, strong or weak, is influenced by the property of the compound under purification, the effect of pH on its charge characteristics, its solubility and stability. Desai et al. (25) have reviewed the use of chromatography in the purification of biotechnology proteins.

3.6. Hydrophobic Interaction Chromatography

The molecular weight, structure, and function of a protein is based on its genomic sequence and is exhibited through its amino acid composition (26). The property of hydrophobicity of proteins is expressed by certain amino acids and of course, by protein molecules that contain these amino acids. Quite often the hydrophobic residues are present as clusters on the surface of the protein molecule. Hydrophobic interaction chromatography is based upon the exploitation of these residues, which permits the preferential adsorption and subsequent elution of the protein in the downstream purification scheme of biotechnology products (27,28). A list of the commercially available hydrophobic matrices and a comparison of their ligand chemistry is presented by Desai et al. (25). The technique has been used both at the research level and on an industrial scale. Manzke et al. (29) describe the purification of murine monoclonal antibodies by this method.

3.7. Size Exclusion Chromatography

The technique, often referred to as gel chromatography or gel filtration (30), relies on the separation of proteins based on their molecular weight differences. The technique is widely employed in biochemical research to purify small quantities of proteins based on their size difference. The separation depends on the ability of a molecule to pen-

etrate porous particles in the stationary phase in a chromatography column. The smaller molecules enter the pores of the column material more frequently. The pores of the support material with a defined diameter do not permit the entry of molecules of a larger diameter and these molecules flow through the column in the void volume of the gel. Thus, during elution, the larger proteins elute first through the column and the smaller ones emerge later in the reverse order of their size, the smallest ones eluting last.

3.8. Affinity Chromatography

A number of purification methods employing membrane-based separations and chromatography procedures are at the disposal of development chemists in the biotechnology industry. However, most of these are rather nonselective methods that depend on physicochemical separation of proteins. The regulatory requirements call for a greater than 99% purity of the products, particularly the injectable proteins, which is not easily achieved by traditional methods. The highly selective immunoaffinity chromatography provides the solution to this problem (31).

Affinity chromatography works on the interaction between two molecules just as in the binding between an enzyme and its coenzyme or between hormones and their receptors. The interaction between an antigen and an antibody is a much stronger and more specific binding. Monoclonal antibodies with low to intermediate affinity are effectively employed in the purification of the therapeutic proteins. The method can be employed on an industrial scale and has helped the industry to come up with high-purity therapeutic proteins, which have received regulatory approval.

A range of matrices of good mechanical strength and other desirable properties are available from several vendors. The general properties of the ideal matrix for specific applications are described by Walters (32) and Jervis (33). Several procedures for immobilizing antibodies on the matrices are also described by other authors (34,35). The choice of the procedure eventually depends on the functional groups on the matrix and the ligand.

3.9. Removal of Endotoxins

Endotoxins, also known as pyrogens, are agents that cause fever when injected intravenously into humans or animals. They are lipopolysaccharides present in the cell wall of Gram negative bacteria and are released into the surrounding liquid when they are killed. They are present as aggregates in solution and their size depends on the composition of the surrounding liquids.

Endotoxins can be effectively removed from process streams by ultrafiltration using membranes with a low-molecular-weight cut off, typically 10 kDa or lower (36–38). The protein being purified, if it has a molecular weight of less than 10 kDa, goes through the membrane while the larger endotoxin molecules stay behind in the retentate. But the method is not suitable for depyrogenating large proteins. However, there are ways to restrict the introduction of pyrogens into the products by following certain procedures as follows:

1. Sterile filtration or heat sterilization of all liquids including buffers, salt solutions, and so on, used in the manufacturing processes to remove the contaminant bioburden.
2. Sterilization of all production equipment including membrane filters, chromatography columns, holding vessels, and other equipment.
3. Maintaining good manufacturing practices in all production areas to avoid the risk of bacterial contamination, post-use cleaning of all equipment to bring it to an endotoxin-free condition, and maintaining the equipment in that condition between successive production runs.

The industry is able to keep the endotoxin levels of products below the permissible levels by rigorously following the above procedures.

4. Final Purification of Biotechnology Products

The purification procedures described above are used in sequences specially adapted to a particular product or process. At this stage the product is very likely to be between 95% and 99% pure. However, it still needs to be put through certain additional steps to further qualify the product for regulatory approval. The special treat-

ments are to remove from the product certain potential contaminants, notably bacterial and viral contaminants.

4.1. Sterile Filtration to Remove Bacterial Contaminants

The products manufactured by the biotechnology industry are mostly injectables and fall in the general classification of parenteral drugs. These need to be dispensed in a sterile form. Using aseptic manufacturing conditions and good manufacturing practices (GMP) are not sufficient to ensure the sterility of these products, since they are almost always proteins and they are thermolabile and cannot be sterilized by terminal heating. The only way to make these products free of bacterial contaminants is by filtering the product solution through sterilizing grade 0.2 μm filters prior to the filling operation. This is an accepted procedure that is rigidly followed in the industry, but the procedure needs to be validated (39) according to the guidelines for parenteral drugs issued by the U.S. Food and Drug Administration (40).

4.2. Virus Removal and Inactivation

Many biotechnology products of high therapeutic value are manufactured by cell culture processes or derived from different starting materials including human plasma, animal tissue, and milk of transgenic animals. The products thus carry an inherent risk of transmitting to the human recipients potential disease-causing viral contaminants coming from the starting material of their manufacturing process. There is also some risk of contaminating viruses coming from certain downstream processing steps, e.g., the use of proteolytic enzymes of animal origin such as porcine pepsin or trypsin and the monoclonal antibodies used during purification by immunoaffinity chromatography. Finally, there always exists the risk of adventitious viruses entering the process stream owing to failure of GMP. Therefore, the regulatory authorities have issued clear guidelines for the removal or inactivation of viruses from biotherapeutic proteins (41).

In 1994, the FDA's Center for Biologics Evaluation and Research (CBER) issued a directive asking the industries to validate the removal and

inactivation of the viruses from all biologicals. Products derived from cell culture processes can carry the murine leukemia virus coming from the cell lines, while products made by extracting animal tissue or from the milk of transgenic animals have the potential risk of viruses from the host animals. The authorities have recommended that the biotechnology products must go through dedicated virus removal or inactivation steps and that these should achieve an overall virus reduction of at least 12 logs. The blood products industry has been using for some years different methods for virus inactivation. These methods are heat inactivation (pasteurization), pH inactivation, solvent/detergent treatment, UV and gamma ray irradiation, and the addition of certain chemical inactivating agents such as β -propiolactone. Claims have been made by some companies that the chromatography steps employed in their normal purification procedure can also reduce the virus content of biologicals.

However, all these methods have some limitations. For example, the solvent/detergent treatment can inactivate the lipid-coated viruses, but the method is innocuous against the nonlipid coated viruses. There is a risk of denaturing therapeutic proteins if the manufacturer employs heat inactivation. With chemical inactivation, the manufacturer has the additional task of removing the added chemical completely from the product, not to mention the fact that sometimes the chemicals fail to inactivate certain target viruses. The chromatography methods give rather low level of virus reduction. Moreover, the results are not always reproducible under different process conditions and are therefore considered as nonrobust methods by the regulatory authorities.

For some years, membrane filtration has emerged as an effective method of virus removal from biologicals. This inert method removes both lipid-enveloped and nonenveloped viruses, because the removal is based on size exclusion and not on the surface characteristics of the virus, as is the case with the inactivating methods. Larger viruses are removed more effectively than the smaller viruses. The technique is well established and accepted as is evident from the pub-

lished literature (42–45). However, an important point to remember is that no single method among those listed above can alone give the level of virus removal expected by the regulatory authorities. Therefore, membrane filtration is used with the other methods in a complementary fashion, and this helps the industry to achieve the overall viral reduction to satisfy regulatory requirements. The subject of virus contamination in biologicals and the merits and drawbacks of the different methods employed for their elimination are topics of a recent review (46). Also Darling (47) has discussed the topic of design and interpretation of viral clearance studies in the biopharmaceutical industry. This is an excellent reference for biotechnology companies planning validation of virus removal.

5. Process Validation

Process validation permits the drug manufacturers to assure themselves that the methods set in-place to manufacture a product work as they are expected to. When performed in a well documented manner, it also permits the drug manufacturer to ensure regulatory agencies (48,49) that the methods that are employed perform reproducibly and as expected to yield the final product of a consistent quality.

In the context of downstream processing, process validation extends to all critical steps, especially membrane-based separation systems (50,51) and chromatographic procedures (52), including those used for the clearance of potential bacterial (39,40) and viral contaminants (47). Kuwahara and Chuan have published an extensive review of the topic of process validation (53), which is an excellent reference to personnel in the validation departments of the industry.

6. Conclusions

The biotechnology industry manufactures numerous therapeutic products that are derived from several source materials. The recovery and downstream purification of biologicals is a combination of diverse purification methods. Well-developed processes at the research bench scale are carefully scaled up to the production level, always bearing in mind that fewer the steps used,

higher is the eventual yield. This is because even if a particular step loses only 5% of the product, the losses add up when the product goes through a multistep procedure for eventually providing a product of the desired level of purity. The production must also proceed following GMP while paying attention to keeping all the procedures within the limits specified in the validation reports of the company.

Early batches are used to make product for different phases of clinical trials for the drug product. By paying attention to all details, the manufacturing personnel can help the company to secure regulatory approval to market the drug ahead of its closest rivals. In today's highly competitive environment in the biopharmaceutical industry, the first company that gets the marketing approval for a new drug stands to benefit immensely from its sales and takes a significant share of the market for that drug and can, in most cases, prevent its competitors from putting a similar product on the market by virtue of its patent protection. Therefore, downstream processing of new drug products is critical to a biotechnology company in achieving a premium position in the industry.

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REIVINDICACIONES

1. Un procedimiento para preparar un conjugado de anticuerpo-maitansinoide caracterizado porque comprende las etapas de:
 - (a) poner en contacto un anticuerpo seleccionado de entre el grupo que consiste en huN901, huMy9-6, HUB4, huC242, trastuzumab, bivatuzumab, sibrotuzumab, CNTO95, huDS6, y rituximab con un reactivo de reticulación bifuncional seleccionado entre el grupo que consiste de N-succinimidil 4-(2-piridilditio)butanoato (SPDB), N-succinimidil 4-(2-piridilditio)pentanoato (SPP), y N-succinimidil 4-(maleimidometil)ciclohexanocarboxilato (SMCC) para unir covalentemente un ligante al anticuerpo y de ese modo preparar una primera mezcla que comprende anticuerpos que tienen enlazantes unidos al mismo,
 - 12 (b) someter la primera mezcla a la filtración de flujo tangencial (TFF) y de ese modo preparar una primera mezcla purificada de anticuerpos que tienen enlazantes unidos al mismo;
 - (c) la conjugación de un maitansinoide que es N2'-deacetil-N2'-(3-mercapto-1-oxopropil)-maitansina (DM1) o N2'-deacetil-N2'-(4-metil-4-mercapto-1-oxopentil)-maitansina (DM4) a los anticuerpos que tienen enlazantes unidos al mismo en la primera mezcla purificada mediante la reacción de los anticuerpos que tienen enlazantes unidos al mismo con un maitansinoide en una solución que tiene un pH de 4 a 9 para preparar una segunda mezcla que comprende (i) anticuerpo acoplado químicamente a través del enlazante a maitansinoide, (ii) maitansinoide libre, y (iii) los subproductos de reacción producidos durante la etapa (c), y
 - 18 (d) someter la segunda mezcla a la filtración de flujo tangencial para purificar los anticuerpos acoplados químicamente a través de los enlazantes para el maitansinoide de los otros componentes de la segunda mezcla y de ese modo preparar una segunda mezcla purificada de anticuerpos acoplados químicamente a través de los enlazantes a maitansinoide.
 - 24
- 30 2. El proceso de la reivindicación 1, caracterizado porque la solución en el paso (c) tiene un pH de 4 a 6,0.
3. El proceso de la reivindicación 1, caracterizado porque la solución en el paso (c) tiene un pH de 6,5 a 9.

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4. El proceso de cualquiera de las reivindicaciones 1-3, caracterizado porque la solución en la etapa (c) comprende sacarosa.

5. El proceso de cualquiera de las reivindicaciones 1-4, caracterizado porque la solución en la etapa (c) comprende un agente regulador seleccionado del grupo que consiste en un regulador de citrato, un regulador de acetato, un regulador de succinato y un regulador de fosfato.

6. Un procedimiento para preparar un conjugado de anticuerpo-maitansinoide caracterizado porque comprende las etapas de:

(a) poner en contacto un anticuerpo seleccionado de entre el grupo que consiste en huN901, huMy9-6, HUB4, huC242, trastuzumab, bivatuzumab, sibrotuzumab, CNTO95, huDS6, y rituximab con un reactivo de reticulación bifuncional seleccionado entre el grupo que consiste de N-succinimidil 4-(2-piridilditio)butanoato (SPDB), N-succinimidil 4-(2-piridilditio)pentanoato (SPP), y N-succinimidil 4-(maleimidometil)ciclohexanocarboxilato (SMCC) para unir covalentemente un ligador al anticuerpo y de ese modo preparar una primera mezcla que comprende anticuerpos que tienen enlazantes unidos al mismo,

(b) conjugar un maitansinoide que es N2'-deacetil-N2'-(3-mercapto-1-oxopropil)-maitansina (DM1) o N2'-deacetil-N2'-(4-metil-4-mercapto-1-oxopentil)-maitansina (DM4) a los anticuerpos que tienen enlazantes unidos al mismo en la primera mezcla por reacción de los anticuerpos que tienen enlazantes unidos al mismo con un maitansinoide en una solución que tiene un pH de 4 a 9 para preparar una segunda mezcla que comprende (i) anticuerpos químicamente acoplado a través del enlazador a maitansinoide, (ii) maitansinoide libre, y (iii) los subproductos de reacción producidos durante la etapa (b), y

(c) someter la segunda mezcla a filtración de flujo tangencial (TFF) o cromatografía de adsorción para purificar los anticuerpos acoplados químicamente a través de los enlazantes para maitansinoide de los otros componentes de la segunda mezcla y de ese modo preparar una segunda mezcla purificada de anticuerpos acoplados químicamente a través de los enlazantes a la maitansinoide en el que la primera mezcla no se sometió a purificación.

7. El proceso de la reivindicación 6, caracterizado porque la cromatografía de adsorción se selecciona del grupo que consiste de cromatografía de hidroxipatita, cromatografía de inducción de carga hidrófoba (HCIC),

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cromatografía de interacción hidrofóbica (HIC), cromatografía de intercambio iónico, cromatografía de intercambio iónico de modo mixto, cromatografía de afinidad de metal inmovilizado (IMAC), cromatografía de ligando de tinción, cromatografía de afinidad, cromatografía de fase inversa, y combinaciones de las mismas.

6

8. El proceso de la reivindicación 6 o 7, caracterizado porque la solución en el paso (b) tiene un pH de 4 a 6,0.

9. El proceso de la reivindicación 6 o 7, caracterizado porque la solución en el paso (b) tiene un pH de 6,5 a 9.

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10. El proceso de cualquiera de las reivindicaciones 6-9, caracterizado porque la solución en la etapa (b) comprende sacarosa.

11. El proceso de cualquiera de las reivindicaciones 6-10, caracterizado porque la solución en la etapa (b) comprende un agente regulador seleccionado del grupo que consiste en un regulador de citrato, un regulador de acetato, un regulador de succinato y un regulador de fosfato.

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12. El proceso de cualquiera de las reivindicaciones 1-11, caracterizado porque el anticuerpo es trastuzumab.

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13. El proceso de cualquiera de las reivindicaciones 1-12, caracterizado porque el maitansinoide es N2'-deacetil-N2'-(3-mercapto-1-oxopropil)-maitansina (DM1).

14. El proceso de cualquiera de las reivindicaciones 1-12, caracterizado porque el maitansinoide es N2'-deacetil-N2'-(4-metil-4-mercapto-1-oxopentil)-maitansina(DM4).

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15. El proceso de cualquiera de las reivindicaciones 1-14, caracterizado porque el

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reactivo de reticulación bifuncional es N-succinimidil 4-(maleimidometil)ciclohexanocarboxilato (SMCC).

16. El proceso de cualquiera de las reivindicaciones 1-11, caracterizado porque en el que el anticuerpo es el trastuzumab , el reactivo de reticulación bifuncional es N-succinimidil 4-(maleimidometil)ciclohexanocarboxilato (SMCC), y el maitansinoide es N2'-deacetil-N2'-(3-mercapto-1-oxopropil)-maitansina (DM1).

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SON: ****CIENTO OCHENTA MIL PESOS MONEDA CORRIENTE****

Responsable: _____
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