

Clarke, Modet & C^o

COLOMBIA

P-1099

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 08-019768- -00009-0000

SUPERINTENDENCIA DE INDUSTRIA
DIVISION DE NUEVAS CREACIONES
E. S.

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Act. 412 REPOSPRESRECU Folios: 70

Re: Expediente No.08.019.768

Solicitud de patente a nombre de IMMUNOGEN INC.

Yo, DILIA MARIA RODRIGUEZ D'ALEMAN, identificada como aparece al pie de mi firma, domiciliada en la carrera 11 A No. 86-53, piso 6, de la sociedad IMMUNOGEN INC., domiciliada en Cambridge, Estados Unidos de América, solicitante de la patente de la referencia, interpongo recurso de reposición contra la resolución No. 40711 emitida el 28 de junio de 2012 por el Superintendente de Industria y Comercio, por medio de la cual se niega el privilegio de patente para la solicitud contenida en el expediente de la referencia.

1. FUNDAMENTOS DEL RECURSO

En relación con los argumentos presentados por ese despacho en la resolución impugnada No. 40711 es necesario señalar que carecen de un enfoque adecuado de conformidad con las normas, jurisprudencia y reglas técnicas que existen para evaluar la altura inventiva de una invención.

1.1 Violación de los artículos 14, 16 y 18 de la Decisión 486 por una indebida aplicación de la norma basada en un error de interpretación de la misma.

Consideramos que la resolución impugnada viola los artículos 14, 16 y 18 al no otorgar el beneficio de patente de invención debido a una supuesta carencia de nivel inventivo argumentando que "...La persona del oficio medianamente versada en la materia se vería motivada a utilizar la filtración de flujo tangencial en la etapa (d) porque, de acuerdo con lo divulgado en los documentos D1 y D2, las operaciones de purificación usando filtración de flujo tangencial o la técnica de cromatografía puede llevarse a cabo en diferentes etapas del proceso, comoquiera que es bien conocido en el estado del arte que la filtración de flujo tangencial es una técnica de separación utilizada para purificar y concentrar proteínas¹" (página 7, últimos 7 renglones del primer párrafo) y "La persona del oficio medianamente versada en la materia se vería motivada a eliminar la etapa de purificación de la mezcla 1 porque es bien conocido en el estado del arte que los procesos de purificación, como filtración de flujo tangencial, afectan el rendimiento del producto, y que un alto rendimiento, calidad y pureza del producto, son objetivos primarios a tener en cuenta en la optimización de procesamiento de proteínas¹, por lo que existía la sugerencia o motivación razonable de excluir

etapas previas a la conjugación del anticuerpo al maytansinoide y el efecto técnico fue previsto en el estado de la técnica.”(página 11, últimas 9 líneas del primer párrafo).

Sobre este particular, es importante señalar que el solicitante no se encuentra de acuerdo con la posición que ha tomado ese despacho en relación con la altura inventiva de la presente solicitud, especialmente con el hecho que las reivindicaciones dependientes carezcan de nivel inventivo a la vista de la Publicación de la Solicitud de Patente Internacional WO 2003/057163 A2 (Mazzola et al.) (D1) y la Publicación de la Solicitud de Patente Internacional WO 2002/098897 A3 (Bander et al.) (D2).

En este sentido, nos permitimos decir que las reivindicaciones dependientes de la presente solicitud están dirigidas a dos modalidades de un proceso para preparar un conjugado maytansinoide-anticuerpo, los cuales se discuten separadamente a continuación.

Discusión de la reivindicación 1 y las reivindicaciones dependientes de ésta

En la modalidad definida por la reivindicación independiente 1, el proceso comprende las etapas (a) – (d), en donde la etapa (d) es una etapa de purificación que comprende someter una mezcla a filtración por flujo tangencial (TFF) para purificar un conjugado maytansinoide-anticuerpo. Como se discute con más detalle a continuación, este proceso es inventivo sobre lo repostado en D1 y en D2, puesto que fue una decisión clara por los inventores de D1 y de D2 no usar TFF para purificar el conjugado. Además, la persona versada en el arte no escogería el uso del TFF para purificar el conjugado porque, al momento de radicar la presente solicitud era del entendimiento general en el arte que la membrana TFF es susceptible a la obstrucción debido a su pequeño tamaño de poro, y por lo tanto, no sería adecuada para purificar el conjugado. Más aún, la invención reivindicada por el solicitante necesariamente tendría un rendimiento más alto y una mejor eficiencia debido a que combina las etapas de purificación y “acabado” enseñadas en D1 y D2 en una sola etapa.



Es claro entonces que D1 y D2 representan una decisión clara por parte de los inventores de no usar TFF para la purificación del conjugado.

En efecto, D1 divulga un proceso para conjuguar un maytansinoide a un anticuerpo mediante el proceso así llamado de “dos etapas”. La primera etapa involucra hacer reaccionar un ligante con el anticuerpo para formar un anticuerpo modificado, y la segunda etapa involucra conjuguar el anticuerpo modificado con el maytansinoide (véase por favor la página 2, líneas 22 a 35, de D1).

En ese orden de ideas, después de la primera etapa, D1 enseña la remoción del ligante que no reaccionó del anticuerpo, modificado por filtración de flujo tangencial. Luego de la segunda etapa, D1 enseña **purificar el conjugado de maytansinoide-anticuerpo modificado por cromatografía de intercambio iónico** (ver, por ejemplo, página 2, líneas 22 a 35).

Así las cosas, al mirar y revisar en detalle, en la etapa 1 de purificación del conjugado maytansinoide-anticuerpo modificado, **la clara y única enseñanza de D1 es hacer ésta purificación por cromatografía de intercambio iónico**. En este sentido, se divulgan dos medios adecuados basados en intercambio iónico: (i) **SP-Sefarosa** (página 2, línea 34; página 13, Ejemplo 7; y la reivindicación 8 de D1); y (ii) **hidroxiapatita cerámica** (página 5, líneas 16 a 17; página 7, líneas 28 a 30; y la reivindicación 7 de D1).

En consideración a la filtración de flujo tangencial (TFF), los inventores de D1 claramente tenían conocimiento de ésta técnica. En particular, se hace referencia a TFF en las páginas 4 y 6 de D1 en el contexto de retirar el ligante que no reaccionó del anticuerpo modificado; y en la página 8 en relación con lo que se puede considerar como una etapa de "acabado", por la cual el conjugado ya purificado se somete a filtración de flujo tangencial para diafiltrar el conjugado en el amortiguador de la formulación a granel y ajustar la concentración. Por lo tanto, la divulgación de D1 **representa una decisión clara por parte de los inventores de D1 de no enseñar o incluso sugerir el uso de TFF en relación con la purificación del conjugado**.

Similar a D1, la anterioridad D2 también está relacionada con un proceso para conjugar un maytansinoide con un anticuerpo por un proceso así llamado de "**dos etapas**". La primera etapa involucra hacer reaccionar un ligante con el anticuerpo, y la segunda etapa involucra conjugar el anticuerpo modificado con el maytansinoide (ver párrafo 00443, Páginas 160-161, las etapas (2) y (4), de D2, respectivamente).

Después de la primera etapa, D2 enseña la remoción del ligante que no reacciona, del anticuerpo modificado por cromatografía de no adsorción (ver párrafo 00443, página 161, etapa (3), de D2). Luego de la segunda etapa, D2 enseña la purificación del conjugado maytansinoide-anticuerpo modificado por cromatografía de no adsorción (ver párrafo 00443, página 161, etapa (5)).

Al revisar y observar detalladamente, la etapa de purificación el conjugado de maytansinoide-anticuerpo modificado, **la única y clara enseñanza de D2 es la de hacer ésta purificación por cromatografía de no adsorción**. A este respecto, se divulgan dos métodos adecuados de cromatografía de no adsorción: (i) **cromatografía de filtración por gel**; y (ii) **una columna de Sephadex G-25™** (ver párrafo 00443, página 161, las etapas (3) y (5), de D2).



De manera similar a D1, los inventores de D2 también tenían claramente el conocimiento de la TFF. En particular, D2 enseña que la TFF se utiliza para concentrar el anticuerpo antes de hacer reaccionar un ligante con el anticuerpo (ver párrafo 00443, página 160, etapa (1)). D2 también enseña el uso de TFF en relación con lo que se puede considerar como una etapa de "acabado" por la cual el conjugado ya purificado se somete a filtración de flujo tangencial para diafiltrar el conjugado en el amortiguador de la formulación a granel y ajustar la concentración (ver párrafo 00443, páginas 161-162, etapa (6)). Por lo tanto, la divulgación de D2 también representa una decisión clara por parte de los inventores de D2 de no enseñar o incluso sugerir el uso de TFF en relación con la purificación del conjugado.

Alguien versado en el arte no escogería el uso de TFF para purificar el conjugado puesto que la membrana de TFF tiene la tendencia a obstruirse debido al pequeño tamaño del poro.

Se entenderá a partir de lo anterior que la diferencia entre el proceso enseñado en D1 y D2 y el proceso descrito en la reivindicación 1 de la presente solicitud es que la reivindicación 1 requiere el uso de TFF para purificar el conjugado. Esta modificación se puede ver como una mejora a los métodos de D1 y D2 puesto que la etapa de purificación se combina efectivamente con la etapa de "acabado", por lo cual se simplifica el método y se mejora la eficiencia, según se discute con más detalle más adelante.

Por lo tanto, la pregunta a ser respondida es si hubiera sido obvio apartarse de la enseñanza clara y no ambigua de D1 (de usar cromatografía de intercambio iónico para purificar el conjugado) y D2 (de usar cromatografía de no adsorción para purificar el conjugado) y en lugar usar TFF.

El solicitante sostiene respetuosamente que no habría sido obvio apartarse de la enseñanza clara y no ambigua de D1 (de usar cromatografía de intercambio iónico para purificar el conjugado) y D2 (de utilizar cromatografía de no adsorción para purificación del conjugado) y en cambio usar TFF.

La TFF es una técnica de ultrafiltración. Usa una membrana que tiene un tamaño de poro muy pequeño, por lo que es particularmente sensible. Como tal, aquellos versados en este arte habrían entendido en la fecha de prioridad de la presente solicitud que la TFF requiere una solución clara a ser aplicada a fin de que el proceso de ultrafiltración trabaje efectivamente. Por el contrario, una solución turbia (con los componentes disueltos sólo parcialmente) se hubiera esperado para obstruir la membrana y hacer el proceso de ultrafiltración inoperable.

La solución que contiene el conjugado junto con el maytansinoide que no reaccionó en D2 y D1 hubiera sido turbia debido a la baja solubilidad del

maytansinoide que no reaccionó, lo cual hubiera empezado a sacarlo de la solución. La persona versada en el arte, por lo tanto, habría esperado que el proceso de TFF solamente hubiera hecho este problema peor (por la remoción del solvente), con el maytansinoide precipitado obstruyendo el sistema TFF y haciéndolo inoperable (ver párrafo 9 de la declaración radicada por Godfrey Amphlett con la respuesta del solicitante del 22 de mayo 2012).

Como tal, uno podría considerar que hubo un prejuicio técnico contra el uso de TFF para purificar el conjugado de D1 y de D2, y fue a causa de éste prejuicio técnico que los inventores de D1 y de D2 tomaron una decisión consciente, de no mencionar la TFF en relación con la purificación del conjugado. Como se mencionó antes, D1 enseña solamente el uso de la cromatografía de intercambio iónico para purificar el conjugado y D2 enseña solamente el uso de cromatografía de no adsorción para purificar el conjugado, a pesar que los inventores tuvieran un conocimiento claro de TFF.

A la vista de una persona normalmente versada en el arte, el conocimiento general común como se describió antes, y a la vista del hecho que la persona versada en el arte no hubiera ido en contra de un prejuicio establecido, el solicitante sostiene que no habría sido obvio apartarse de la enseñanza clara de D1, a saber, el uso de cromatografía de intercambio iónico para purificar el conjugado, o D2, a saber, el uso de cromatografía de no adsorción para purificar el conjugado. En resumen, la comprensión abrumadora de aquellos versados en este arte fue que la solución turbia del conjugado junto con el maytansinoide simplemente no podría ser puesta efectivamente a través de un sistema TFF con el propósito de purificar el conjugado.

El proceso reivindicado necesariamente tendría un rendimiento más alto y mejor eficiencia, ya que combina las etapas de purificación y "acabado" en una etapa única.

Esto fue solamente porque los presentes inventores pensando inventivamente decidieron ir en contra de esta opinión generalizada. Los resultados obtenidos por los inventores fueron totalmente inesperados en la medida en que no solamente hicieron el proceso de trabajo sino que el proceso trabajado, así como también, los proceso en los cuales las etapas de purificación y "acabado" se hicieron separadamente, utilizando técnicas diferentes, como se evidencia en los resultados mostrados en el Ejemplo 7 de la presente solicitud.

Los presentes inventores hallaron inesperadamente que la TFF se puede usar para purificar directamente el conjugado, elimina la necesidad de las etapas de cromatografía de intercambio iónico o de la cromatografía de no adsorción enseñadas por D1 y D2. Por lo tanto, el proceso descrito en la reivindicación dependiente 1 se puede realizar en menos tiempo que los

procesos divulgados en D1 y D2, y los costos son menores que en los procesos divulgados en D1 y D2 puesto que el conjugado se somete a TFF, en lugar de cromatografía de intercambio iónico seguida por TFF, como lo enseñado por D1, o cromatografía de no adsorción seguida por TFF, como se muestra en D2.

Es bien conocido en el arte que cada etapa de purificación produce una cierta reducción en el rendimiento. Por Ejemplo, D2 divulga que el rendimiento del conjugado obtenido por la etapa de cromatografía de no adsorción está entre 60% y 65%, y que el rendimiento del conjugado obtenido de la etapa posterior TFF está entre 92% y 100% (ver párrafo 00443, Páginas 161-162, etapas (6) y (7)). Puesto que los procesos descritos en la reivindicación dependiente 1 elimina la necesidad de las etapas de cromatografía de intercambio iónico o cromatografía de no adsorción, como lo enseña D1 y D2, el rendimiento del conjugado producido por el proceso descrito en reivindicación dependiente 1 sería necesariamente más alto que el rendimiento del conjugado producido por los procesos divulgados en D1 y D2.

Además para mejorar el rendimiento, la eliminación de la etapa de purificación cromatográfica conduce a otras ventajas. Se evita el tiempo significativo requerido para una puesta en marcha y ejecutar la etapa de cromatografía, así como los costos asociados (por ejemplo, mano de obra y materiales). Así la eficiencia operacional se mejora y los costos de fabricación se reducen.

A la vista de lo anterior, los presentes inventores hallaron inesperado que la TFF se puede utilizar para purificar directamente el conjugado, como se describió en la reivindicación dependiente 1, dando como resultado un proceso que es (1) más rápido que los procesos divulgados en D1 y D2, (2) costos menores que los de los procesos divulgados en D1 y D2, y (3) produce un rendimiento más alto del conjugado que los procesos divulgados en D1 y D2, los cuales son ventajas comerciales muy importantes para la producción de grandes lotes de conjugados maytansinoide-anticuerpo.

Discusión de la reivindicación 15 y las reivindicaciones dependientes de ésta

En modalidad definida por la reivindicación independiente 15, el procedimiento comprende las etapas (a) - (c), en donde la mezcla no se somete a filtración de flujo tangencial, precipitación selectiva, filtración por adsorción, o cromatografía de adsorción entre las etapas (a) y (b), y en donde la etapa (c) es una etapa de purificación que comprende someter la mezcla a filtración de flujo tangencial, precipitación selectiva, filtración adsorbente, cromatografía de adsorción, o una combinación de éstos.

Como se discutió anteriormente, D1 y D2 cada una enseña un proceso así denominado de "dos etapas" proceso en donde se usa una etapa de purificación después de la primera etapa para eliminar el ligante que no reaccionó del anticuerpo modificado. En particular, D1 enseña la eliminación del ligante que no reacciona del anticuerpo modificado por TFF (ver, por ejemplo, la página 4, líneas 19-21; página 5, líneas 3-6; y página 6, líneas 34-36), y D2 enseña la eliminación del ligante que no reacciona del anticuerpo modificado mediante cromatografía de no adsorción (ver párrafo 00443, página 161, etapa (3), de D2).

La diferencia entre los procesos que se enseñan en D1 y D2 y el proceso descrito en la reivindicación 15 de la presente solicitud es que la reivindicación 15 requiere que el anticuerpo modificado no se someta a filtración de flujo tangencial, precipitación selectiva, filtración por adsorción, cromatografía por adsorción después de la primera etapa. Esta modificación se puede ver como una mejora a los procesos de D1 y D2 puesto que el anticuerpo modificado se puede conjugar directamente con el maytansinoide sin la necesidad de una etapa de purificación, por lo cual se simplifica el método y se mejora la eficiencia, como se discute en mayor detalle más adelante.

Por lo tanto, la pregunta a contestar es si hubiera sido obvio apartarse de la enseñanza clara y no ambigua de D1 (al usar TFF para purificar el conjugado) y D2 (para usar la cromatografía de no adsorción para purificación el conjugado) y en su lugar eliminar completamente las etapas de purificación antes mencionadas.

De este modo, es necesario hacer hincapié sobre el hecho que no habría sido evidente una eliminación de las etapas de purificación clara y rotundamente enseñadas por D1 (para usar TFF para purificar el anticuerpo modificado) y D2 (para usar cromatografía de no adsorción para purificar el anticuerpo modificado).

Antes de la presente invención, se entendía por aquellos versados en este arte que la eliminación de componentes que no reaccionaron, solvente, y productos de la reacción de modificación, tales como la eliminación del ligante que no reaccionó y el etanol del anticuerpo modificado, como se divulgó en D1 y D2, era necesaria para hacer un proceso eficiente que proporcionara un conjugado maytansinoide-anticuerpo de buena pureza (ver párrafo 8 de la declaración presentada por Godfrey Amphlett con la respuesta del solicitante del 22 de mayo 2012).

Fue solamente por el pensamiento inventivo de los presentes inventores decididos a ir contra esta opinión generalizada y omitir el uso de filtración de flujo tangencial, precipitación selectiva, filtración de adsorción, o cromatografía de adsorción entre la etapa de modificación y la etapa de conjugación, como se describe en la reivindicación corregida 15. Los resultados



obtenidos por los presentes inventores fueron completamente inesperados en la medida en que no solamente, hicieron el proceso de trabajo sino el proceso trabajado, así como también los procesos que comprenden el uso de filtración de flujo tangencial, precipitación selectiva, filtración por adsorción, o cromatografía por adsorción entre la etapa de modificación y la etapa de conjugación, como se evidencia en los resultados mostrados en el Ejemplo 6 de la presente solicitud.

Más específicamente, los inventores hallaron de manera inesperada que la **omisión** de filtración de flujo tangencial, precipitación selectiva, filtración por adsorción, o cromatografía por adsorción entre la etapa de modificación y la etapa de conjugación, como se expone en la reivindicación corregida 15, aumenta significativamente el rendimiento del conjugado, sin afectar la calidad del conjugado. Como se muestra en el Ejemplo 6 de la presente solicitud, el conjugado producido por un proceso que comprende una única etapa de purificación, como se cita en la reivindicación dependiente 15, produjo **91%** del conjugado, al compararlo con un rendimiento de sólo **79%** cuando el conjugado se produjo por un proceso que comprende dos etapas de purificación, de forma similar a los procesos enseñados por D1 y D2 (ver Tabla 7).

Los datos presentados en el Ejemplo 6 también demuestran que la calidad del conjugado producido por un proceso que comprende una única etapa de purificación, como se cita en la reivindicación dependiente 15, fue similar a la calidad de un conjugado de la producida por un proceso que comprende dos etapas de purificación, similar a los procesos enseñados por D1 y D2 (ver Tabla 7). Por lo tanto, los datos presentados en el Ejemplo 6 demuestran que el proceso divulgado en la reivindicación 15, en donde el anticuerpo modificado no se somete a filtración de flujo tangencial, precipitación selectiva, filtración por adsorción, o cromatografía por adsorción, produce un aumento en el rendimiento del conjugado que es de la misma calidad que un conjugado preparado con tal purificación de los anticuerpos modificados, al tiempo que reduce tiempo total y el costo para preparar el conjugado. Además algunas mejoras en el rendimiento, la eliminación de la etapa de purificación para el anticuerpo modificado conducen a otras ventajas.

En efecto, se evita el tiempo significativo que se requiere para la puesta en marcha y ejecución de la etapa de purificación, como son los costos asociados (por ejemplo, para la mano de obra y materiales). La eficiencia operativa se mejora y el costo de fabricación de se reduce.

A la vista de lo anterior, el hallazgo inesperado de los presentes inventores que se puede omitir la filtración de flujo tangencial, precipitación selectiva, filtración por adsorción, o cromatografía por adsorción y entre la etapa de modificación y la etapa de conjugación, como se describe en la reivindicación corregida 15, resulta en un proceso que es (1) **más rápido** que los procesos divulgados en D1 y D2,



(2) con costos menores que los de los procesos divulgados en D1 y D2, y (3) produce un rendimiento más alto del conjugado que los procesos divulgados en D1 y D2, los cuales son ventajas comerciales muy importantes para la producción de grandes lotes de conjugados maytansinoide-anticuerpo.

En este sentido, es claro que la evaluación de la altura inventiva de la presente invención no fue la adecuada y resulta ser un análisis de tipo hindsight de la solución al problema técnico que se proporciona en la presente invención. Por lo tanto, consideramos que la resolución impugnada viola los artículos 14, 16 y 18 de la Decisión 486 de la Comunidad Andina.

Aunado a lo anterior y sin apartarse del conocimiento del principio de independencia entre las oficinas de Propiedad Intelectual en el Mundo, es deseo del solicitante informar a ese despacho que solicitudes que corresponden a la misma materia objeto de la solicitud en Colombia, han sido permitidas en un número de países los cuales también realizan evaluaciones de fondo.

A continuación en la Tabla 1, se pone en consideración un resumen de las correspondientes solicitudes de patentes otorgadas y permitidas. Además, se adjuntan para su referencia (Anexo 1) las correspondientes copias de las reivindicaciones permitidas y la Notificación de Asignación para cada una de las solicitudes australiana, canadiense, china y Europea.

TABLA 1

País	Aplicación/Patente No.	Estado
AUSTRALIA	2006283726	Permitido - Aviso de Aceptación de fecha 19 de junio 2012
CANADÁ	2620343	Permitido - Aviso de Asignación de fecha 20 de abril 2012
CHINA	200680034242.6	Permitido - Aviso de la Fecha de Concesión de fecha 11 de junio 2012
EURASIA	013327	Concedido 30 de abril 2010 (validada en AM, AZ, BY, KZ, MD, RU, TJ, TM)
EUROPA	06801436.4-1216	Permitido - Aviso de Intención de Concesión de fecha 08 de marzo 2012
MÉXICO	285364	Concedido 05 de abril 2011
MÉXICO	298205	Concedido 13 de abril 2012
NEW ZELANDA	565964	Concedido 05 de marzo 2012
ESTADOS UNIDOS DE AMÉRICA	7,811,572	Concedido 12 de octubre 2010



La información pertinente en consideración con la acción judicial de la solicitud en China

En el caso de la solicitud en dicho país, durante la acción judicial correspondiente, que ahora está concedida; se presentaron argumentos similares a los consignados en este escrito, manifestando que no hubiera sido obvio para alguien versado en el arte usar TFF para purificar el conjugado, como se expone en la reivindicación dependiente 1 de la solicitud de patente anteriormente mencionada.

En este sentido, creemos necesario reiterar que la TFF es una técnica de ultrafiltración. Usa una membrana que tiene un tamaño de poro muy pequeño, por lo que es particularmente sensible. Como tal, aquellos versados en este arte habrían entendido en la fecha de prioridad de la presente solicitud que la TFF requiere una solución clara a ser aplicada a fin de que el proceso de ultrafiltración trabaje efectivamente. Por el contrario, una solución turbia (con los componentes disueltos sólo parcialmente) se hubiera esperado para obstruir la membrana y hacer el proceso de ultrafiltración inoperable.

En ese orden de ideas, alguna persona normalmente versada en la técnica ha apreciado que la solución que contiene el conjugado junto con maytansinoide que no reaccionó (DM1), DMA, y los agregados descritos en D1 hubieran estado turbios debido a la baja solubilidad del maytansinoide que no reacciona, lo cual hubiera empezado a sacarlos de la solución.

Por lo tanto, la persona versada en el arte, habría esperado que el proceso de TFF solamente hubiera hecho que este problema se acentuara (por la remoción del solvente), con el maytansinoide precipitado obstruyendo el sistema TFF y haciéndolo inoperable como método de purificación (ver párrafo 9 de la declaración radicada por Godfrey Amphlett con la respuesta previa de la Acción de la Oficina). De hecho, es bien conocido en el arte a partir de la fecha de prioridad de la solicitud que las incrustaciones de la membrana eran un problema importante asociado con el uso de la TFF. Ver, por Ejemplo, *Huisman et al., Journal de Membrana Science*, 179:79-90 (2000) (**ANEXO 2**), describe que el problema principal que restringe la aplicación práctica de la ultrafiltración es la incrustación de la membrana y el comportamiento resultante dependiente del tiempo de flujo y de rechazo (ver página 79).

Huisman et al. También describe que el flujo observado y el comportamiento de retención en la ultrafiltración puede estar relacionado con muchos mecanismos de incrustaciones tales como interacciones hidrofóbicas, interacciones electrostáticas, propiedades de proteínas tales como el tamaño y comportamiento de agregación (ver páginas 80-81).



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

En conclusión, teniendo en cuenta lo anterior que fue expuesto en el caso de la patente china y los argumentos presentados en este recurso, es evidente que únicamente la invención en estudio podría ser deducida por una persona medianamente versada en la materia técnica correspondiente a través de un análisis retrospectivo o hindsight de los documentos D1 y D2 dado que D1 enseñaba la eliminación del ligante que no reacciona con el anticuerpo modificado por TFF y D2 enseñaba la eliminación del ligante que no reacciona con el anticuerpo modificado mediante cromatografía de no adsorción pero que, de ninguna manera, enseñaban o sugerían una eliminación de las etapas de purificación o que se pudiera emplear la TFF para purificar directamente el conjugado, eliminando la necesidad de las etapas de cromatografía de intercambio iónico o de la cromatografía de no adsorción.

Sobre este particular, nos permitimos manifestar que la Decisión 486 en el artículo 45 establece que “*Cuando la oficina nacional competente estimara que ello es necesario para los fines del examen de patentabilidad podrá notificar al solicitante dos o más veces conforme al párrafo precedente*” (El resaltado es nuestro).

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Clarke, Modet & Cº

COLOMBIA

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De otra parte, es evidente la violación de este artículo por parte de la Resolución impugnada, toda vez que si el examinador consideró que la solicitud a pesar de las modificaciones restrictivas que se hicieron en el capítulo reivindicatorio, la invención aún necesitaba ser más aclarada, ha debido de darle la otra oportunidad al Solicitante de acuerdo con lo indicado en el artículo 45 de la Decisión 486 de la Comunidad Andina, para que se resolvieran dichas cuestiones.

2. Teniendo en cuenta lo dicho anteriormente, solicito a usted lo siguiente:

a) Que de conformidad con lo previsto por los artículos 3 (inciso 3); 35 (inciso 2) y 59 (inciso 2) del Código Contencioso Administrativo se consideren y se resuelvan TODOS los argumentos y TODAS las cuestiones planteadas así como las que aparezcan con motivo de este recurso.

b) Que se revoque la resolución **No. 40711** emitida el 28 de junio de 2012 por el Superintendente de Industria y Comercio, y se proceda a conceder el privilegio de patente de invención a la solicitud de mi mandante.

Señor Superintendente,

DILIA MARIA RODRIGUEZ D'ALEMAN
C.C. 41.654.882 de Bogotá
T.P. 20824 del C.S.J.

PRESENTACION PERSONAL
El anterior escrito fue presentado ante el
NOTARIO QUE SE DEL CIRCULO DE BOGOTA, D.C.
personalmente por Diego Rodriguez
quien exhibió la c.c. 1631082 B1
y Tarjeta Profesional No. 20824 03 AGO 2012
Fecha [Signature]
Guillermo Chavez Cristancho
NOTARIO

[Signature]
REPUBLICA DE COLOMBIA
Guillermo Chavez Cristancho
NOTARIO DEL CIRCULO DE BOGOTA, D.C.

ANEXO 1

Correspondientes copias de las reivindicaciones permitidas y la Notificación de
Asignación para cada una de las solicitudes australiana, canadiense, china y
Europea.

Chinese Patent Application No. 200680034242.6
ALLOWED CLAIMS

1. A process for preparing an antibody-maytansinoid conjugate comprising the steps of:
 - (a) contacting an antibody with a bifunctional crosslinking reagent to covalently attach a linker to the antibody and thereby prepare a first mixture comprising antibodies having linkers bound thereto,
 - (b) subjecting the first mixture to tangential flow filtration and thereby prepare a purified first mixture of antibodies having linkers bound thereto,
 - (c) conjugating a maytansinoid to the antibodies having linkers bound thereto in the purified first mixture by reacting the antibodies having linkers bound thereto with a maytansinoid in a solution having a pH of 4 to 9 to prepare a second mixture comprising (i) antibody chemically coupled through the linker to the maytansinoid, (ii) free maytansinoid, and (iii) reaction by-products, and
 - (d) subjecting the second mixture to tangential flow filtration to purify the antibodies chemically coupled through the linkers to the maytansinoid from the other components of the second mixture and thereby prepare a purified second mixture of antibodies chemically coupled through the linkers to the maytansinoid.
2. The process of claim 1, wherein the solution in step (c) has a pH of from 4 to 6.0.
3. The process of claim 1, wherein the solution in step (c) has a pH of from 6.5 to 9.
4. The process of any of claims 1-3, wherein the solution in step (c) comprises sucrose.
5. The process of any of claims 1-3, wherein the solution in step (c) comprises a buffering agent selected from the group consisting of a citrate buffer, an acetate buffer, a succinate buffer, and a phosphate buffer.
6. The process of any of claims 1-3, wherein the antibody is a monoclonal antibody.

7. The process of claim 6, wherein the monoclonal antibody is a humanized monoclonal antibody.
8. The process of claim 7, wherein the humanized monoclonal antibody is selected from the group consisting of huN901, huMy9-6, huB4, huC242, trastuzumab, bivatuzumab, sibrotuzumab, CNTO95, huDS6, and rituximab.
9. The process of any of claims 1-3, wherein the maytansinoid comprises a thiol group.
10. The process of claim 9, wherein the maytansinoid is N^{2'}-deacetyl-N^{2'}-(3-mercapto-1-oxopropyl)-maytansine (DM1).
11. The process of claim 9, wherein the maytansinoid is N^{2'}-deacetyl-N^{2'}-(4-methyl-4-mercapto-1-oxopentyl)-maytansine (DM4).
12. The process of any of claims 1-3, wherein the antibody is chemically coupled to the cytotoxic agent via chemical bonds selected from the group consisting of disulfide bonds, acid labile bonds, photolabile bonds, peptidase labile bonds, thioether bonds, and esterase labile bonds.
13. The process of any of claims 1-3, wherein the bifunctional crosslinking reagent is selected from the group consisting of N-succinimidyl 4-(2-pyridyldithio)butanoate (SPDB), N-succinimidyl 4-(2-pyridyldithio)pentanoate (SPP), and N-succinimidyl 4-(maleimidomethyl)cyclohexanecarboxylate (SMCC).
14. The process of any of claims 1-3, wherein the antibody is trastuzumab.
15. The process of claim 14, wherein the bifunctional crosslinking reagent is N-succinimidyl 4-(maleimidomethyl)cyclohexanecarboxylate (SMCC).
16. The processes of claim 15, wherein the maytansinoid is N^{2'}-deacetyl-N^{2'}-(3-mercapto-1-oxopropyl)-maytansine (DM1).



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June 11, 2012

Via E-mail only: CHICAGO-DOCKETINGPATENT@LEYDIG.COM

Attn.: JOHN KILYK, JR.

Y/R : 702460 (CHINA)

O/R : IIC080292

Re : Chinese Patent for Invention No. ZL200680034242.6

In the name of IMMUNOGEN, INC.

Title: PROCESS FOR PREPARING MAYTANSINOID ANTIBODY CONJUGATES

Dear Sir,

We are pleased to inform you that the Chinese Patent Office has decided to grant a patent right for invention to the above-captioned application. With the confirmation of this letter, we will enclose the notification to grant the patent right dated Jun 11, 2012 for your records.

Pursuant to Rule 54, the applicant is requested to pay the patent certificate fee and the annual fee to effect the registration of the patent, otherwise it will be deemed that the applicant has abandoned his right to obtain the patent right.

We will attend to the payment of the above-mentioned fees before the due date of Aug 27, 2012 and send our debit note to you thereafter unless we receive your instructions to the contrary.

For your information, please be informed that the applicant may voluntarily file divisional application(s) before the above non-extendible due date. If you would like to pursue any divisional application from this case, please kindly inform us at least three days before the said non-extendible due date.

In addition, The 8th annuity will be due on Aug 14, 2013 and the total amount is RMB¥2655.00.

Again, thank you very much for your trust and we look forward to cooperating with you in future.

Please kindly acknowledge safe receipt of this letter.

Sincerely yours,

Lili Wu

Director of Application Department

Encl. Notification to grant the patent right (Via airmail only)

Estimated Fees for Registration

Y/R : 702460 (CHINA)

O/R : IIC080292

Application No. : 200680034242.6

Items	Official Fee RMB	Attorney Fee RMB	Disbursement RMB
Certificate fee, including printing and stamp	255.00	700.00	
The 7 year annuity:	2000.00	630.00	
Reviewing and forwarding patent certificate and granted specification		450.00	
Courier			250.00
Facsimile			64.00
Total	RMB4349.00 about USD684.88		

Note: Exchange Rate : 1 USD = 6.35 RMB



100037

北京市阜成门外大街2号万通新世界广场8层 中国国际贸易促进委员会专利商标事务所
袁志明

发文日:

2012年06月11日

284



申请号或专利号: 200680034242.6

发文序号: 2012060600347650

申请人或专利权人: 免疫原公司

发明创造名称: 制备美登木素生物碱抗体缀合物的方法

办理登记手续通知书

根据专利法实施细则第54条及国家知识产权局第75号公告的规定, 申请人应当于2012年08月27日之前缴纳以下费用:

专利登记费	250.0 元	
第7年度年费	2000.0 元	无费减 (减缓标记)
专利证书印花税	5.0 元	
已缴费用	0 元	
应缴费用	2255.0 元	

申请人按期缴纳上述费用的, 国家知识产权局将在专利登记簿上登记专利权的授予, 颁发专利证书, 并予以公告。专利权自公告之日起生效。

申请人期满未缴纳或者未缴足上述费用的, 视为放弃取得专利权的权利。

提示:

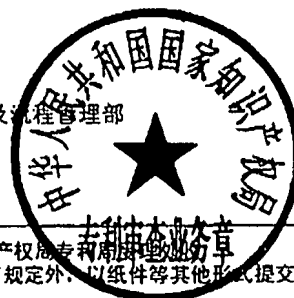
费用可以直接到国家知识产权局缴纳, 也可以通过邮局或者银行汇付。如通过邮局汇付, 收款人姓名: 国家知识产权局专利局收费处; 商户客户号: 110000860。如通过银行汇付, 开户银行: 中信银行北京知春路支行; 户名: 中华人民共和国国家知识产权局专利局; 账号: 7111710182600166032。

汇款时应当准确写明申请号、费用名称(或简称)及分项金额。未写明申请号和费用名称(或简称)的视为未办理缴费手续。

审查员: 姚燕

审查部门: 专利局初审及流程管理部

联系电话: 62356655



200602
2010.4

纸件申请, 回函请寄: 100088 北京市海淀区衙门桥西土城路6号 国家知识产权局专利局受理处
电子申请, 应当通过电子专利申请系统以电子文件形式提交相关文件。除另有规定外, 以纸件等其他形式提交的文件视为未提交。



100037

北京市阜成门外大街2号万通新世界广场8层 中国国际贸易促进委
员会专利商标事务所
袁志明

发文日:

2012年06月11日

194



申请号或专利号: 200680034242.6

发文序号: 2012060400425650

申请人或专利权人: 免疫原公司

发明创造名称: 制备美登木素生物碱抗体缀合物的方法

授予发明专利权通知书

(进入国家阶段的 PCT 申请)

1. 根据专利法第 39 条及实施细则第 54 条的规定, 上述发明专利申请经实质审查, 没有发现驳回理由, 现作出授予专利权的通知。

申请人收到本通知书后, 还应当依照办理登记手续通知书的内容办理登记手续。

申请人按期办理登记手续后, 国家知识产权局将作出授予专利权的决定, 颁发发明专利证书, 并予以登记和公告。期满未办理登记手续的, 视为放弃取得专利权的权利。

2. 授予专利权的上述发明专利申请是以下列申请文件为基础的:

☐ 原始提交的国际申请的中文文本或中文译文进行的。

☒ 审查是针对下列申请文件进行的:

2008 年 3 月 18 日提交的说明书摘要;

2010 年 4 月 19 日提交的说明书第 1-119 段;

2012 年 5 月 2 日提交的权利要求第 1-16 项。

3. 授予专利权的上述发明专利申请的名称:

☒ 未变更。

☐ 由__变更为上述发明创造名称。

4. ☐ 申请人于__年__月__日提交专利号为__的“放弃专利权声明”, 经审查:

☐ 进入放弃专利权的程序。

☐ 未进入放弃专利权的程序。理由是: 申请人声明放弃的专利与本发明专利申请不属于相同的发明创造。

5. ☐ 审查员依职权对申请文件修改如下:

6. 在本通知书发出后收到的申请人主动修改的申请文件, 不予考虑。

审查员: 韦轶

联系电话: 62412193

审查部门: 医药生物发明审查部



210414
2010.2

纸件申请, 回函请寄: 100088 北京市海淀区蓟门桥西土城路 6 号 国家知识产权局专利局受理处
电子申请, 应当通过电子专利申请系统以电子文件形式提交相关文件。除另有规定外, 以纸件等其他形式提交的文件视为未提交。

WE CLAIM:

1. A process for preparing an antibody-maytansinoid conjugate comprising the steps of:
 - a. contacting an antibody with a bifunctional crosslinking reagent to covalently attach a linker to the antibody and thereby prepare a first mixture comprising antibodies having linkers bound thereto,
 - b. subjecting the first mixture to tangential flow filtration, selective precipitation, adsorptive filtration, or adsorptive chromatography and thereby prepare a purified first mixture of antibodies having linkers bound thereto,
 - c. conjugating a maytansinoid to the antibodies having linkers bound thereto in the purified first mixture by reacting the antibodies having linkers bound thereto with the maytansinoid in a solution having a pH of 4 to 9 to prepare a second mixture comprising (i) antibody chemically coupled through the linker to the maytansinoid, (ii) free maytansinoid, and (iii) reaction by-products, and
 - d. subjecting the second mixture to tangential flow filtration to purify the antibodies chemically coupled through the linkers to the maytansinoid from the other components of the second mixture and thereby prepare a purified second mixture of antibodies chemically coupled through the linkers to the maytansinoid.
2. The process of claim 1, wherein the adsorptive chromatography is selected from the group consisting of hydroxyapatite chromatography, hydrophobic charge induction chromatography (HCIC), hydrophobic interaction chromatography (HIC), ion exchange chromatography, mixed mode ion exchange chromatography, immobilized metal affinity chromatography (IMAC), dye ligand chromatography, affinity chromatography, reversed phase chromatography, and combinations thereof.
3. The process of claim 1, wherein tangential flow filtration is utilized in step (b).
4. The process of claim 1, wherein selective precipitation is utilized in step (b).

5. The process of claim 1, wherein adsorptive filtration is utilized in step (b).
6. The process of claim 1 or 2, wherein adsorptive chromatography is utilized in step (b).
7. The process of claim 6, wherein the adsorptive chromatography is hydroxyapatite chromatography.
8. The process of any one of claims 1-7, wherein the solution in step (c) has a pH of from 4 to 6.
9. The process of any one of claims 1-7, wherein the solution in step (c) has a pH of from 6.5 to 9.
10. The process of any one of claims 1-9, wherein the solution in step (c) comprises sucrose.
11. The process of any one of claims 1-10, wherein the solution in step (c) further comprises a buffering agent selected from the group consisting of a citrate buffer, an acetate buffer, a succinate buffer, and a phosphate buffer.
12. The process of any one of claims 1-11, wherein the antibody is a monoclonal antibody.
13. The process of claim 12, wherein the antibody is a humanized monoclonal antibody.
14. The process of claim 13, wherein the antibody is selected from the group consisting of huN901, huMy9-6, huB4, huC242, trastuzumab, bivatuzumab, sibrotuzumab, CNTO95, huDS6, and rituximab.
15. The process of any one of claims 1-11, wherein the antibody is trastuzumab.
16. The process of any one of claims 1-11, wherein the antibody is a chimeric antibody.
17. The process of any one of claims 1-11, wherein the antibody is a humanized antibody.
18. The process of any one of claims 1-11, wherein the antibody is a human monoclonal antibody.

19. The process of any one of claims 1-11, wherein the antibody is a mouse monoclonal antibody.
20. The process of any one of claims 1-19, wherein the maytansinoid comprises a thiol group.
21. The process of claim 20, wherein the maytansinoid is N^{2'}-deacetyl-N^{2'}-(3-mercapto-1-oxopropyl)-maytansine (DM1).
22. The process of claim 20, wherein the maytansinoid is N^{2'}-deacetyl-N^{2'}-(4-methyl-4-mercapto-1-oxopentyl)-maytansine (DM4).
23. The process of any one of claims 1-22, wherein the antibody having linkers bound thereto is chemically coupled to the maytansinoid via chemical bonds selected from the group consisting of disulfide bonds, acid labile bonds, photolabile bonds, peptidase labile bonds, thioether bonds, and esterase labile bonds.
24. The process of any one of claims 1-22, wherein the bifunctional crosslinking reagent comprises an N-succinimidyl ester moiety.
25. The process of any one of claims 1-22, wherein the bifunctional crosslinking reagent comprises an N-sulfosuccinimidyl ester moiety.
26. The process of any one of claims 1-22, wherein the bifunctional crosslinking reagent comprises a maleimido-based moiety.
27. The process of any one of claims 1-22, wherein the bifunctional crosslinking reagent comprises a haloacetyl-based moiety.
28. The process of any one of claims 1-22, wherein the bifunctional crosslinking reagent is selected from the group consisting of N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP), N-succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxy-(6-amidocaproate), (LC-SMCC), κ -maleimidoundecanoic acid N-succinimidyl ester (KMUA), γ -maleimidobutyric acid N-succinimidyl ester (GMBS), ϵ -maleimidocaproic

acid N-hydroxysuccinimide ester (EMCS), m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), N-(α -maleimidoacetoxy)-succinimide ester (AMAS), succinimidyl-6-(β -maleimidopropionamido)hexanoate (SMPH), N-succinimidyl 4-(p-maleimidophenyl)-butyrate (SMPB), N-(p-maleimidophenyl)isocyanate (PMPI), N-succinimidyl-4-(iodoacetyl)-aminobenzoate (SIAB), N-succinimidyl iodoacetate (SIA), N-succinimidyl bromoacetate (SBA), and N-succinimidyl 3-(bromoacetamido)propionate (SBAP).

29. The process of any one of claims 1-22, wherein the bifunctional crosslinking reagent is N-succinimidyl 4-(2-pyridyldithio)butanoate (SPDB).
30. The process of any one of claims 1-22, wherein the bifunctional crosslinking reagent is N-succinimidyl 4-(2-pyridyldithio)pentanoate (SPP).
31. The process of any one of claims 1-22, wherein the bifunctional crosslinking reagent is N-succinimidyl 4-(maleimidomethyl)cyclohexanecarboxylate (SMCC).
32. The process of any one of claims 1-11, wherein the antibody is huN901 and the maytansinoid is N^{2'}-deacetyl-N^{2'}-(3-mercapto-1-oxopropyl)-maytansine (DM1).
33. The process of claim 32, wherein the bifunctional crosslinking reagent is N-succinimidyl 4-(2-pyridyldithio)pentanoate (SPP).
34. The process of any one of claims 1-11, wherein the antibody is trastuzumab and the maytansinoid is N^{2'}-deacetyl-N^{2'}-(4-methyl-4-mercapto-1-oxopentyl)-maytansine (DM4).
35. The process of claim 34, wherein the bifunctional crosslinking reagent is N-succinimidyl 4-(2-pyridyldithio)butanoate (SPDB).
36. The process of any one of claims 1-11, wherein the antibody is trastuzumab and the maytansinoid is N^{2'}-deacetyl-N^{2'}-(3-mercapto-1-oxopropyl)-maytansine (DM1).
37. The process of claim 36, wherein the bifunctional crosslinking reagent is N-succinimidyl 4-(maleimidomethyl)cyclohexanecarboxylate (SMCC).

38. The process of any one of claims 1-11, wherein the antibody is huB4 and the maytansinoid is N^{2'}-deacetyl-N^{2'}-(4-methyl-4-mercapto-1-oxopentyl)-maytansine (DM4).
39. The process of claim 38, wherein the bifunctional crosslinking reagent is N-succinimidyl 4-(2-pyridyldithio)butanoate (SPDB).
40. The process of any one of claims 1-11, wherein the antibody is huDS6 and the maytansinoid is N^{2'}-deacetyl-N^{2'}-(4-methyl-4-mercapto-1-oxopentyl)-maytansine (DM4).
41. The process of claim 40, wherein the bifunctional crosslinking reagent is N-succinimidyl 4-(2-pyridyldithio)butanoate (SPDB).
42. The process of any one of claims 1-11, wherein the antibody is huC242 and the maytansinoid is N^{2'}-deacetyl-N^{2'}-(3-mercapto-1-oxopropyl)-maytansine (DM1).
43. The process of claim 42, wherein the bifunctional crosslinking reagent is N-succinimidyl 4-(2-pyridyldithio)pentanoate (SPP).
44. The process of any one of claims 1-11, wherein the antibody is huC242 and the maytansinoid is N^{2'}-deacetyl-N^{2'}-(4-methyl-4-mercapto-1-oxopentyl)-maytansine (DM4).
45. The process of claim 44, wherein the bifunctional crosslinking reagent is N-succinimidyl 4-(2-pyridyldithio)butanoate (SPDB).
46. The process of any one of claims 1-11, wherein the antibody is trastuzumab, the bifunctional crosslinking reagent is N-succinimidyl 4-(maleimidomethyl)cyclohexanecarboxylate (SMCC), and the maytansinoid is N^{2'}-deacetyl-N^{2'}-(3-mercapto-1-oxopropyl)-maytansine (DM1).

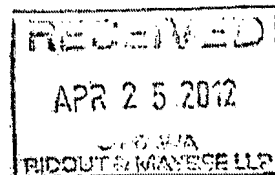


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Date : 2012/04/20

Classification :
A61K 47/48

AVIS D'ACCEPTATION/NOTICE OF ALLOWANCE

N° de demande/Application No. : 2,620,343

Date de dépôt/Filing date : 2006/08/14

Votre référence/
Your Reference : 33170-0122 SJH:KK

Titre de l'invention/
Title of Invention : PROCESS FOR PREPARING ANTIBODY MAYTANSINOID CONJUGATES

Propriétaire(s)/Owner(s) : IMMUNOGEN, INC.

Revendications/Claims : 046

Examiné tel que modifié/
Examined as amended : 2012/03/22

La demande de brevet susmentionnée a été jugée acceptable.

Il faut acquitter la taxe finale de TROIS CENTS DOLLARS (300\$), ou CENT CINQUANTE DOLLARS (150\$) si le requérant a le droit de revendiquer le statut de petite entité et a soumis une déclaration de petite entité, dans les six mois suivant la date du présent avis. Faute de quoi la demande sera réputée abandonnée conformément à l'alinéa 73(1)f) de la Loi sur les brevets.

Une taxe additionnelle de six dollars (6\$) par page excédant 100 pages du mémoire descriptif et dessins devra aussi être payée.

Le brevet sera délivré au nom du dernier propriétaire inscrit à nos dossiers à qui a fourni une documentation acceptable, au plus tard la date du paiement de la taxe finale, conformément à l'article 41 des Règles sur les brevets.

La réponse au présent avis doit comprendre l'identification complète de la demande et la date de l'avis.

La publication des brevets canadiens délivrés dans la Gazette du Bureau des brevets peut comprendre aussi une note concernant la mise en vente d'un brevet ou de sa licence. Si vous désirez profiter de ce service gratuit, veuillez l'indiquer au moment de payer la taxe finale.

The above application for patent has been found allowable.

The final fee of THREE HUNDRED DOLLARS (\$300), or ONE HUNDRED AND FIFTY DOLLARS (\$150) where the applicant is entitled to claim small entity status and has submitted a small entity declaration, must be paid within six months following the date of this notice. Otherwise, the application will be deemed to be abandoned pursuant to paragraph 73(1)f) of the Patent Act.

An additional fee of six dollars (\$6.00) per page over 100 pages of specification and drawings must also be paid.

The patent shall issue to the last registered owner who has submitted acceptable documentation on or before the date that the final fee is paid (as pursuant to Section 41 of the Patent Rules).

A reply to this notice must include full identification of the application including the date of the notice.

The publication of issued Canadian patents in the Patent Office Record can also include an indication that the patent is available for licence or sale. If you wish to take advantage of this free service, please indicate this when paying the final fee.

Commissaire aux brevets/Commissioner of Patents

Canada

OPIC  CIPO

WHAT IS CLAIMED IS:

1. A process for preparing an antibody-maytansinoid conjugate comprising the steps of:
 - (a) contacting an antibody with a bifunctional crosslinking reagent to covalently attach a linker to the antibody and thereby prepare a first mixture comprising antibodies having linkers bound thereto,
 - (b) subjecting the first mixture to tangential flow filtration, selective precipitation, adsorptive filtration, or adsorptive chromatography and thereby prepare a purified first mixture of antibodies having linkers bound thereto,
 - (c) conjugating a maytansinoid to the antibodies having linkers bound thereto in the purified first mixture by reacting the antibodies having linkers bound thereto with a maytansinoid in a solution having a pH of about 4 to about 9 to prepare a second mixture comprising (i) antibody chemically coupled through the linker to the maytansinoid, (ii) free maytansinoid, and (iii) reaction by-products produced during step (c), and
 - (d) subjecting the second mixture to tangential flow filtration to purify the antibodies chemically coupled through the linkers to the maytansinoid from the other components of the second mixture and thereby prepare a purified second mixture of antibodies chemically coupled through the linkers to the maytansinoid.
2. The process of claim 1, wherein the adsorptive chromatography is selected from the group consisting of hydroxyapatite chromatography, hydrophobic charge induction chromatography (HCIC), hydrophobic interaction chromatography (HIC), ion exchange chromatography, mixed mode ion exchange chromatography, immobilized metal affinity chromatography (IMAC), dye ligand chromatography, affinity chromatography, reversed phase chromatography, and combinations thereof.
3. The process of claim 1, wherein tangential flow filtration is utilized in step (b).
4. The process of claim 1, wherein selective precipitation is utilized in step (b).
5. The process of claim 1, wherein adsorptive filtration is utilized in step (b).
6. The process of claim 1 or 2, wherein adsorptive chromatography is utilized in step (b).
7. The process of any of claims 1-6, wherein the solution in step (c) has a pH of from about 4 to about 6.
8. The process of any of claims 1-6, wherein the solution in step (c) has a pH of from about 6.5 to about 9.

9. The process of any of claims 1-8, wherein the solution in step (c) comprises sucrose.

10. The process of any of claims 1-9, wherein the solution in step (c) comprises a buffering agent selected from the group consisting of a citrate buffer, an acetate buffer, a succinate buffer, and a phosphate buffer.

11. A process for preparing an antibody-maytansinoid conjugate comprising the steps of:

(a) contacting an antibody with a bifunctional crosslinking reagent to covalently attach a linker to the antibody and thereby prepare a first mixture comprising antibodies having linkers bound thereto,

(b) conjugating a maytansinoid to the antibodies having linkers bound thereto in the first mixture by reacting the antibody having linkers bound thereto with a maytansinoid in a solution having a pH of about 4 to about 9 to prepare a second mixture comprising (i) antibody chemically coupled through the linker to the maytansinoid, (ii) free maytansinoid, and (iii) reaction by-products produced during step (b), and

(c) subjecting the second mixture to tangential flow filtration, selective precipitation, adsorptive filtration, adsorptive chromatography, or a combination thereof, to purify the antibodies chemically coupled through the linkers to the maytansinoid from the other components of the second mixture and thereby prepare a purified second mixture of antibodies chemically coupled through the linkers to the maytansinoid

with the proviso that the first mixture is not subjected to tangential flow filtration, selective precipitation, adsorptive filtration, or adsorptive chromatography.

12. The process of claim 11, wherein the first mixture is not subjected to purification.

13. The process of claim 11 or 12, wherein the adsorptive chromatography is selected from the group consisting of hydroxyapatite chromatography, hydrophobic charge induction chromatography (HCIC), hydrophobic interaction chromatography (HIC), ion exchange chromatography, mixed mode ion exchange chromatography, immobilized metal affinity chromatography (IMAC), dye ligand chromatography, affinity chromatography, reversed phase chromatography, and combinations thereof.

14. The process of any of claims 11-13, wherein the solution in step (b) has a pH of from about 4 to about 6.

15. The process of any of claims 11-13, wherein the solution in step (b) has a pH of from about 6.5 to about 9.

16. The process of any of claims 11-13, wherein the solution in step (b) has a pH of less than 6 or a pH of greater than 6.5.

17. The process of any of claims 11-16, wherein the solution in step (b) comprises sucrose.

18. The process of any of claims 11-17, wherein the solution in step (b) comprises a buffering agent selected from the group consisting of a citrate buffer, an acetate buffer, a succinate buffer, and a phosphate buffer.

19. The process of any of claims 1-18, wherein the antibody is a monoclonal antibody.

20. The process of any of claims 1-18, wherein the antibody is a humanized monoclonal antibody.

21. The process of any of claims 1-18, wherein the antibody is selected from the group consisting of a chimeric antibody, a humanized antibody, a human monoclonal antibody, and a mouse monoclonal antibody.

22. The process of any of claims 1-18, wherein the antibody is selected from the group consisting of huN901, huMy9-6, huB4, huC242, trastuzumab, bivatuzumab, sibrotuzumab, CNTO95, huDS6, and rituximab.

23. The process of any of claims 1-18, wherein the antibody is trastuzumab.

24. The process of any of claims 1-23, wherein the maytansinoid comprises a thiol group.

25. The process of claim 24 wherein the maytansinoid is N^{2'}-deacetyl-N^{2'}-(3-mercapto-1-oxopropyl)-maytansine (DM1).

26. The process of claim 24, wherein the maytansinoid is N^{2'}-deacetyl-N^{2'}-(4-methyl-4-mercapto-1-oxopentyl)-maytansine (DM4).

27. The process of any of claims 1-26, wherein the antibody is chemically coupled to the maytansinoid via chemical bonds selected from the group consisting of disulfide bonds, acid labile bonds, photolabile bonds, peptidase labile bonds, thioether bonds, and esterase labile bonds.

28. The process of any of claims 1-27, wherein the bifunctional crosslinking reagent is selected from the group consisting of N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP), N-succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxy-(6-amidocaproate), (LC-SMCC), κ -maleimidoundecanoic acid N-succinimidyl ester (KMUA), γ -maleimidobutyric acid N-succinimidyl ester (GMBS), ϵ -maleimidocaproic acid N-hydroxysuccinimide ester (EMCS), m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), N-(α -maleimidoacetoxy)-succinimide ester (AMAS), succinimidyl-6-(β -maleimidopropionamido)hexanoate (SMPH), N-succinimidyl 4-(p-maleimidophenyl)-butyrate (SMPB), N-(p-maleimidophenyl)isocyanate (PMPI),

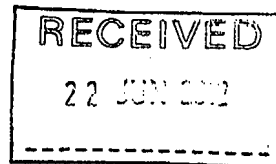
N-succinimidyl-4-(iodoacetyl)-aminobenzoate (SIAB), N-succinimidyl iodoacetate (SIA), N-succinimidyl bromoacetate (SBA), and N-succinimidyl 3-(bromoacetamido)propionate (SBAP).

29. The process of any of claims 1-27, wherein the bifunctional crosslinking reagent is selected from the group consisting of N-succinimidyl 4-(2-pyridyldithio)butanoate (SPDB), N-succinimidyl 4-(2-pyridyldithio)pentanoate (SPP), and N-succinimidyl 4-(maleimidomethyl)cyclohexanecarboxylate (SMCC).

30. The process of any of claims 1-27, wherein the bifunctional crosslinking reagent is N-succinimidyl 4-(maleimidomethyl)cyclohexanecarboxylate (SMCC).

31. The process of any of claims 1-18, wherein the antibody is trastuzumab, the bifunctional crosslinking reagent is N-succinimidyl 4-(maleimidomethyl)cyclohexanecarboxylate (SMCC), and the maytansinoid is N^{2'}-deacetyl-N^{2'}-(3-mercapto-1-oxopropyl)-maytansine (DM1).

19 June 2012



Australian Government

IP Australia

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RE: Patent Application number **2006283726**
in the name(s) of **ImmunoGen, Inc.**

Your Ref: **18314AU1-PDWPDW/JRL**

Dear Sir/Madam

Notice of Acceptance

I am pleased to advise that there are no objections to the application. The Examiner has incorporated into the complete specification amendments made under PCT Article 34 and/or Section 104 based on the following:

PCT: - Not Applicable

S104 amendments up to and including item number: - 3

The application and complete specification were accepted on 18 June 2012 and a notice of the acceptance will appear in the Supplement to the Australian Official Journal of Patents on 28 June 2012.

For applications filed after 31 August 2002 or that entered National Phase after 31 August 2002, a fee for acceptance of an application applies. This fee includes a component determined by the number of claims in excess of 20. If the acceptance fee applies and has not been paid, an Invitation to Pay (ITP) will issue. If the amount is paid by the due date, your patent will be sealed as soon as practicable after the 3 month period for opposition has expired. The total number of claims at acceptance has been reported as: 31

If you need any further information please contact (02) 6283 2898. Alternatively, you may contact us by email at assist@ipaustalia.gov.au.

Yours faithfully

Electronic Records Administration

Bibliographic Details at Acceptance

PAMS Details for Patent Application No. 2006283726

Your Reference: 18314AU1-PDWPDW/JRL

Acceptance Date: 18 June 2012

Acceptance to be Advertised: 28 June 2012

Complete Filing Date: 14 August 2006

OPI Date: 1 March 2007

National Phase Entry Date: 12 February 2008

Applicant Name: ImmunoGen, Inc.

Address: 830 Winter Street
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United States of America

Inventor Name(s): Amphlett, Godfrey W.
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Dai, Yong
Meshulam, Deborah H.
Wang, Yong

Title: Process for preparing maytansinoid antibody conjugates

Agent Name: Fisher Adams Kelly

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Prior Art Documents:
WO 2002/098897 A2
WO 2003/057163 A2

Priority Details:		
Date	Application Number	Country
4 May 2006	60/797,713	United States of America
24 August 2005	60/710,858	United States of America

Int. Cl.
A61K 47/48 (2006.01)

Continuation Fee Due Date: 14 August 2012



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Date
05.07.12

Reference P38206EP-PCT	Application No./Patent No. 06801436.4 - 1216 / 1928503
Applicant/Proprietor ImmunoGen, Inc.	

Amendment or correction of the text intended for grant

Your request of 16.05.12 for amendment or correction of the text intended for grant pursuant to Rule 71(4) or (5) EPC in force until 31.03.2012 has been allowed.

- ☒ The replacement pages have been used.
- ☒ A copy of the requested amendments or corrections made is attached hereto. (desc. p.12,21,22)
- ☐ Invitation to pay additional claims fees

The text intended for grant as amended/corrected comprises more additional claims than the text enclosed with the communication under Rule 71(3) EPC. You are therefore invited under Rule 71(6) EPC in force until 31.03.2012 to pay

within **TWO** months

of notification of this communication claims fees in respect of additional claims (Ref. 016).

If the claims fees are not paid in due time, the European patent application shall be deemed to be withdrawn (R. 71(7) EPC).

Note to users of the automatic debiting procedure

The fees for the additional claim(s) as mentioned above will be debited automatically on the last day of the period of this communication. For further details see the Arrangements for the automatic debiting procedure (AAD) and accompanying information from the EPO concerning the automatic debiting procedure (Annexes A.1 and A.2 to the Arrangements for deposit accounts (ADA) in Supplement to OJ EPO 3/2009.

For the Examining Division



The total number of pages is now 38, you are hereby invited to pay the printing fee for 1 additional page.

☒ Registered letter

EPO Form 2098A 06.12 (02/07/12)

ACOR

AM01984

4

18. The process of claim 17, wherein the cytotoxic agent is a maytansinoid.

any of 1 to 10
11 19. The process of claim 18, wherein the maytansinoid comprises a thiol group.

11
12 20. The process of claim 19, wherein the maytansinoid is DM1.

11
13 21. The process of claim 19, wherein the maytansinoid is DM4.

13
14 22. The process of any of claims 1-21, wherein the antibody is chemically coupled to the ~~cytotoxic agent~~ ^{maytansinoid} via chemical bonds selected from the group consisting of disulfide bonds, acid labile bonds, photolabile bonds, peptidase labile bonds, thioether bonds, and esterase labile bonds.

15. The process of any of Claims 1 to 14, wherein the first mixture is subjected to tangential flow filtration in step (b).

Y

10. The process of claim 9, wherein the antibody is trastuzumab.



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European Patent Office
Erhardtstrasse 27
D-80298 Munich
Germany

Our Ref: P38206EP-PCT

Date: 16 May 2012

E-Filing

Dear Sirs

European Patent Application No. 06801436.4
Process for preparing maytansinoid antibody conjugates
ImmunoGen, Inc.

Filed herewith are translations of the claims of the above-mentioned application in the French and German languages.

The Applicant also files herewith amended pages 3, 5, 6, 7, 9, 12, 22, 32, and 35 of the description and page 39 of the claims to replace the corresponding pages in the Drukexemplar. In addition, the Applicant files herewith an amended page 21 (in the Drukexemplar this page was proposed to be deleted in its entirety). The amendments filed herewith were discussed with the Examiner in a telephone conversation on 18 April 2012 and it is understood that the Examiner consents to them.

The Applicant approves the text proposed for grant subject to the inclusion of the amended pages filed herewith.

Grant and printing fees are being paid by way of Form 1038e accompanying this letter.

You are authorised to deduct any shortfall of fees from our deposit account number 2805-0049.

Yours faithfully

Evenson, Jane Harriet
For and on behalf of
MATHYS & SQUIRE LLP
Association No 171

Encs: Claims translations
Amended pages 3, 5, 6, 7, 9, 12, 21, 22, 32, and 35 of the description
Amended page 39 of the claims

EUROPEAN PATENT ATTORNEYS | CHARTERED PATENT ATTORNEYS | EUROPEAN TRADEMARK ATTORNEYS | REGISTERED TRADEMARK AGENTS
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A list of the members of the LLP is open to inspection at the registered office. We use the word "partner" to refer to a member of the LLP or an employee or consultant of similar seniority.



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Application No. 06 801 436.4 - 1216	Ref. P38206EP-PCT	Date 08.03.2012
Applicant ImmunoGen, Inc.		

Communication under Rule 71(3) EPC

You are informed that the Examining Division intends to grant a European patent on the basis of the above application with the text and drawings as indicated below:

In the text for the Contracting States:

AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IS IT LI LT LU LV MC NL PL PT RO SE SI SK TR

Description, Pages

1, 2, 4, 5, 7-14, 16-18, 22-34 as originally filed

15, 19	received on	21-05-2008	with letter of	20.05.2008
3, 6, 6a	received on	16-10-2009	with letter of	15-10-2009

Claims, Numbers

1-14	filed in electronic form on	03-02-2012
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With the following amendments to the above-mentioned documents by the division

Description, Pages 1-7, 11-13, 22-24, 31, 34
Delete Previous: 6b, 20, 21, 35

Comments

Amendments on pages 1-6,7,11-13,22-24,31,34 by the Examining Division to bring the description into conformity with the claims and to remove superfluous passages. The amendments resulted in

Registered Letter

EPO Form 2004 04.11TRI

the deletion of pages 6b,20,21,35.

A copy of the relevant documents is enclosed

The title of the invention in the three official languages of the European Patent Office, the international patent classification, the designated Contracting States, the registered name of the applicant and the bibliographic data are shown on the attached EPO Form 2056.

You are requested within a non-extendable period of **four months** of notification of this communication

1.	to file 1 set of translations of the claim(s) in the two other EPO official languages;		EUR
2a.	to pay the fee for grant including the fee for printing up to and including 35 pages; Reference 007		830.00
2b.	to pay the printing fee for the 36th and each subsequent page; number of pages: 2 Reference 008		26.00
3.	to pay the additional claim fee(s) (R. 71(6) EPC); number of claims fees payable: 0 Reference 016		0.00
	Total amount		856.00

The mention of the grant of the patent shall be published in the European Patent Bulletin as soon as possible after the requirements concerning the translation of the claims and the payment of the fees for grant and printing, claims fees, designation fees and renewal fees as laid down in Rule 71(3), (4), (6) and (8) and (9) EPC are fulfilled.

If you do not approve the text intended for grant but wish to request amendments or corrections, the procedure described in Rule 71(4) EPC is to be followed.

If filing amendments, you must identify them and indicate the basis for them in the application as filed. Failure to meet either requirement may lead to a communication from the Examining Division requesting that you correct this deficiency (R. 137(4) EPC).

If this communication is based upon an auxiliary request, and you reply within the time limit set that you maintain the main or a higher ranking request which is not allowable, the application will be refused (Art. 97(2) EPC).

If the enclosed claims contain amendments proposed by the Examining Division, and you reply within the time limit set that you cannot accept these amendments, refusal of the application under Article 97(2) EPC will result if agreement cannot be reached on the text for grant.

In all cases except those of the previous two paragraphs, if the fees for grant and printing or claims fees are not paid, or the translations are not filed, in due time, the European patent application will be deemed to be withdrawn (R. 71(7) EPC).

For all payments you are requested to use EPO Form 1010 or EPO Form 1010E or to refer to the relevant reference number.

After publication, the European patent specification can be downloaded free of charge from the EPO publication server <https://data.epo.org/publication-server/> or ordered from the Vienna sub-office upon payment of a fee (OJ EPO 2005, 126).

Upon request in writing each proprietor will receive the certificate for the European patent **together with one copy** of the patent specification provided that the request is filed within the time limit of Rule 71(3) EPC. If such request has been previously filed, it has to be confirmed within the time limit of Rule 71(3) EPC. The requested copy is free of charge. If the request is filed after expiry of the Rule 71(3) EPC time limit, the certificate will be delivered without a copy of the patent specification (R.74 EPC, Decision of the President of the EPO, Special edition No.3, OJ EPO 2007, D.2).

Filing of a divisional application

Any divisional application relating to this European patent application must be filed directly with the European Patent Office in Munich, The Hague or Berlin and shall be in the language of the proceedings relating to the present application (cf. Article 76(1) and Rule 36(2) EPC). Any such divisional application must be filed while the present application is still pending and the time limit for filing divisional applications must be observed (Rule 36(1) EPC; Guidelines for Examination in the EPO, A-IV, 1.1.1).

Note on payment of renewal fees

If a renewal fee falls due between notification of the present communication and the proposed date of publication of the mention of the grant of the European patent, publication will be effected only after the renewal fee and any additional fee have been paid (R. 71(9) EPC).

Under Article 86(2) EPC, the obligation to pay renewal fees to the European Patent Office terminates with the payment of the renewal fee due in respect of the year in which the mention of the grant of the European patent is published.

Filing of translations in the Contracting States

As regards translation requirements prescribed by the Contracting States under Article 65(1) EPC, please consult the website of the European Patent Office
www.epo.org → Patents → Law → Legal texts → National law relating to the EPC
www.epo.org → Patents → Law → Legal texts → London Agreement

In case of a valid extension

As regards translation requirements prescribed by the Extension States, please consult the website of the European Patent Office
www.epo.org → Patents → Law → Legal texts → National law relating to the EPC

Failure to supply a prescribed translation in a Contracting State or an Extension State may result in the patent being deemed to be void *ab initio* in the State concerned (Article 65(3) EPC).

Important note to users of the automatic debiting procedure

The fees for grant and printing and also any additional claims fees due under Rule 71(6) EPC will be debited automatically on the date of filing of the translation of the (relevant) claims, or on the last day of the period of this communication. However, if the designation fees become due as set out in Rule 71(8) EPC and/or a renewal fee becomes due as set out in Rule 71(9) EPC, these should be paid separately by another permitted means of payment in order not to delay the publication of the mention of grant. The same applies in these circumstances to the payment of extension fees. For further details see the Arrangements for the automatic debiting procedure (AAD) and accompanying Information from the EPO concerning the automatic debiting

procedure (Annexes A.1 and A.2 to the Arrangements for deposit accounts (ADA) in Supplement to OJ EPO 3/2009).

Important information concerning fee amounts

Following any amendment to the Rules relating to Fees, the amount(s) mentioned in this communication may be different from the amount(s) **actually due on the date of payment**. The latest version of the Schedule of fees and expenses, published as a Supplement to the Official Journal of the EPO, is also available on the EPO website (www.epo.org) and can be found under www.epoline.org, which allows the viewing, downloading and searching for individual fee amounts, both current and previous.

Payments by cheque delivered or sent direct to the EPO are no longer accepted as from 1 April 2008 (see OJ EPO 2007, 626).

Examining Division:

Chairman:	Hoff, Philippe
2nd Examiner:	Birikaki, Lemonia
1st Examiner:	Kukolka, Florian



Hübner, Werner
For the Examining Division
Tel. No.: +31 70 340 - 2665

Branch at The Hague

Enclosure(s): Form 2056
 37 Copies of the relevant documents

Application No.:

06 801 436.4

IV.2. Patent classification

The classification indicated on the published patent application remains unchanged. It is as follows:

INV. A61K47/48

IV.3. Title of the invention

The title has been changed. It now is as follows:

VERFAHREN ZUR HERSTELLUNG VON MAYTANSINOID-ANTIKÖRPER-
KONJUGATEN

PROCESS FOR PREPARING MAYTANSINOID ANTIBODY CONJUGATES

Procédé de preparation de conjuguées de maytansinoïde et d'anticorps

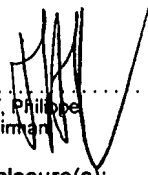
IV.4. Documentation

STIN The file contains technical information submitted after the application was filed and not included in this specification.

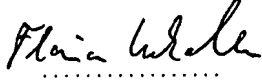
CDOC The following documents not mentioned in the Search Report were cited during the Examination Procedure:

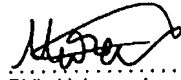
US 2005/175619 A1 (DUFFY ROBERT [US] ET AL) 11 August 2005
(2005-08-11)

28.02.2012
Date


Hoff, Philippe
Chairman

Enclosure(s):


Kukolka, Florian
1st examiner


Birikaki, LEMONIA
2nd examiner

Amended Claims

1. A process for preparing an antibody-^{maytansinoid}~~cytotoxic agent~~ conjugate comprising the steps of:
- (a) contacting an antibody with a bifunctional crosslinking reagent to covalently attach a linker to the antibody and thereby prepare a first mixture comprising antibodies having linkers bound thereto,
 - (b) subjecting the first mixture to tangential flow filtration and thereby prepare a purified first mixture of antibodies having linkers bound thereto,
 - (c) conjugating a ^{maytansinoid}~~cytotoxic agent~~ to the antibodies having linkers bound thereto in the purified first mixture by reacting the antibodies having linkers bound thereto with a ^{maytansinoid}~~cytotoxic agent~~ in a solution having a pH of ~~about~~ 4 to ~~about~~ 9 to prepare a second mixture comprising (i) antibody chemically coupled through the linker to the ^{maytansinoid}~~cytotoxic agent~~, (ii) free ^{maytansinoid}~~cytotoxic agent~~, and (iii) reaction by-products, and
 - (d) subjecting the second mixture to a tangential flow filtration to purify the antibodies chemically coupled through the linkers to the ^{maytansinoid}~~cytotoxic agent~~ from the other components of the second mixture and thereby prepare a purified second mixture of antibodies chemically coupled through the linkers to the ^{maytansinoid}~~cytotoxic agent~~.
2. The process of claim 1, wherein the solution in step (c) has a pH of from ~~about~~ 4 to 6.0.
3. The process of claim 1, wherein the solution in step (c) has a pH of from 6.5 to ~~about~~ 9.
4. The process of claim 1, wherein the solution in step (c) has a pH of less than 6.0 or a pH of greater than 6.5.
- (X) , selective precipitation, adsorptive filtration, or an adsorptive chromatography resin

5. The process of any of claims 1-4, wherein the solution in step (c) comprises sucrose.

6. The process of any of claims 1-5, wherein the solution in step (c) further comprises a buffering agent selected from the group consisting of a citrate buffer, an acetate buffer, a succinate buffer, and a phosphate buffer.

7. A process for preparing an antibody-cytotoxic agent conjugate comprising the steps of:

(a) contacting an antibody with a bifunctional crosslinking reagent to covalently attach a linker to the antibody and thereby prepare a first mixture comprising antibodies having linkers bound thereto,

(b) conjugating a cytotoxic agent to the antibodies having linkers bound thereto in the first mixture by reacting the antibody having linkers bound thereto with a cytotoxic agent in a solution having a pH of about 4 to about 9 to prepare a second mixture comprising (i) antibody chemically coupled through the linker to the cytotoxic agent, (ii) free cytotoxic agent, and (iii) reaction by-products produced during step (b), and

(c) subjecting the second mixture to tangential flow filtration, selective precipitation, adsorptive filtration, adsorptive chromatography, or a combination thereof, to purify the antibodies chemically coupled through the linkers to the cytotoxic agent from the other components of the second mixture and thereby prepare a purified second mixture of antibodies chemically coupled through the linkers to the cytotoxic agent wherein the first mixture is not subjected to purification.

8. The process of claim 7, wherein the adsorptive chromatography is selected from the group consisting of hydroxyapatite chromatography, hydrophobic charge induction

chromatography (HCIC), hydrophobic interaction chromatography (HIC), ion exchange chromatography, mixed mode ion exchange chromatography, immobilized metal affinity chromatography (IMAC), dye ligand chromatography, affinity chromatography, reversed phase chromatography, and combinations thereof.

9. The process of claim 7 or 8, wherein the solution in step (b) has a pH of from about 4 to about 6.0.

10. The process of claim 7 or 8, wherein the solution in step (b) has a pH of from about 6.5 to about 9.

11. The process of claim 7 or 8, wherein the solution in step (b) has a pH of less than 6.0 or a pH of greater than 6.5.

12. The process of any of claims 7-11, wherein the solution in step (b) comprises sucrose.

13. The process of any of claims 7-12, wherein the solution in step (b) further comprises a buffering agent selected from the group consisting of a citrate buffer, an acetate buffer, a succinate buffer, and a phosphate buffer.

~~7~~⁶14. The process of any of claims 1-~~13~~⁶, wherein the antibody is a monoclonal antibody.

~~8~~⁷15. The process of claim ~~14~~⁷, wherein the antibody is a humanized monoclonal antibody.

~~9~~⁸16. The process of claim ~~15~~⁸, wherein the antibody is selected from the group consisting of huN901, huMy9-6, huB4, huC242, trastuzumab, bivatuzumab, sibrotuzumab, CNT095, huDS6, and rituximab.

①→ 17. The process of any of claims 1-16, wherein the cytotoxic agent is selected from the group consisting of maytansinoids, taxanes, and CC1065.

18. The process of claim 17, wherein the cytotoxic agent is a maytansinoid.

any one of 1 to 10
11 19. The process of claims 18, wherein the maytansinoid comprises a thiol group.

11 20. The process of claim 19, wherein the maytansinoid is DM1.

11 21. The process of claim 19, wherein the maytansinoid is DM4.

13 22. The process of any of claims 1-21, wherein the antibody is chemically coupled to the ~~cytotoxic agent~~ ^{maytansinoid} via chemical bonds selected from the group consisting of disulfide bonds, acid labile bonds, photolabile bonds, peptidase labile bonds, thioether bonds, and esterase labile bonds.

Y

10. The process of claim 9, wherein the antibody is trastuzumab.

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ANEXO 2

Artículo de *Huisman et al.*, *Journal de Membrana Science*, 179:79-90 (2000)

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

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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

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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

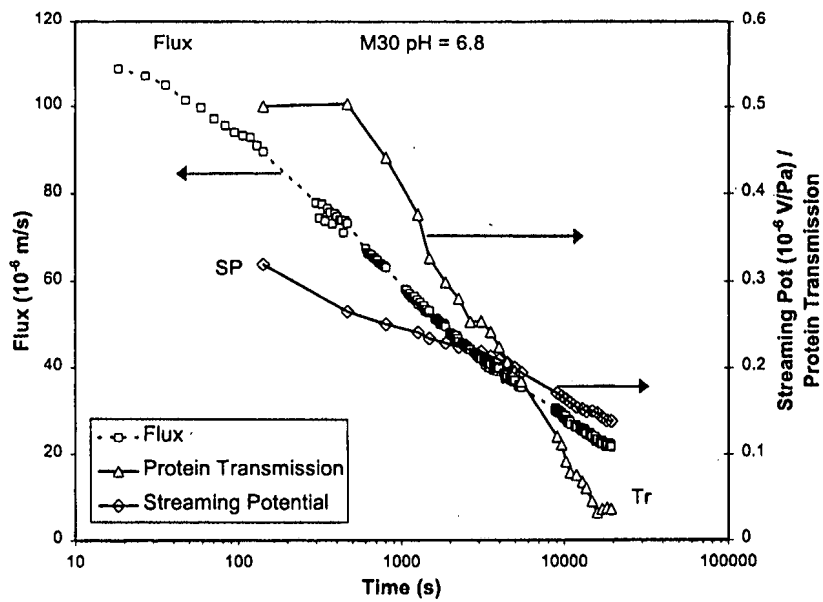


Fig. 1. Permeate flux, streaming potential, and protein transmission vs. time during the ultrafiltration of 0.5 g/dm³ BSA solutions using a 30 kDa membrane at neutral pH.

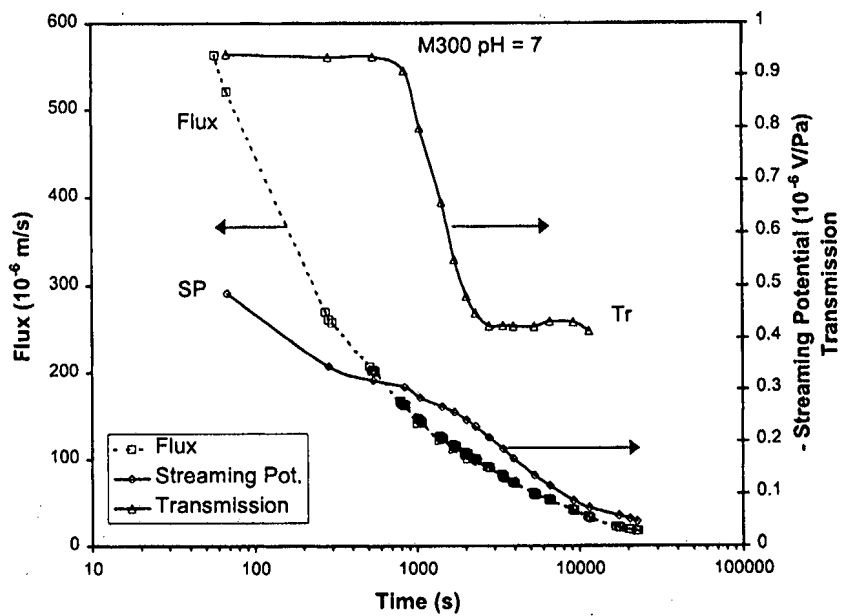


Fig. 2. Permeate flux, streaming potential, and protein transmission vs. time during the ultrafiltration of 0.5 g/dm³ BSA solutions using a 300 kDa membrane at neutral pH.

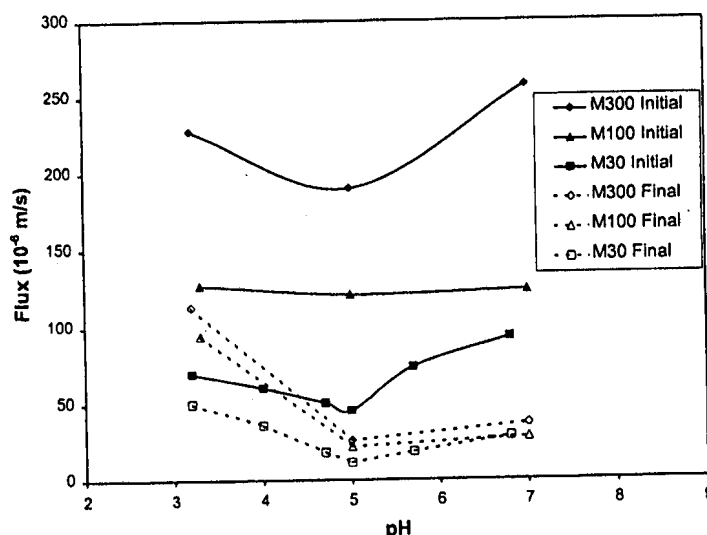


Fig. 3. Initial flux (after 100 s of fouling) and final flux (after 10000 s of fouling) during the ultrafiltration of 0.5 g/dm³ BSA solutions vs. pH of the feed solution. Results are shown for membranes with cut-off values of 300, 100, and 30 kDa.

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

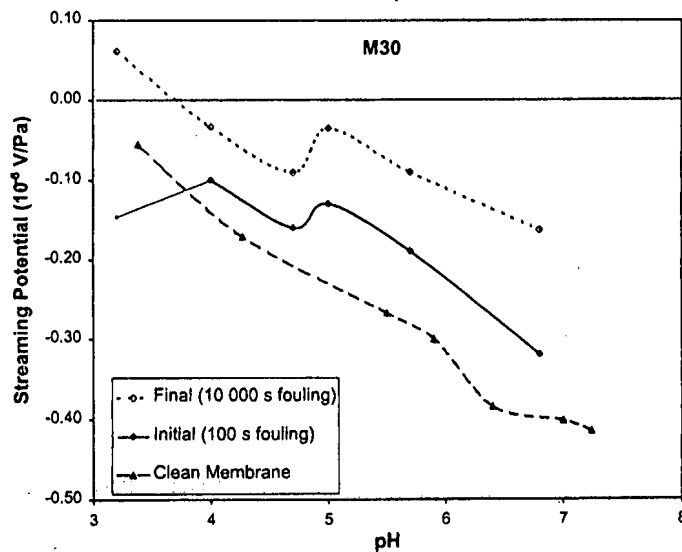


Fig. 4. Initial streaming potential (after 100 s of fouling) and final streaming potential (after 10 000 s of fouling) during the ultrafiltration of 0.5 g/dm³ BSA solutions using a 30 kDa membrane vs. pH of the feed solution. The streaming potential of the clean membrane, before filtration, is also shown.

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

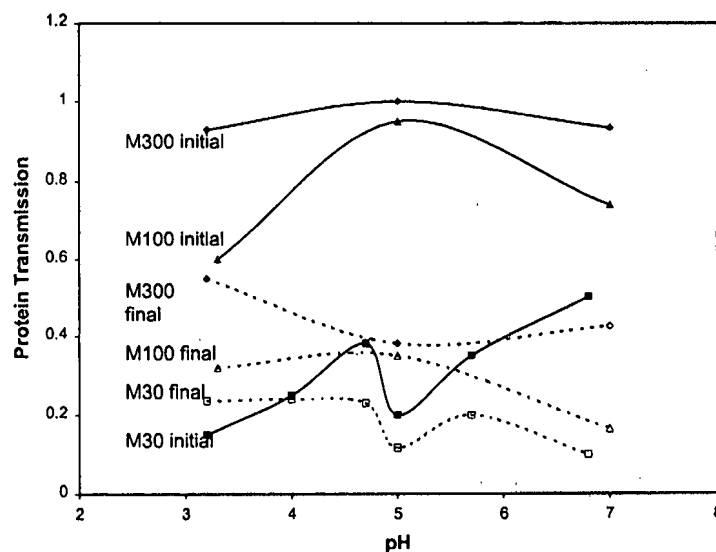


Fig. 5. Initial protein transmission (after 100 s of fouling) and final protein transmission (after 10000 s of fouling) during the ultrafiltration of 0.5 g/dm³ BSA solutions vs. pH of the feed solution. Results are shown for membranes with cut-off values of 300, 100, and 30 kDa.

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

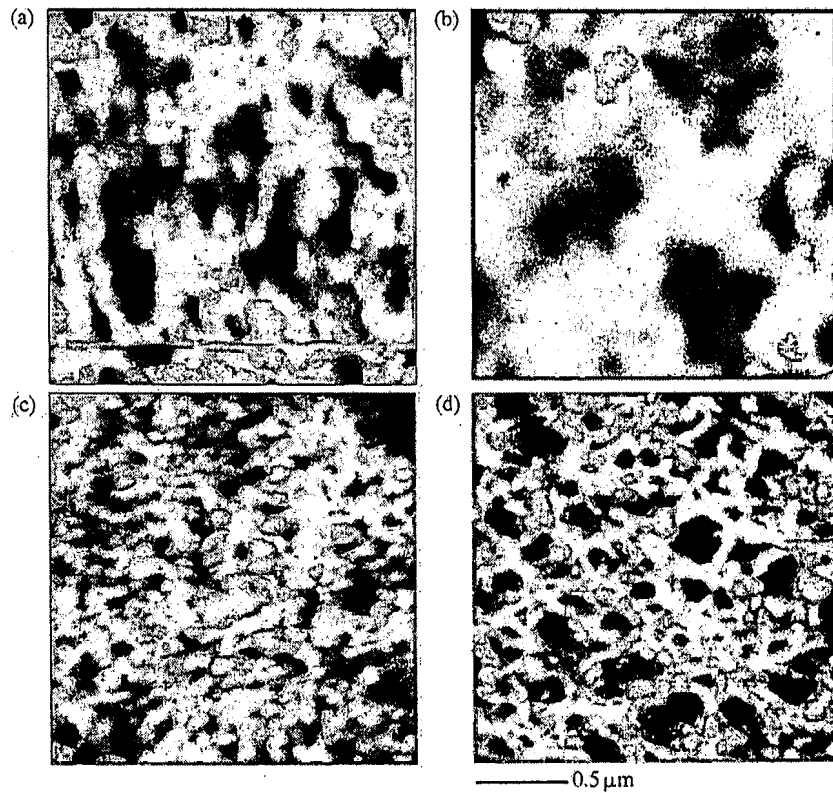


Fig. 6. AFM images of the M300 membrane taken before fouling experiments (d) and after fouling for 20 000 s with a 0.5 mol/dm³ BSA solution at (a) pH = 3, (b) pH = 5, and (c) pH = 7: scan size — 1.72 μm × 1.72 μm; z-range — 150 nm; average roughness obtained from 15 μm × 15 μm — (a) 37.9 nm, (b) 53.4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

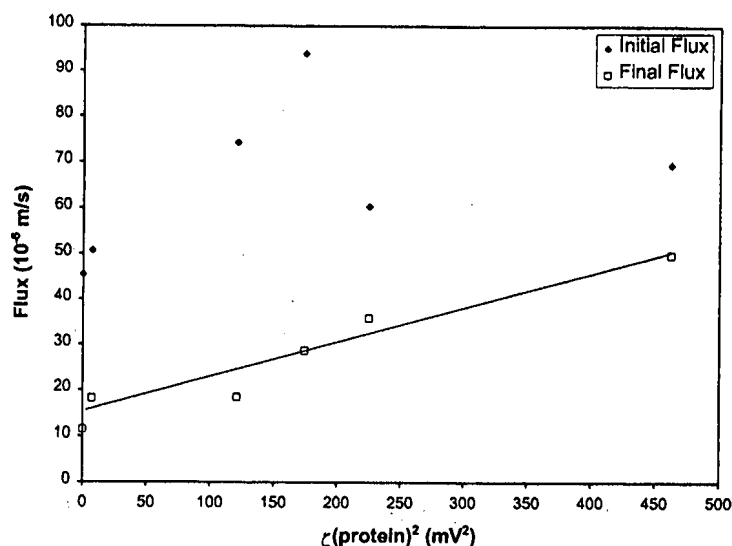


Fig. 7. Initial flux (after 100 s of fouling) and final flux (after 10 000 s of fouling) while filtering a 0.5 mol/dm³ BSA solution over an M30 membrane vs. the square of the zeta potential of the protein. The solid line is a guide for the eye.

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

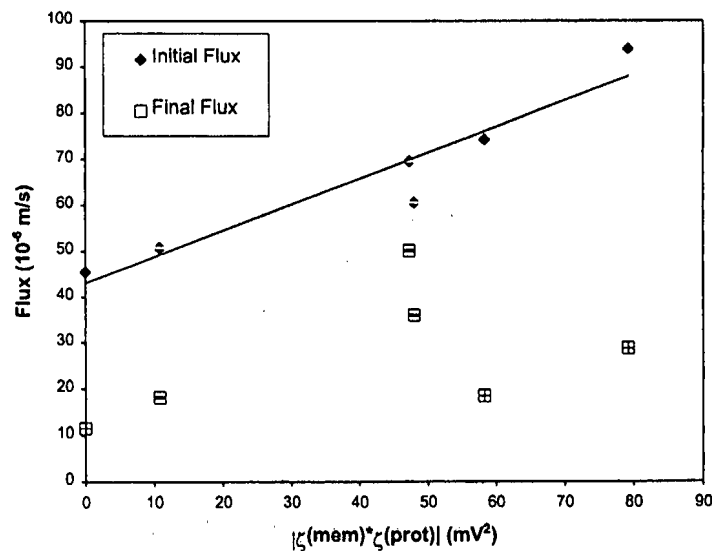


Fig. 8. Initial flux (after 100 s of fouling) and final flux (after 10 000 s of fouling) while filtering a 0.5 mol/dm³ BSA solution over an M30 membrane vs. the absolute value of the product of the zeta potential of the protein and the zeta potential of the clean membrane: data points for which $\zeta_p \cdot \zeta_m < 0$ 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.

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ANEXO 3

Artículo de Kalyanpur, *Molecular Biotechnology* 22:87-98 (2002)

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

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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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