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**“ESTRATEGIAS PARA LA PURIFICACIÓN DE PROTEÍNAS PEGILADAS
UTILIZANDO CROMATOGRAFÍA DE INTERACCIÓN HIDROFÓBICA:
RIBONUCLEASA A COMO MODELO DE ESTUDIO”**

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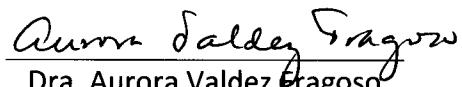
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Resumen

La PEGilación es la unión covalente de una proteína a una o varias cadenas de poli(etilen glicol) (PEG). Esta técnica ha sido utilizada para mejorar las propiedades fisicoquímicas de varias proteínas utilizadas como drogas terapéuticas. Durante la reacción de PEGilación se forman diferentes bioconjugados variando en el número de cadenas de PEG añadidas y el sitio de unión. La purificación de proteínas PEGiladas consiste en remover todas las especies que no formen parte del producto de interés, lo que involucra dos retos principalmente: 1) la separación de las proteínas PEGiladas del resto de los productos de la reacción y 2) el sub-fraccionamiento de las proteínas PEGiladas en base al grado de PEGilación y a los isómeros posicionales. Los métodos cromatográficos han sido frecuentemente utilizados para resolver las mezclas de la reacción de PEGilación, la cromatografía de exclusión molecular (SEC) y de intercambio iónico (IEC) son las más utilizadas. Comparativamente muy poco trabajo ha sido realizado para explorar otras técnicas como la cromatografía de interacción hidrofóbica (HIC). Por otro lado, una revisión detallada de la literatura muestra que las técnicas no-cromatográficas (ultrafiltración, sistemas de dos fases acuosas, electroforesis, etc.) son básicamente utilizadas para la caracterización de los productos de la reacción de PEGilación.

Dentro de este contexto, en este trabajo se presenta el estudio de la separación de los productos de la reacción de PEGilación de RNasa A utilizando diferentes condiciones y tipos de resinas en HIC. Se demuestra que el uso de un soporte ligeramente hidrofóbico como CH sefarosa 4B cubierta con Tris, puede ser utilizado como una alternativa para la separación de las proteínas PEGiladas de la proteína nativa.

Adicionalmente, los productos de la reacción de PEGilación fueron separados utilizando tres resinas con diferente grado de hidrofobicidad: butil, octil y fenil sefarosa. Se evaluaron los efectos del tipo de resina, el tipo y la concentración de sal (sulfato de amonio y NaCl) y la longitud del gradiente sobre el proceso de separación. Se calcularon la pureza y el rendimiento empleando el modelo de platos. Bajo todas las condiciones analizadas, la proteína nativa es completamente separada de las especies PEGiladas. Las mejores condiciones para la purificación de RNasa A monoPEGilada se dan cuando se utiliza la resina butil sefarosa, sulfato de amonio 1M y un gradiente de elución de 35 CVs. Con esto es posible obtener un rendimiento del 85% y una pureza del 97%. Este proceso representa una alternativa viable para la separación de proteínas PEGiladas.

Abstract

PEGylation is the covalent attachment of protein to one or more chains of poly(ethylene glycol) (PEG). This technique has been used to improve the physicochemical properties of some proteins and therapeutic drugs. During the PEGylation reaction the formation of several conjugated forms varying in the number of grafted chains and their grafting site usually occurs. PEGylation of proteins creates two basic challenges for purification. The first involves separation of PEGylated proteins from other reaction products including non-reacted PEG and protein. The second is the sub-fractionation of PEGylated proteins on the basis of their degree of PEGylation and positional isomerism (PEGamers). Chromatographic operations are often used to resolve PEGylation reaction mixtures, size exclusion and charge-based ion exchange modes most frequently used; comparatively little work has been done to explore other modes such as the hydrophobic interaction chromatography (HIC). Furthermore, a detailed revision of the literature showed that non-chromatographic techniques (ultrafiltration, aqueous two-phase systems, electrophoresis, etc.) are basically used for the characterization of the products of the PEGylation reaction.

In this context, this work presents the study of the separation of the products of the RNase A PEGylation reaction using different conditions and resins types of HIC. The use of a mildly hydrophobic support, Tris-capped CH Sepharose 4B as an alternative for separating PEGylated proteins from their unmodified counterparts was demonstrated. The effects of parameters such as pH, salt type and salt concentration upon the chromatographic behavior of native, monoPEGylated and di-PEGylated RNase A on this media were characterized.

In addition, the products of the PEGylations reaction were separated using three resins with different degrees of hydrophobicity: butyl, octyl and phenyl sepharose. The effects of resin type, concentration and salt type (ammonium sulphate or sodium chloride), and gradient length on the separation performance were evaluated. Yield and purity were calculated using the plate model. Under all conditions assayed the native protein was completely separated from PEGylated species. The best conditions for the purification of monoPEGylated RNase A were: Butyl sepharose, 1 M ammonium sulphate and 35 column volumes (CVs); this resulted in a yield as high as 85% with a purity of 97%. This process represents a viable alternative for the separation of PEGylated proteins.

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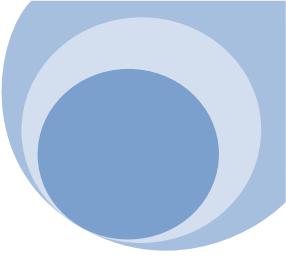
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Capítulo 1.

Introducción



1

Introducción

En la última década la inversión de la industria farmacéutica en la investigación y el desarrollo de nuevas drogas se ha duplicado debido al interés de reducir el tiempo y los costos de los procesos de producción y purificación.

Avances recientes en biotecnología han conducido a la utilización de proteínas terapéuticas para el tratamiento de numerosas enfermedades. Sin embargo, la aplicación de dichas proteínas se ha visto obstaculizada principalmente por dos problemas; el primero, la dificultad de obtener cantidades suficientes del material biológico y el segundo, la necesidad de dirigir las moléculas hacia un área específica del cuerpo, pues se sabe de la inestabilidad intrínseca de las proteínas ya que pueden sufrir proteólisis, eliminación celular o ser excretadas por filtración renal.¹ En consecuencia, existe la necesidad de optimizar los procesos de producción y separación de estos productos biológicos con el objetivo de incrementar su estabilidad y eficiencia.

Algunas proteínas terapéuticas han sido conjugadas con un polímero sintético llamado poli (etilen glicol) (PEG) para incrementar su actividad farmacológica. El PEG ha sido aprobado por la FDA para ser utilizado como vehículo en alimentos, cosméticos y farmacéuticos. No es tóxico, es soluble en agua y diversos solventes orgánicos y puede ser eliminado del organismo por procesos renales.²

En general, la conjugación de proteínas y PEG, llamada PEGilación, es capaz de prolongar el tiempo de circulación en la sangre aunque en algunas ocasiones también reduce la actividad biológica. Sin embargo, la prolongación de la vida media *in vivo* de las proteínas PEGiladas compensa la reducción de la actividad biológica, dando como resultado un incremento en los efectos terapéuticos de la proteína.³ La PEGilación de una proteína pura genera dos grandes retos en su purificación. El primero, incluye la separación de los conjugados

proteína-PEG de otros productos, que no están limitados necesariamente a la proteína y al PEG sin reaccionar. El segundo es separar los conjugados dependiendo de su grado de PEGilación (mono-, di-, tri-PEGilados, etc.) y de los isómeros que existan.⁴

Debido a que el proceso de PEGilación aumenta el peso molecular de la proteína, la cromatografía de exclusión (*Size Exclusion Chromatography*, SEC) es uno de los métodos más utilizados para separar la proteína nativa de las PEGiladas.⁴ Diversos estudios han demostrado que esta técnica presenta baja resolución conforme aumenta el número de moléculas de PEG ligadas a la proteína además de presentar desventajas en el tiempo de separación.⁵ Por otro lado, es prácticamente imposible separar mezclas de isómeros posicionales, debido a que los conjugados tienen el mismo peso molecular.⁶

Otra técnica comúnmente utilizada es la Cromatografía de Intercambio Iónico (IEX).⁷ El método IEX más común es intercambio catiónico pues las proteínas PEGiladas contienen una carga positiva menor por cada molécula de PEG adherida cuando las proteínas son PEGiladas en el grupo amino. Es claro que esta técnica depende de las diferencias de carga entre las proteínas no modificadas y las conjugadas con PEG, lo cual no siempre es suficiente para asegurar una separación predecible en base a la carga.⁴

La Cromatografía de Interacción Hidrofóbica (HIC) se ha aplicado con poca frecuencia en la separación de proteínas PEGiladas⁶, sin embargo es un método que podría ser ampliamente explotado debido a que el proceso de PEGilación puede afectar la hidrofobicidad de la proteína. En HIC, las proteínas se ligan a una fase estacionaria hidrofóbica bajo concentraciones ligeramente altas de sales cosmotrópicas. La elución se lleva a cabo modificando la fuerza iónica en la fase móvil a través de un gradiente. El rendimiento de HIC depende de las características del sistema y las propiedades fisicoquímicas de las proteínas. Las principales propiedades de las proteínas que afectan su retención en HIC son su hidrofobicidad, la distribución de la superficie hidrofóbica y el tamaño de la proteína. Mientras que las características del sistema dependen del tipo y concentración de la sal y las propiedades de la matriz.⁸

Adicionalmente, métodos no-cromatográficos han sido utilizados y desarrollados con el objetivo de fraccionar y/o caracterizar los conjugados PEGilados. Estas metodologías representan una alternativa atractiva a las estrategias cromatográficas por razones técnicas y económicas. Sin embargo, muchas de estas estrategias no han sido totalmente analizadas y optimizadas para el fraccionamiento de proteínas PEGiladas.

Se han utilizado diversas proteínas como modelos de estudio en los procesos de purificación de proteínas PEGiladas; entre las que se encuentran Albúmina de suero bovino, lisozima, α -lactoalbúmina, β -lactoglobulina, etc.⁴ En nuestro grupo de investigación se ha seleccionado como modelo de estudio a Ribonucleasa A.

Ribonucleasa A (RNasa A; EC 3.1.27.5) es una proteína pequeña, secretada por las células exócrinas del páncreas de bovinos. Está formada por 124 residuos de aminoácidos, contiene 19 de los 20 aminoácidos naturales, careciendo solo de triptófano. Su fórmula molecular es $C_{575}H_{907}N_{171}O_{192}S_{12}$ correspondiendo a un peso molecular de 13,686 Da y tiene un pI de 9.3.⁹ In vitro, RNasa A ha mostrado tener un efecto citotóxico específico para células de tumores malignos y – a través de un mecanismo aún desconocido – exhibe efectos terapéuticos en tumores que son resistentes a drogas quimioterapéuticas convencionales. Algunos estudios han demostrado el aumento de su efecto terapéutico cuando es conjugada con PEG en comparación con la RNasa A no modificada.¹⁰

De acuerdo a lo anterior, en este trabajo de investigación se propuso el estudio de las condiciones de purificación de proteínas PEGiladas a través de cromatografía de interacción hidrofóbica utilizando como modelo RNasa A.

Objetivo General

Establecer las condiciones óptimas para la purificación de proteínas PEGiladas explotando las propiedades de la Cromatografía de Interacción Hidrofóbica, utilizando Ribonucleasa A como modelo de estudio.

Objetivos específicos

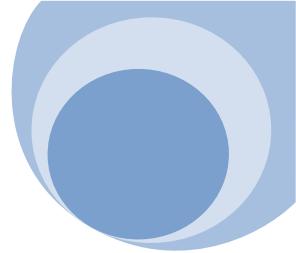
1. Producir los conjugados mono- y di-PEGilados a través de la reacción de PEGilación del grupo amino N-terminal de RNasa A.
2. Evaluar el efecto del tipo de resina en la separación de los productos PEGilados utilizando Cromatografía de Interacción Hidrofóbica.
3. Evaluar los efectos del tipo de sal y su concentración en la separación de los conjugados PEGilados a través de Cromatografía de Interacción Hidrofóbica.
4. Optimizar el proceso de purificación de RNasa A monoPEGilada.

Estructura de Tesis

La tesis se encuentra dividida en 6 capítulos. En el capítulo 1 se da una breve introducción sobre el tema de tesis. Los capítulo 2 y 3 están descritos a través de dos artículos de revisión publicados como: 1) **Mayolo-Deloisa K y Rito-Palomares M, Proteínas PEGiladas: Producción, purificación y aplicaciones.** *Revista Mexicana de Ingenieria Química* 9: 17-27 (2010); y 2) **Mayolo-Deloisa K, Gonzalez-Valdez J, Guajardo-Flores D, Aguilar O, Benavides J and Rito-Palomares M, Current advances in the non-chromatographic fractionation and characterization of PEGylated proteins.** *Journal of Chemical Technology and Biotechnology* 86: 18-25 (2011). En los capítulos 4 y 5 se reportan los resultados experimentales obtenidos en dos artículos publicados como: 1) **Cisneros-Ruiz M, Mayolo-Deloisa K, Przybycien TM and Rito-Palomares M, Separation of PEGylated from unmodified ribonuclease A using sepharose media.** *Separation and Purification Technology* 65: 105-109 (2009); y 2) **Mayolo-Deloisa K, Lienqueo ME, Andrews B, Rito-Palomares M and Asenjo JA, Hydrophobic Interaction Chromatography for Purification of monoPEGylated RNase A.** *Journal of Chromatography A* (2012) *In press*. Todos los artículos se presentan en formato de manuscrito científico de acuerdo a la revista en la que fueron publicados. Los números de las figuras y las tablas fueron adaptados al formato de tesis. Finalmente, en el capítulo 6 se resumen las conclusiones globales de la tesis y las perspectivas para el trabajo futuro.

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Capítulo 2.

Proteínas PEGiladas: producción, purificación y aplicaciones

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Proteínas PEGiladas: producción, purificación y aplicaciones

Resumen

La PEGilación es la conjugación de una proteína y/o péptido con una o más moléculas de poli(etilen glicol). El poli(etilen glicol) es un polímero no tóxico, no inmunogénico y está aprobado por la FDA (Food and Drug Administration, USA). En los últimos años, la PEGilación ha sido utilizada para mejorar las propiedades fisicoquímicas de proteínas y drogas terapéuticas, por lo que esta tecnología ha impactado fuertemente a la industria bio-farmacéutica. La PEGilación permite prolongar el tiempo de residencia en el cuerpo, mejorar la estabilidad, aumentar la solubilidad, disminuir la proteólisis y excreción renal. Desde el surgimiento de esta tecnología, diferentes proteínas han sido PEGiladas para el tratamiento de enfermedades como: hepatitis C, leucemia, artritis reumatoide, etc. Este artículo de revisión presenta una descripción del desarrollo de la PEGilación en los últimos años, así como de los procedimientos usados para la producción de bio-conjugados. Además, se revisan las estrategias de purificación utilizadas para la recuperación de proteínas PEGiladas, siendo este uno de los grandes retos en el proceso debido a que la reacción de PEGilación puede generar bio-conjugados con diferentes grados de PEGilación. Por último, se presentan las aplicaciones de dichos bio-conjugados y los retos futuros que se identifican para su aplicación genérica.

Palabras clave: *PEG, PEGilación, proteínas terapéuticas, bio-conjugados.*

Abstract

PEGylation is the covalent attachment of protein and/or peptide to poly(ethylene glycol). The poly(ethylene glycol) is a polymer, non toxic, non immunogenic, and FDA (Food and Drug Administration, USA) approved. In the last years, PEGylation has been used to improve the physicochemical properties of some proteins and therapeutic drugs; this technology has impacted heavily on the bio-pharmaceutical industry. PEGylation prolongs the body-residence time and stability, decreases the proteolysis and renal excretion. Since the emergence of this technology, some proteins have been PEGylated for the treatment of diseases including hepatitis C, leukemia, rheumatoid arthritis, etc. This review presents a description of the PEGylation development in the last years and the chemical procedures used to obtain some bio-conjugated products. Strategies of purification used to obtain PEGylated proteins are reviewed; purification is one of the major problems to establish suitable processes due to the fact that the reaction can generate bio-conjugates with different degree of PEGylation. Finally the applications of PEGylated proteins and the future challenges that are identified for generic application are presented.

Keywords: *PEG, PEGylation, therapeutic proteins, bio-conjugates.*

Introducción

La revolución biotecnológica y nanotecnológica ha producido novedosos péptidos y proteínas que están siendo utilizados como nuevas drogas para el tratamiento del cáncer y de diversas enfermedades (Harris y Chess, 2003; Parveen y Sahoo, 2006). Algunas técnicas han sido desarrolladas para mejorar las propiedades terapéuticas de dichas macromoléculas, éstas incluyen la alteración de la secuencia de aminoácidos para reducir su degradación o la fusión de péptidos con inmunoglobulina o albúmina para incrementar la vida media. Hasta ahora, la técnica más exitosa ha sido la conjugación de péptidos y/o proteínas a una o varias cadenas de poli(etilen glicol) (PEG), llamada PEGilación (Ryan y col., 2008).

El término PEGilación ha sido utilizado desde 1977 después de que Abuchowsky y colaboradores describieran por primera vez un método para adherir covalentemente una o varias moléculas de PEG a una proteína (Abuchowsky y col., 1977). El PEG es un poliéster lineal o ramificado con un grupo hidroxilo en cada extremo (Figura 2.1), este polímero es altamente

soluble en agua así como en varios solventes orgánicos y está aprobado por la FDA para su administración en seres humanos (Morar y col., 2006; Wattendorf y Merkle, 2008). Muchos de los beneficios de la PEGilación de proteínas están ligados a las propiedades del PEG. El PEG es inerte, no tóxico y no inmunogénico, además es fácilmente desechado por el cuerpo a través del riñón (pesos moleculares del polímero menores a 20 kDa), o del hígado (pesos moleculares arriba de 20 kDa) (Morar y col., 2006).

La conjugación de PEG con una proteína generalmente mejora sus propiedades debido a que aumenta su vida media, causa una reducción del reconocimiento de la proteína por el sistema inmune, aumenta su resistencia al ataque proteolítico, aumenta su solubilidad y estabilidad (Figura 2.2). La mayoría de estos fenómenos pueden ser explicados debido a la expansión del radio hidrodinámico del conjugado proteína-PEG como un resultado de la capacidad del PEG de coordinar numerosas moléculas de agua y de la alta flexibilidad de la cadena polimérica (Gaberc-Porekar y col., 2008).

Existen varios métodos químicos y enzimáticos para llevar a cabo la PEGilación (Veronese y Pasut, 2005). El primer paso en el proceso es la “activación” de la molécula de PEG, la modificación de PEG más utilizada es el metoxi-PEG (mPEG) (Figura 2.1) (Hamidi y col., 2006). El PEG activado puede ser ligado a un sitio específico de las proteínas, frecuentemente sobre un grupo amino, sulfidrilo u otro grupo nucleofílico. En muchos casos, el sitio preferido para la modificación es el grupo amino de la lisina o el grupo amino N-terminal de la cadena polipeptídica (Veronese y Pasut, 2005; Hamidi y col., 2006). Sin embargo, la PEGilación del grupo amino genera un alto número de isómeros lo que dificulta en gran medida el siguiente paso en el proceso de PEGilación, la purificación de los conjugados (Veronese y Pasut, 2005). Debido a los costos extremadamente altos de los procesos de producción de proteínas terapéuticas, uno de los retos en la ingeniería de la reacción de PEGilación es generar reacciones sitio-específicas lo más eficientes posibles, que produzcan un solo conjugado sin alterar las propiedades fisicoquímicas de la proteína de interés.

La purificación de proteínas PEGiladas envuelve la remoción de todas las especies moleculares que no sean parte del producto de interés, que pueden incluir a la proteína no

modificada y a la proteína con diferentes grados de PEGilación (mono-, di-, tri-, etc.). Actualmente los procesos de purificación de proteínas PEGiladas están dominados por la cromatografía de exclusión molecular e intercambio iónico. Otros métodos también han sido utilizados aunque con menor frecuencia, ejemplos de ellos son la cromatografía en fase reversa y la cromatografía de interacción hidrofóbica, ultrafiltración, electroforesis, electroforesis capilar, diálisis, sistemas de dos fases acuosas, etc. (Delgado y col., 1997; Fee y Van Alstine, 2006).

Desde el surgimiento de la PEGilación, un gran número de proteínas han sido PEGiladas en ellas se incluyen: factores de crecimiento, adenosin desaminasa, asparaginasa, interferones, ribonucleasa A, albúmina de suero bovino, α -albúmina, entre otras. Muchas de ellas están siendo utilizadas en el tratamiento de enfermedades como: leucemia, artritis reumatoide, hepatitis C, acromegalia, etc. (Veronese y Pasut, 2005; Gaberc-Porekar y col., 2008).

A pesar de que en los últimos años los trabajos en el desarrollo de los procesos de PEGilación se han intensificado notablemente, aún no se han encontrado métodos eficientes que eleven el rendimiento en la recuperación de los productos bio-conjugados y disminuyan los altos costos de producción. La ingeniería química y bioquímica juegan un papel fundamental en el diseño de reacciones en donde se controle el sitio de PEGilación de manera que se evite la generación de proteínas multiPEGiladas. Otra área de oportunidad es la ingeniería de bioseparaciones de proteínas PEGiladas, puesto que aún no se han encontrado procesos que puedan ser aplicados de manera genérica en la etapa de purificación.

El objetivo de este artículo es el de presentar un panorama general del estado del arte en cuanto a la reacción de PEGilación, las estrategias de purificación utilizadas para la recuperación de proteínas PEGiladas, las aplicaciones y los retos futuros que se identifican en el desarrollo de dichos conjugados proteína-polímero.

Reacción de PEGilación: producción de proteínas PEGiladas

Para llevar a cabo la reacción de PEGilación, se deben tomar en cuenta varios factores que incluyen el objetivo por el cual una proteína debe de ser PEGilada. La estructura y el tamaño del PEG son dos variables que limitan el proceso; por ejemplo, PEGs ramificados

incrementan el peso molecular de la proteína mono-PEGilada, pero también pueden limitar la disponibilidad estérica del sitio de PEGilación. Además, otros factores como el tiempo de reacción, pH, temperatura, concentración de PEG y proteína deben ser tomados en cuenta (Gaberc-Porekar y col., 2008).

En una reacción típica, un PEG activado se hace reaccionar con uno o más residuos de lisina o con el grupo amino N-terminal. La PEGilación de otros sitios nucleofílicos tales como cisteína, histidina, arginina o tirosina también son posibles. Por otro lado, es posible utilizar enzimas que se encarguen de la conjugación de la proteína con el PEG.

Al llevar a cabo la reacción, la solución de proteína es mezclada con el PEG activado bajo condiciones de pH, temperatura y agitación controladas. Las moléculas de proteína mono-PEGiladas con los sitios más reactivos son las primeras en formarse, los sitios menos reactivos forman las especies di-PEGiladas y así sucesivamente (Morar y col., 2006).

Modificación del grupo amino.

La modificación química más común para llevar a cabo la reacción de PEGilación se da en los grupo ϵ -amino de los residuos de lisina, a través de alquilación o acilación (Fee y Van Alstine, 2006; Veronese y Mero, 2008). La alquilación mantiene la carga positiva del grupo amino, mientras que la acilación genera una pérdida de la carga debido a la formación de una amida (Veronese y Mero, 2008). En una proteína típica las lisinas constituyen el 10% del total de los aminoácidos, su disponibilidad hace que la conjugación sea sencilla; sin embargo, el gran número de sitios presentes para la conjugación dificulta la posibilidad de obtener un número específico de aductos por lo que es muy común que se generen mezclas de PEGámeros. Una forma de controlar la reacción es cambiar el pH, a valores altos de pH (arriba de 8.0) se favorece la conjugación con los grupos ϵ -amino de las lisinas presentes, mientras que una reacción a pH ácido favorece el enlace con el grupo amino N-terminal (Gaberc-Porekar y col., 2008). Los agentes para la modificación de grupos amino incluyen: mPEG-diclorotriazina, mPEG-tresilato, mPEG-succimidil carbonato, mPEG-N-hidroxisuccimida, mPEG-propilaldehído, mPEG-p-nitrofenilcarbonato, etc. (Roberts y col., 2002; Veronese y Mero, 2008). En la Figura 2.3, se muestra una de las reacciones más utilizadas para llevar a cabo la modificación del grupo amino

N-terminal, en dicha reacción el aldehído interacciona con la amina para producir una base de Schiff que finalmente es reducida a una amina secundaria estable (Veronese y Mero, 2008).

Modificación de residuos de cisteínas.

Los polímeros utilizados para la modificación de cisteínas incluyen a: mPEG-maleimido (Figura 2.4), mPEG-iodoacetato, mPEG-tiol, mPEG-vinilsulfona y mPEG-piridildisulfido. La PEGilación sitio-específica de residuos de cisteína rara vez se lleva a cabo, debido a que este aminoácido, cuando está presente, se encuentra participando en los enlaces disulfuro o es requerido para la actividad biológica (Veronese y Mero, 2008). En ausencia de cisteínas libres en la proteína nativa, una o más cisteínas pueden ser insertadas por ingeniería genética; sin embargo, en ocasiones se pueden generar puentes disulfuro incorrectos y por lo tanto la dimerización de la proteína (Roberts y col., 2002; Veronese y Mero 2008).

PEGilación específica utilizando enzimas.

La conjugación específica de PEG al grupo amido de una glutamina o al grupo hidroxilo de las serinas y treoninas es solo posible utilizando enzimas. Existen enzimas que reconocen a la glutamina como sustrato, llamadas transglutaminasas (Veronese y Pasut, 2005). Sato (2002) reportó que la glutamina de las proteínas puede ser el sustrato de la enzima transglutaminasa, si un PEG-amino es usado como donador nucleofílico, por lo que el PEG puede ser ligado a la proteína a través de un residuo de glutamina.

La reacción para la producción de proteínas PEGiladas juega un papel fundamental en el proceso de PEGilación, los productos que se obtienen son a) la proteína en sus diferentes grados de PEGilación, b) el PEG en exceso y c) la proteína que no logró reaccionar. El conocimiento de la estructura primaria de la proteína es fundamental, el uso de herramientas como la bioinformática pueden ayudar a predecir los sitios de PEGilación y las posibles consecuencias sobre la estructura tridimensional, además de facilitar el proceso de purificación. A pesar de que la PEGilación de cisteínas genera mezclas menos complejas, sigue siendo más utilizada la PEGilación de los grupos amino debido a que las cisteínas son aminoácidos que generalmente participan en el sitio activo o en la conformación de la estructura tridimensional, lo que en

muchas ocasiones afecta negativamente a la proteína. El diseño de la reacción debe de ser específico para la proteína de interés, dependiendo de sus propiedades fisicoquímicas y de su aplicación.

Estrategias de purificación de proteínas PEGiladas.

La purificación de proteínas PEGiladas consiste en remover todas las especies que no formen parte del producto de interés, lo que involucra dos retos principalmente: 1) la separación de las proteínas PEGiladas del resto de los productos de la reacción y 2) el sub-fraccionamiento de las proteínas PEGiladas en base al grado de PEGilación y a los isómeros posicionales o PEGámeros. La purificación se complica debido a que no solo se deben tomar en cuenta las características de la proteína, también la naturaleza amfipática del PEG afecta fuertemente al proceso de separación (Fee y Van Alstine, 2006). En la Tabla 2.1.1 se muestran algunos de los métodos utilizados para la separación de proteínas PEGiladas. En esta Tabla 2.1.1 es evidente que PEG de distintos pesos moleculares han sido utilizados para la obtención de productos PEGilados. Adicionalmente, se ha documentado que para la recuperación de las proteínas PEGilados, métodos cromatográficos y no-cromatográficos han demostrado su factibilidad.

Métodos cromatográficos.

Históricamente, la cromatografía de exclusión molecular (*Size Exclusion Chromatography*, SEC) ha sido ampliamente usada para la separación de productos PEGilados debido al significativo incremento del radio hidrodinámico de los conjugados comparado con las especies nativas. El poder de resolución de la cromatografía de exclusión para diferentes especies PEGiladas no es muy alto (Figura 2.5). Esta técnica es inherentemente inadecuada para resolver mezclas de isómeros que tienen el mismo número de cadenas de PEG ligadas a la proteína, pero en diferentes sitios (Garberc-Porekar y col., 2008).

La cromatografía de intercambio iónico (*Ion-Exchange Chromatography*, IEC) ofrece la posibilidad de efectuar la separación del PEG, la proteína nativa y las especies PEGiladas en un solo paso. Debido a esto, IEC es comúnmente utilizada para separar proteínas PEGiladas; sin embargo, el método requiere ser optimizado. Para ello, debe tomarse en cuenta que el PEG es

un polímero neutral pero puede afectar la carga de las proteínas en tres maneras diferentes. Primero, la presencia del PEG conjugado puede proteger la carga superficial de la proteína y de este modo debilitar el enlace con la resina de intercambio iónico. Segundo, la conjugación a residuos de aminoácidos que altera la carga neta de la proteína o cambia a ciertos valores de pH altera la carga potencial y por lo tanto el punto isoeléctrico (pI). Tercero, la superficie de la proteína en donde se localiza el PEG puede formar puentes de hidrógeno (Fee y Van Alstine, 2006).

La cromatografía de intercambio catiónico es especialmente ventajosa y parece ser el método más fino para separar mezclas de PEGilados (Kinstler y col., 2002; Fee y Van Alstine, 2006; Garberc-Porekar y col., 2008). En la PEGilación aleatoria (básicamente grupos amino), el orden usual es que eluyan primero las especies altamente PEGiladas, después las di-PEGiladas seguidas de las mono-PEGiladas, la proteína no PEGilada eluye al final; sin embargo, el mismo orden de elución se puede obtener cuando se requiere separar proteínas PEGiladas sobre un residuo de cisteína (Seely y col., 2005). Aunque la cromatografía de intercambio catiónico es altamente efectiva para resolver mezclas complejas de proteínas PEGiladas, cargar demasiado la columna puede disminuir la resolución. La mayoría de los espacios de la columna están ocupados por el PEG resultando en una baja capacidad del medio en términos de masa de proteína por volumen de resina. El tiempo de vida útil del medio cromatográfico usado es relativamente corto, por lo que se requiere empacar la columna en repetidas ocasiones. Todos estos factores contribuyen a generar altos costos de separación, a pesar de el uso de resinas de intercambio iónico relativamente económicas (Garberc-Porekar y col., 2008).

La cromatografía de fase reversa (*Reverse Phase Chromatography*, RPC) es una opción atractiva para la resolución de conjugados proteína-polímero debido a que la cadena del PEG sobresale del conjugado actuando como un sitio hidrofóbico y de esa manera dominar la interacción con la superficie hidrofóbica (Daly y col., 2005; Cisneros-Ruiz, 2006). Sin embargo, RPC puede estar limitada por diferentes factores, incluyendo cambios estructurales en los productos proteícos de interés, además de bajos niveles de recuperación debido a la desnaturización causada por el uso de solventes orgánicos (Cisneros-Ruiz, 2006).

La cromatografía de interacción hidrofóbica (*Hydrophobic Interaction Chromatography*, HIC) ha sido aplicada con menos frecuencia para la separación de proteínas PEGiladas. Esta técnica generalmente trabaja pobremente debido a que el PEG por sí mismo también se liga al medio, lo que interviene en la separación (Fee y Van Alstine, 2006; Garberc-Porekar y col., 2008). Cisneros-Ruiz y colaboradores (2009) reportaron que bajo ciertas condiciones es posible separar ribonucleasa A nativa de sus especies PEGiladas; sin embargo, no es posible separar las proteína mono-PEGilada de la di-PEGilada. Algunos autores consideran que esta técnica no ha sido debidamente explotada, por lo que es necesario realizar más investigación al respecto (Fee y Van Alstine, 2006).

En términos generales, las técnicas cromatográficas clásicas para la separación de proteínas, no ofrecen de manera individual un desempeño óptimo para la purificación de conjugados proteína-polímero. En muchas ocasiones es necesario utilizar un conjunto de las técnicas antes mencionadas, algunos autores han sugerido que la cromatografía de exclusión molecular seguida por intercambio iónico e interacción hidrofóbica podrían ser la mejor propuesta de una aplicación genérica para la purificación de proteínas PEGiladas (Fee y Van Alstine, 2006; Garberc-Porekar y col., 2008).

Métodos no cromatográficos.

El incremento en tamaño de los conjugados proteína-PEG ha sido explotado para llevar a cabo su separación haciendo uso de membranas de ultrafiltración. Con mayor frecuencia, dicha técnica ha sido empleada para remover el agua y la solución buffer del resto de los componentes de la reacción de PEGilación; sin embargo, también es posible separar la proteína nativa de las moléculas PEGiladas así como retirar el PEG remanente (Lee y Park, 2002; Pabst y col., 2007; Molek y Zydney, 2007), además ha sido examinado su uso potencial para la separación de los conjugados de manera cuantitativa. El diseño y optimización para la aplicación de la ultrafiltración en la recuperación de proteínas PEGiladas requiere de la adecuada selección del tamaño de poro de la membrana, el pH, fuerza iónica y el flujo del filtrado (Molek y Zydney, 2007).

Sistemas de dos fases acuosas (SDFA) también han sido utilizados para la separación de

proteínas PEGiladas; sin embargo, reportes que documenten la caracterización de proteínas PEGiladas en estos sistemas no son muy comunes. SDFA es un método de separación líquido-líquido en donde la separación está basada en la diferencia de partición de los solutos entre las fases (Rito-Palomares, 2004), que se visualiza como una alternativa atractiva para la recuperación de proteínas PEGiladas. En este contexto, estudios previos mostraron que los conjugados PEG-proteína de las proteínas albúmina, factor de estimulación de colonias de granulocitos y macrófagos e inmunoglobulina G, se comportan diferente que sus equivalentes proteínas nativas en sistemas que utilizan PEG y dextrano como formadores de fases. Los resultados muestran que el coeficiente de partición (K) incrementa con el número de moléculas de PEG ligadas a la proteína (Delgado y col., 1994; Delgado y col., 1997). Si bien estos estudios demostraron el potencial de utilizar SDFA para la recuperación de conjugados proteína-PEG, la falta de una caracterización extensa del comportamiento de partición es evidente. Alternativamente, Sookkumnerd y Hsu (2000) explotaron la distribución a contracorriente en sistemas de dos fases acuosas (PEG-fosfatos) como técnica para purificar conjugados PEG-lisozima. Los resultados de esta investigación mostraron que a través de esta técnica es posible separar cada una de las especies PEGiladas y la proteína nativa. A pesar de los estudios realizados, una extensa caracterización de los conjugados proteína-polímero utilizando estrategias que explotan los mecanismos de partición en dos fases acuosas es necesaria.

Como se ha podido apreciar, se han realizado diversos esfuerzos por mejorar los procesos de purificación de proteínas PEGiladas, la mayoría de ellos utilizando métodos cromatográficos. Sin embargo, los procesos siguen llevándose a cabo en varias etapas, lo que prolonga el tiempo de recuperación de los bio-conjugados e impacta negativamente en el rendimiento del proceso de PEGilación. Es en este punto donde herramientas de ingeniería química y bioquímica puede ser de gran impacto en el desarrollo de métodos de separación más rápidos y efectivos. Lo ideal sería el diseño de procesos con dos etapas, una de recuperación primaria (utilizando métodos como ultrafiltración, diálisis o sistemas de dos fases acuosas) en donde se separen el PEG y la proteína que no reaccionaron y una segunda etapa (cromatográfica) que permita la separación de los diferentes bio-conjugados (mono-PEGilados, di-PEGilados, etc.).

Aunque los procesos cromatográficos son los más utilizados en la separación de bio-

conjugados, hasta ahora no se conocen modelos matemáticos que ayuden a predecir el comportamiento de las proteínas PEGiladas en el proceso de purificación, lo cual sería de gran ayuda en la optimización e intensificación de los métodos de separación.

Aplicaciones, tendencias y retos futuros.

Diferentes clases de drogas proteicas, como enzimas, citoquinas y anticuerpos han sido significativamente mejoradas debido al proceso de PEGilación. La Tabla 2.1.2 compila los ejemplos más importantes de bio-conjugados aprobados por la FDA, que explotando las ventajas de la PEGilación, han sido utilizados en la terapia de diversas enfermedades (Veronese y Pasut, 2005; Fishburn, 2008). PEGamadasa bovina (PEG-adenosin deaminasa, Adagen®, Enzon Inc.) fue la primera proteína PEGilada en ser comercializada satisfactoriamente. Fue aprobada por la FDA en 1990 para tratar la enfermedad de inmunodeficiencia combinada severa (*Severe Combined Immunodeficiency*, SCID). Adenosin desaminasa fue PEGilada aleatoriamente con PEG 5 kDa para extender el tiempo que permanece en el plasma y reducir su inmunogenicidad. Este fue un paso predominante en el desarrollo de la PEGilación porque se demostró por primera vez la viabilidad de esta tecnología (Veronese y Mero, 2008).

La segunda proteína biológicamente activa en ser conjugada con PEG fue L-asparaginasa (PEGaspargasa; Oncaspar®, Enzon Inc.). PEGaspargasa fue aprobada por la FDA en 1994 para pacientes en los cuales la proteína no modificada (o nativa) provocaba una reacción alérgica. La conjugación de la proteína con múltiples cadenas de PEG 5 kDa incrementó el tiempo de eliminación tres veces más comparado con la proteína nativa. El producto PEGilado fue tan efectivo como la droga nativa en el tratamiento de pacientes con leucemia linfoblástica aguda, además mostró un bajo grado de inmunogenicidad (Graham, 2003).

El factor de crecimiento de colonias de granulocitos (*Granulocyte Colony-Stimulating Factor*, G-CSF) es el mayor regulador de la granulopoyesis *in vivo*. Su tiempo de vida media es relativamente corto (3.5-3.8 h) por lo que a diario se requieren de múltiples dosis. El conjugado PEG-G-CSF, pegfilgrastim (Neulasta®, Amgen Inc.), fue producido por la unión de una molécula de PEG 20 kDa a el grupo α -amino del residuo N-terminal de metionina. Pegfilgrastim permanece en el plasma el tiempo suficiente para permitir una simple inyección subcutánea para

tratamientos de quimioterapia (Kinstler y col., 2002; Veronese y Mero, 2008).

Los interferones combinados con ribavirina son los tratamientos más usados para tratar infecciones virales, en su forma nativa, tienen un tiempo de vida muy corto (4-5 h). PEG-interferón α 2a (Pegasys®, Hoffman La Roche Inc.) fue obtenido por el acoplamiento covalente de PEG-N-hidroxisuccimida 40 kDa a un residuo de lisina. Dicha reacción produce una mezcla de cuatro isómeros mono-PEGilados en Lys³¹, Lys²¹, Lys¹³¹ y Lys¹³⁴. Recientemente, los isómeros de PEG-interferón α 2a fueron separados cromatográficamente y su actividad fue evaluada; los isómeros Lys³¹ y Lys¹³⁴, fueron los más activos. Lo que demuestra que es posible diseñar nuevos PEG-interferones que retengan mayor actividad biológica que la proteína nativa, además de que se evidencia que aún hace falta investigación sobre estrategias de purificación más eficientes (Veronese y Mero, 2008).

Las ventajas de la PEGilación no están limitadas a su aplicación en proteínas terapéuticas, también han sido utilizadas para mejorar la estabilidad en solventes orgánicos y la eficiencia catalítica de proteínas como la lacasa, utilizada en procesos de biorremediación debido a su capacidad de oxidar un amplio rango de compuestos fenólicos y poliaromáticos (Vandertol-Vanier y col., 2002; López-Cruz y col., 2006). Otra proteína utilizada también como biocatalizador en reacciones de oxidación de compuestos de estructura química diversa y que representan un problema de contaminación ambiental, es el citocromo C. El citocromo C ha sido modificado químicamente mediante PEGilación, lo que ha permitido aumentar su estabilidad térmica a temperaturas mayores a 100°C (García-Arellano y col., 2002). La PEGilación también podría ser utilizada para mejorar la estabilidad de proteínas de afinidad (v.gr. proteína A) utilizadas en lechos cromatográficos para la separación de anticuerpos.

Además de proteínas y péptidos, otras moléculas como: cofactores, oligonucleótidos, lípidos sacáridos y bio-materiales están siendo PEGilados, lo que representa un área de oportunidad para el desarrollo de diversas investigaciones en el área.

Hasta ahora, el desarrollo de la PEGilación de proteínas ha estado enfocado en su aplicación terapéutica, que sin duda ha sido de gran impacto para el desarrollo de nuevas drogas.

Sin embargo, esta técnica puede mejorar la estabilidad de prácticamente cualquier proteína, de ahí la necesidad de profundizar la investigación en este campo pues aún existen diversos retos por superar.

En cuanto a la producción (reacción) de bio-conjugados, es necesario diseñar reacciones de PEGilación sitio-específicas que eviten la formación de PEGámeros sin dañar el sitio catalítico; además de conocer a detalle las características intramoleculares de las especies PEGiladas que ayuden a entender su comportamiento bajo diferentes condiciones. La optimización de la reacción de PEGilación es crucial, puesto que el exceso tanto del PEG como de la proteína que no reaccionan aumenta la viscosidad de la solución, lo que complica el proceso de purificación. Sería ideal contar con procesos compuestos por una etapa de recuperación primaria (v.gr. ultrafiltración, fases acuosas, etc.) y una de purificación (utilizando métodos cromatográficos). Otra alternativa es llevar a cabo la reacción de PEGilación y la separación en un solo paso utilizando cromatografía de exclusión molecular, esta es una técnica que ha sido muy poco explorada por lo cual tiene que ser afinada, pero ofrece la posibilidad de realizar tanto el proceso de producción como el de purificación a través de una misma etapa con la ayuda de sistemas cromatográficos.

Conclusiones.

La PEGilación es una técnica versátil que permite superar muchas de las limitaciones farmacológicas de las proteínas terapéuticas. Durante el desarrollo de esta técnica han surgido importantes avances en cuanto a la reacción de PEGilación, la generación de más bioconjugados, el entendimiento de su comportamiento y las estrategias para su purificación. Sin embargo, aún se presentan nuevos retos en materia de ingeniería, tanto en la preparación como en la purificación de las moléculas PEGiladas. Los métodos clásicos de separación han sido útiles pero no ofrecen una resolución óptima; siguen siendo procesos en varias etapas, de alto costo y con rendimientos bajos, además de que en casos muy específicos no ha sido posible separar los isómeros conformacionales. De manera que es necesario profundizar en la investigación de métodos no convencionales de separación que puedan ser aplicados en la purificación de proteínas PEGiladas. Los productos aprobados por la FDA son una clara demostración del éxito de la PEGilación tanto en el mejoramiento de las propiedades de las

proteínas terapéuticas como de su aplicación en el tratamiento de diversas enfermedades; sin embargo, aún hace falta profundizar sobre la aplicación de proteínas PEGiladas en diversas áreas de la biotecnología.

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Leyendas de Figuras

Figura 2.1 Fórmulas estructurales del poli(etilen)glicol (PEG). a) PEG, b) metoxi-poli(etilen glicol) lineal (mPEG) y c) mPEG ramificado (tomado de Hamidi y col., 2006).

Figura 2.2 Ventajas de la PEGilación. La figura representa la conjugación de una proteína con varias moléculas de PEG (modificado de Veronese y Pasut, 2005).

Figura 2.3 Modificación del grupo amino que mantiene la carga positiva del residuo de la proteína (tomada de Veronese y Mero, 2008).

Figura 2.4 Modificación sitio-específica de cisteína utilizando mPEG-maleimido (tomada de Veronese y Mero, 2008).

Figura 2.5 Separación de Ribonucleasa A nativa de sus formas mono- y di-PEGiladas utilizando Cromatografía de Exclusión Molecular (tomado de Cisneros-Ruiz, 2006).

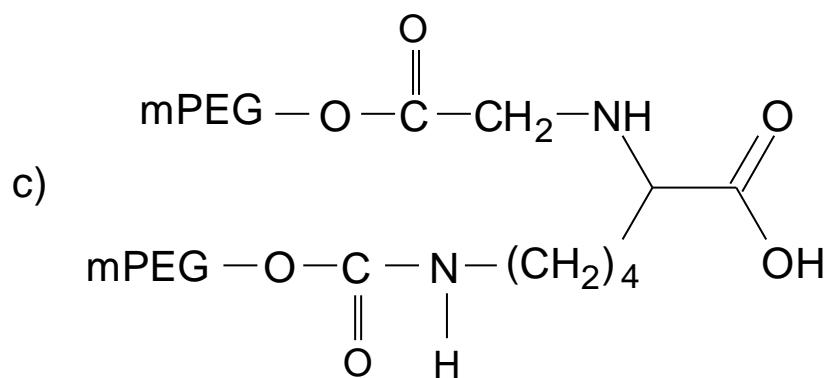


Figura 2.1

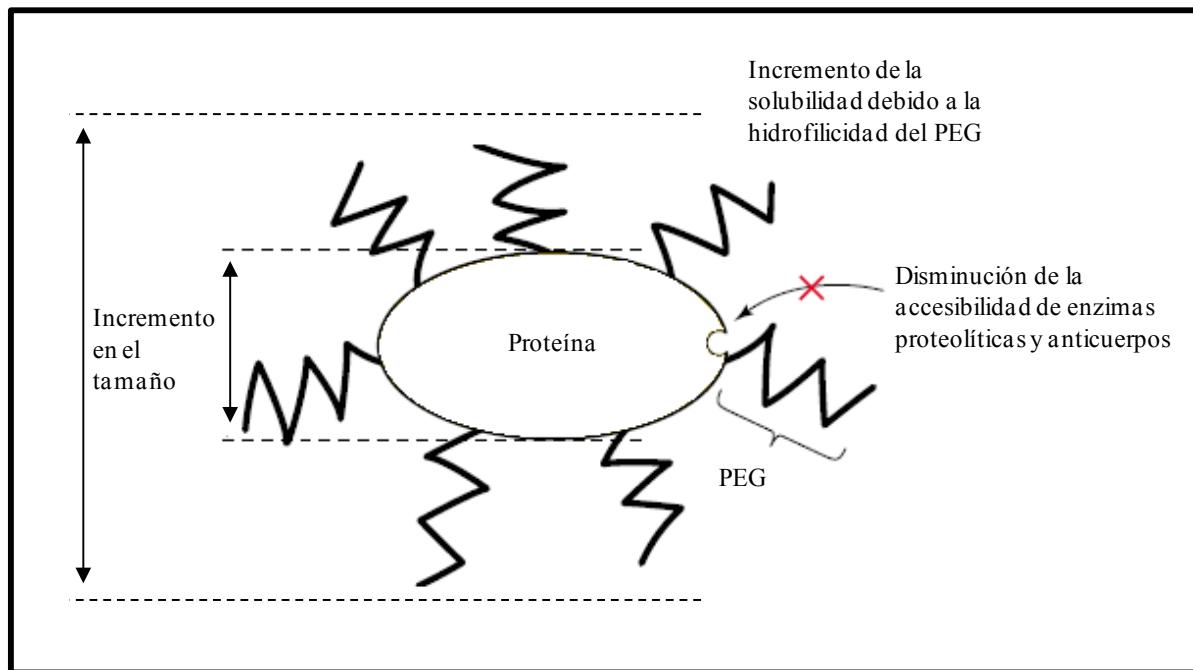
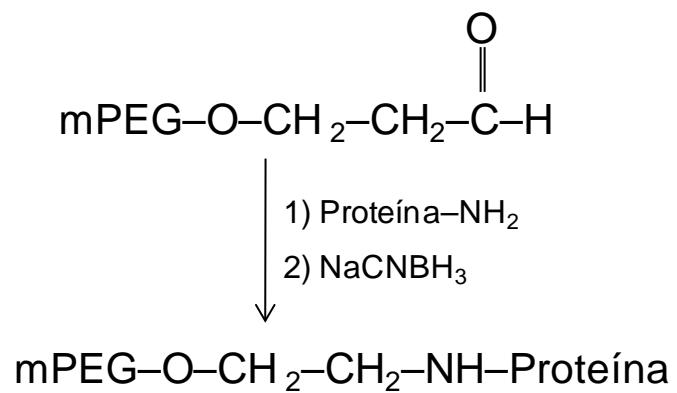


Figura 2.2

**Figura 2.3**

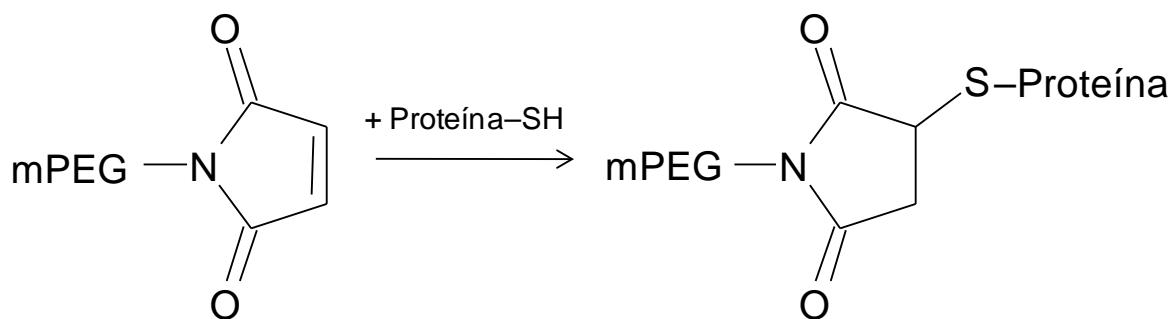


Figura 2.4

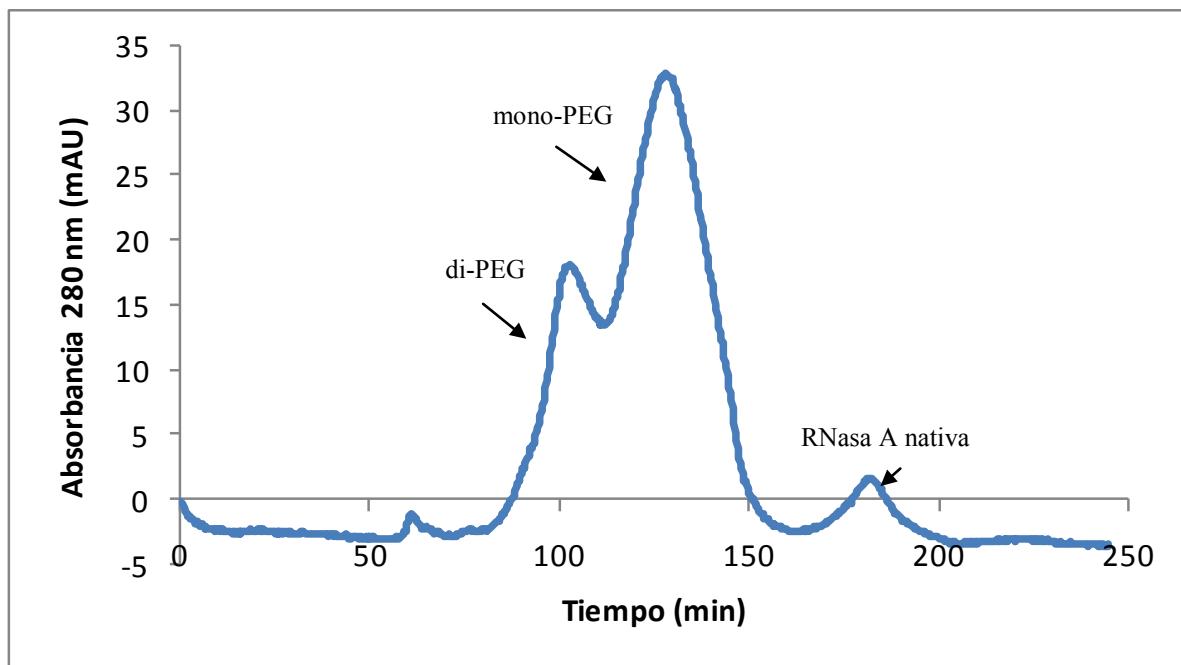


Figura 2.5

Tablas

Tabla 2.1 Métodos de separación utilizados para la purificación de proteínas PEGiladas.

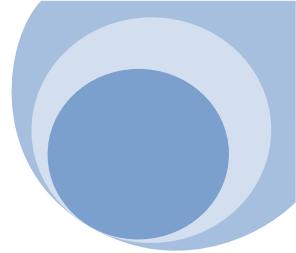
Proteína	PEG (PM, kDa)	Método(s) de purificación	Referencia
Factor de crecimiento epidérmico	3.4	Exclusión molecular , fase reversa y ultrafiltración	Lee y Park, 2002
Factor de crecimiento epidérmico	2.5	Diálisis y liofilización	Kim y col., 2002
α -interferon 2b	12	Intercambio catiónico y exclusión molecular	Wang y col., 2002
PEG Intron® (Shering-Plough)			
α -interferon 2a PEGasys® (Hoffman-La Roche Inc.)	40	Intercambio catiónico	Reddy y col., 2002
β -interferon	20	Intercambio catiónico, ultrafiltración y exclusión molecular	Arduini y col., 2004
Insulina	750 Da	Exclusión molecular	Calceti y col., 2004
α -lactoalbúmina	2,5	Exclusión molecular	Fee y Van Alstine, 2004
β -lactoglobulina	10		
Albúmina de suero bovino	20, 40		
Hemoglobina	5	Exclusión molecular y fase reversa	Li y col., 2006
Insulina	2 y 5	Diálisis, intercarbio catiónico y fase reversa	Dou y col., 2007
Ribonucleasa A	20	Interacción hidrofóbica	Cisneros-Ruiz y col., 2009
Factor estimulador de colonias de granulocitos	10, 20, 30	Exclusión molecular, fase reversa e intercambio iónico.	Zhai y col., 2009

PM, peso molecular

Tabla 2.2 Conjugados proteína-polímero aprobados por la FDA.

Nombre comercial	Conjugado PEG - proteína	PM PEG (kDa)	Indicación	Año de aprobación
Andagen®	PEG-adenosin deaminasa	5	SCID	1990
Oncaspar®	PEG-asparaginasa	5	Leucemia	1994
PEG-Intron®	PEG-interferon α2b	12	Hepatitis C	2001
Pegasys®	PEG-interferon α2a	40	Hepatitis C	2002
Neulasta®	PEG-G-CSF	20	Neutropenia	2002
Somavert®	PEG-GH (antagonista)	4-5 x 5	Acromegalia	2003
Mircera®	PEG-eritropoyetina	40	Anemia	2007
Cimzia®	PEG-TNF	40	Artritis reumatoide y enfermedad de Crohn	2008

FDA, *Food and Drug Administration* (USA); PM, peso molecular; SCID, enfermedad de inmunodeficiencia combinada severa; G-CSF, factor de estimulación de colonias de granulocitos; GH, hormona del crecimiento. (Hamidi y col., 2006; Fishburn, 2008; Veronese y Mero, 2008).



Capítulo 3.

Current advances in the non-chromatographic fractionation and characterization of PEGylated proteins

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3

Current advances in the non-chromatographic fractionation and characterization of PEGylated proteins

Abstract

For more than 30 years, PEGylation has been used to improve the physicochemical properties of several proteins and therapeutic drugs having a major impact in the biopharmaceutical industry. The purification of PEGylated proteins usually involves two basic challenges: 1) the separation of PEG-proteins from other reaction products and 2) the sub-fractionation of PEG-proteins on the basis of their degree of PEGylation and positional isomerism. Currently, most of the purification processes of PEGylated proteins are based on chromatographic techniques, especially Size Exclusion Chromatography (SEC) and Ion Exchange Chromatography (IEX). Nonetheless, other less frequently used strategies based on non-chromatographic techniques such as ultrafiltration, electrophoresis, capillary electrophoresis, and aqueous two-phase systems have been developed in order to fractionate and analyze PEGylated derivates. This review presents the current advances in some of the most used non-chromatographic strategies for the fractionation and analysis of PEG – protein conjugates.

Keywords: *PEGylation, PEGylated proteins, fractionation and analysis, non-chromatographic methods.*

Introduction

The use of peptides and proteins in the pharmaceutical industry has increased significantly during the last few years. Therefore, the development of strategies oriented towards the improvement of the pharmacological function and kinetics of therapeutic bioactives once inside the human body has drawn attention from research groups from industry and academia. In this context, PEGylation has been playing a major role in the new proposed strategies. PEGylation consists in the modification of a molecule or protein attaching at least one chain of polyethylene glycol (PEG).¹ Nowadays, the PEGylation of proteins or certain groups in the membranes of different organelles and cells has been reported, increasing the application range of this novel technique.^{2,3}

After the reaction, the coupled PEG chains improve the properties of the modified molecules, and once administered, slow down their degradation by proteolytic enzymes, delay their absorption in the kidneys improving their circulation times, lower the production of antibodies towards them and increase their solubility.⁴ However, during the PEGylation reaction the formation of several conjugated forms varying in the number of grafted chains, their length and their grafting site usually occurs.¹ It must be noted that during the early years of PEGylation, the optimization efforts were focused in the directed grafting of PEG chains mainly using residual amino groups as objective sites. Nevertheless, problems exist due to the large number of derivatives produced as consequence of the PEGylation reaction. This results in the need of purifying these mixtures for their commercial approval.¹

The purification of PEGylated conjugates consists in removing from the obtained mixture, all the species that because of their characteristics are not desired (contaminants). This implies two main challenges: 1) the separation of the PEGylated proteins from the rest of the reaction products, and 2) the sub fractionation of these proteins according to their degree of PEGylation and their positional isomerism.⁵ These challenges are not a trivial task, due to the nature of PEG polymers and the fact that PEGylated molecules are hybrid and have properties that do not necessarily imitate the properties of the protein or the PEG from which they come from. The fractionation and characterization of the positional isomers becomes of great importance due to

the fact that usually only one or very few species exhibit optimal biological properties.^{6,7}

Individual and combined chromatographic approaches are currently used to separate PEGylated proteins. Because of the increment in the hydrodynamic radius, Size Exclusion Chromatography (SEC) separates these molecules reducing their accessibility through the porous support and altering their elution times. However, the differences between the species sizes are not enough to achieve positional isomer separation.⁸ On its part, Ion Exchange Chromatography (IEX) is capable of separating these proteins because for each PEG molecule grafted to an amino group a PEGylated protein has one less positive charge. However, PEG chains sterically interfere in the interaction of the charged residues in the polypeptide and the ionic exchange support or can produce a masking effect of the charges.⁹ Together, both SEC and IEX are the most commonly used chromatographic techniques in the separation of polymer conjugates.⁵ Hydrophobic Interaction Chromatography (HIC) and Reversed Phase Chromatography (RPC) have also been studied, although not extensively, for this purpose. These methods result interesting because PEGylation may also affect hydrophobicity in the protein. For RPC low resolutions have been reported while trying to separate conjugates and it has been proved that this technique can produce structural changes in the separated species. HIC on its part presents a low capacity and poor resolution between adjacent peaks.⁹

To date, it is not possible to conclude which type of chromatography or which combination of techniques has better separation yields or analytical resolutions. Furthermore, there is no generalized chromatographic protocol to study the different PEGylated molecules making it necessary to empirically describe the separation strategies on a case by case basis. An extensive analysis of chromatographic methods for separation and purification of PEGylated proteins has been reported before.⁵ Additionally, non-chromatographic methods have been used and developed in order to fractionate and analyze PEGylated conjugates. These methodologies represent an attractive alternative to chromatographic strategies due to economical and technical reasons. However, many of these non-chromatographic strategies have not been fully characterized for the fractionation and analysis of PEGylated conjugates. The Figure 3.1 presents a simplified diagram depicting the main differences between a fully chromatographic and a non-chromatographic approach to fractionate PEGylated proteins. It becomes evident that in the

chromatographic strategy at least two different types of chromatography must be used while the more robust non-chromatographic techniques offer the potential to eliminate these time consuming steps. For example, exploiting the differences in molecular size, membrane separations (i.e. ultrafiltration, diafiltration, dialysis) has been used to separate different PEGylated species from their unmodified precursors.^{5, 10} New advances have also been reported in the use of Aqueous Two-Phase Systems (ATPS) to separate native from PEGylated proteins. Regarding this liquid-liquid fractionation strategy it has been reported that one single grafted chain is enough to promote a selective fractionation towards a specific system phase.¹¹ Also, the use of Polyacrylamide Gel Electrophoresis (PAGE) and Capillary Electrophoresis (CE) has been reported for the analysis and separation of PEGylated proteins.¹²⁻¹⁵ PAGE has been widely used for the characterization of the PEGylation reaction. By its part, CE has applications that include monitoring and optimization of the PEGylation reaction and the separation and purification of products. In addition to PAGE and CE, analysis techniques such as Mass Spectrometry (MS) and Circular Dichroism (CD) are used to evaluate the impact of PEGylation on the secondary and tertiary structure of proteins.

The aim of this review is to present current advances and future trends available on the non-chromatographic fractionation and analysis strategies for PEGylated conjugates, including membrane operations, aqueous two-phase systems, electrophoresis and capillary electrophoresis. The advantages, disadvantages and the impact of the selected strategies on the efficiency of PEGylated conjugate purification processes are discussed.

Membrane separations

The membrane separation techniques are the simplest non-chromatographic methods currently used for PEGylated conjugates fractionation and processing based on their molecular weight and hydrodynamic radius. It was reported by Fee and Van Alstine⁵ that unit operations such as ultrafiltration, diafiltration, and dialysis may be used for the removal of low molecular weight species, buffer exchange and concentration.⁵ Additionally, the authors also mention that in some instances the use of ultrafiltration may generate unacceptable product losses, particularly when using membranes with pores considerably smaller than the PEGylated protein

hydrodynamic radius.⁵ This problem specifically refers to a report regarding the concentration of mono-PEGylated tumor necrosis factor receptor type I (~85 kDa) using 10 kDa molecular weight cut-off membranes.¹⁶ However, current research regarding the different uses and characteristics of ultrafiltration membranes and PEGylated proteins performed mainly by the research group directed by Dr. Andrew L. Zydny^{10, 17-19} confirms that such technique can be used to separate the native from the PEGylated proteins in a reaction mixture (see Table 3.1) and explain the reasons why this was not achieved previously.^{10, 17-19} PEGylated α -Lactalbumin (α -Lac) was separated from its precursor and other byproducts using a two-stage ultrafiltration strategy that exploits the differences in molecular size and electrical charge between the conjugates and the other molecules in the reaction mixture. This was achieved using a regenerated cellulose membrane to remove the native protein followed by a negatively charged membrane to remove the PEG.¹⁰ Additionally, their reports indicate that the hydrodynamic volume cannot be used to predict the sieving coefficients for PEGylated proteins, since these values depend not only on the total molecular weight of the specie but also on the number and length of the grafted polymer branches.¹⁷ Furthermore, it has been proved that due to the presence of the grafted PEG chains the conjugates exhibit a significant flexibility that cannot be described using rigid sphere models and that this flexibility is responsible for molecular elongation during ultrafiltration processes.¹⁸ In fact, this elongation increases as filtrate flux and grafted PEG chain length increases, indicating a higher hydrodynamic drag through the membrane pores as can be deducted from the increments in the sieving coefficients.¹⁸ At low filtration fluxes PEGylated proteins behave as spherical molecules and therefore present lower sieving through different membranes.¹⁸ The effects of using chemically charged membranes to achieve a larger retention of PEGylated products, has been also explained with models that take into consideration the increments in the effective protein size, the increase of the net negative charge and the alterations in the electrostatic potential field of a PEGylated protein.¹⁹ The simple use of charged composite regenerated cellulose membranes presents a reduction in the sieving coefficients. However, a higher reduction in sieving coefficients is observed when decreasing the ionic strength of the ultrafiltration buffer. This behavior is attributed to the increment in the effective size of the protein and the effect of strong electrostatic interactions due to the displacement of the effective protein charge to the outer surface of the PEGylated species.¹⁹ Overall results show that PEGylated species can be fractionated and recovered efficiently using ultrafiltration and

diafiltration achieving high yields and purification factors.¹⁰ Furthermore, these unit operations seem to present advantages over SEC and IEX where proteins of the same molecular size but different polymer lengths and number of grafting sites cannot be separated or fully resolved¹⁸ and the grafted PEG layer apparently shields or blocks the electrostatic binding interactions, respectively.¹⁹ A summary of recent scientific reports addressing the fractionation of protein PEGylated conjugates using membrane unit operations is presented in Table 3.1. No current work known by the authors to separate protein – polymer conjugates using dialysis has been reported. As mentioned earlier, Fee and Van Alstine reported different projects that used this technique to stop the PEGylation reaction by buffer exchange, concentration and removal of byproducts rather than analytical separations⁵.

Aqueous two-phase systems

Aqueous Two-Phase Systems (ATPS) is a well-known and useful technique for the fractionation, recovery and partial purification of several biological, particularly proteins.^{5, 20-23} Chemical compounds such salts (phosphates, sulfates, citrates, etc.) and polymers (PEG, dextran and other copolymers) are used for the construction of ATPS. In this liquid-liquid fractionation technique, two immiscible phases are formed due the electrostatic repulsion and hydrophobicity differences between the phase forming chemicals.^{5, 21} Biomolecules fractionate due to the differences in their physicochemical properties such as molecular weight, superficial electrochemical charge and relative hydrophobicity. ATPS provide favorable conditions to manipulate fractionation in a cost effective and non-denaturing manner due the high contents of water in the system.²⁴ The use of ATPS may service as a preliminary purification strategy of PEGylated proteins in a reaction mixture.⁵ Table 3.2 presents information regarding reports addressing the fractionation, recovery and partial purification of PEGylated conjugates using ATPS.

Delgado *et al.*²⁵ used PEG / dextran ATPS as a feasible technique for the characterization of PEG in PEGylated bovine serum albumin (PEG-BSA) and PEGylated granulocyte-macrophage colony stimulating factor (PEG-GM-CSF) in mixtures of PEG-protein conjugates. They reported a progressive increase in partition coefficient (K , defined as the ratio of the

concentration of a target molecule at the top and bottom phase)²⁰ with the increase in concentration of the grafted PEG molecule due to the increments on the degrees of substitution of the species present in the resulting mixtures. However, at high grafted polymer concentrations a precipitation of a fraction of the conjugates to the bottom phase was observed, resulting in a decrease in $\log K$. This is attributed to the exclusion of bulky molecules from the PEG rich phase. The authors determined a correlation between the partition behavior of the proteins derivates and the degree of modification (n). As a result, evidence that supported that ATPS could be used for the quantitative measure for the degree of modification of PEG-protein conjugates at low concentrations and in mixtures with carrier proteins was stated.

A similar approach to protein purification using ATPS, was also used by Delgado *et al.*²⁶ who identified a simple correlation between the number of grafted polymer chains and the partition coefficient in three different proteins: granulocyte-macrophage colony stimulator factor (GM-CSF), bovine serum albumin (BSA) and immunoglobulin G (IgG) (see Table 3.2). They used NaCl enriched PEG-dextran ATPS to assess a minimal electrostatic chemical potential and to increase sensitivity to polymer grafting. Despite using slightly different polymers, it was proved that the interfacial region of the conjugates would be favored to be polymer-enriched as the polymer-grafting ratio increased. Furthermore, it was stated that $\log K$ vary directly with n as function of the interfacial properties of the conjugate. However, this relationship reached a plateau at high n values (> 20). The authors concluded that further studies were needed to obtain specific data regarding the influence of the protein molecular weight, phase system composition and system parameters to develop an accurate model of PEG-protein partition.

In this context, González-Valdez *et al.*¹¹ evaluated the effect of PEG molecular weight (PEG MW), tie-line length (TLL) and volume ratio (V_R) of PEG-phosphate ATPS on the partition of native RNase A and native α -Lac and their PEGylated conjugates to establish the optimal conditions for their fractionation in a single stage. The use of ATPS constructed with high PEG molecular weight (8,000 g/mol), tie-line lengths of 25 and 35% w/w, and V_R values of 1 and 3 allowed a single step selective fractionation of native RNase A and α -Lactalbumin, respectively, from their PEGylated conjugates on opposite phases. Such conditions resulted in an RNase A bottom phase recovery of 99%, while a top phase recovery greater than 88% was

achieved for both conjugates. On its part, α -Lac had a bottom phase recovery of 92% while its mono and di-PEGylated conjugates were recovered at the top phase with yields superior to 76%. The results presented by the authors demonstrate the potential application of ATPS for the fractionation of PEGylated conjugates from their unreacted precursors. The extended application of ATPS approach for PEGylated proteins separation was reported by Sookkumnerd and Hsu²⁷. This report documented the successful separation of unmodified, mono and di-PEGylated lysozymes using Counter Current Distribution Aqueous Two-Phase Systems (CCD-ATPS). CCD-ATPS is a technique that allows the separation of proteins that like PEGylated conjugates, present minor differences in their partition behaviors or are structurally close related.²⁸ Their experiments were conducted manually using PEG – potassium phosphate systems and as a result three peaks corresponding to each of the species were clearly identified. However, no complete separation between the native and the mono-PEGylated lysozyme was achieved. Furthermore, the authors suggested the use of high-speed countercurrent chromatography or thin layer countercurrent distribution to achieve higher purities of each conjugate at preparative scale.²⁷

Despite the studies presented above, it is actually not possible to create a generic separation process for the purification of PEGylated conjugates using ATPS fractionation. A better understanding of the mechanisms related to the partition of protein conjugates is needed to optimize this technique. Therefore, even when the great potential of this technique has been demonstrated, more experimental studies must be conducted in order to generate partition behavior data to elucidate a robust predictive model for the fractionation of PEGylated conjugates on ATPS.

Electrophoresis

Polyacrylamide Gel Electrophoresis (PAGE) can be used as well for the analysis and separation of PEGylated proteins²⁹ (see Table 3.3). However, unlike the case of native proteins where standard protein ladders can be used to make quantitative determinations of molecular weight, no current correlation exists to determine PEGylated protein molecular weight by this method. Since the migration rates of PEGylated proteins through porous gels are slowed by the large, heavily hydrated and uncharged PEG chains attached to the proteins, their molecular

masses cannot be determined from their positions relative to standard protein ladders.²⁹ On the other hand, PAGE has been used to analyze the extent of PEG conjugation, to characterize the PEGylated products and to indirectly estimate the molecular weight of some PEGylated proteins.^{14, 15, 30, 31}

It was reported by Na *et al.* that PEG moieties in PEGylated conjugates may be specifically stained with a barium iodide solution facilitating the visualization of free PEG and PEGylated derivates on SDS-PAGE.¹³ Jeng *et al.* reported the use of SDS-PAGE to evaluate the efficiency of conjugation in the PEGylation reaction of Horseradish peroxidase (HRP).³² The authors followed the PEG conjugation of HRP with stepwise addition of activated PEG in order to determine the molar ratio leading to an optimal conversion of HRP to PEGylated HRP. In this case, conversion was assayed using SDS-PAGE (12% polyacrylamide gel stained with Commassie Blue) and quantification was carried out in a densitometer.³¹

As mentioned before, protein-bound and free PEG in PAGE may be visualized with barium iodine staining, whereas the protein component either free or conjugated is dyed with Commassie Blue. Unfortunately, the barium iodine and Commassie Blue stains are not suitable to isolate specific conjugate species from gels for subsequent experimentation. Therefore, different techniques of detection of PEGylated proteins in polyacrylamide gels have been developed. For example, it has been shown that a reverse staining technique using zinc and imidazol salts is applicable for detecting gel-separated PEGylated proteins with minimal risk of modification.³² Hardy and coworkers demonstrated that PEGylated proteins (i.e. interferon α 2b) could be straightforwardly reverse stained in analytical (<1 mm) gels. Furthermore, they additionally proved that the properties of PEG-interferon conjugates recovered from gels remain unchanged.³² SDS-PAGE has been used to analyze and characterize the PEGylation reaction of recombinant human granulocyte colony stimulating factor (rhG-CSF) and its purification with size exclusion and cation exchange chromatography. In this case, Zhai *et al.* silver stained the proteins and used barium iodine for PEGs. Their results were consistent with the results obtained with chromatographic techniques.¹⁵

Recently, microchip-based electrophoresis, has successfully demonstrated its capabilities

as a high-performance analytical technique for PEGylated proteins. This technique provides several advantages, including high speed (10 samples within 25 min), small sample volumes (only 4 μ L of protein sample required) and reduced reagent consumption.³³ In 2006, Yu and collaborators reported the application of microchip electrophoresis for measuring the extent of PEGylation of proteins.³⁴ Lately, Park and Na used the microchip electrophoretic method to monitor and characterize the PEGylation reaction of α -Lac and BSA with several grafted PEG chains of different molecular weights. They demonstrated that this method is indeed a very useful tool for protein PEGylation studies that involve reaction monitoring, purity checks, and characterization of polymer conjugates.³³

As seen, SDS-PAGE has been mainly used in the analysis and characterization of PEGylated proteins. The versatility of this technique allows the visualization of both, the reaction products and the ungrafted PEG in several reaction mixtures through different stains. Additionally, it has proved to be extremely useful for the separation of PEGylated conjugates for subsequent mass spectrometry (MS) analysis.

Capillary electrophoresis

Although capillary electrophoresis (CE) has been little used for separation of PEGylated proteins, there are reports where this technique has been successfully applied for this purpose. CE has proved to be a powerful technique for the high resolution separation of biomolecules such as peptides and proteins.³⁵ The sodium dodecyl sulfate-capillary gel electrophoresis (SDS-CE) method using a hydrophilic replaceable polymer network matrix, which combines the principles of SDS-PAGE with the instrumentation and small-diameter capillaries of CE, provides faster and more efficient separations than SDS-PAGE in the slab-gel format.³⁶ Table 3.4 shows recent studies for the separation and analysis of PEGylated proteins using CE.

Na and coworkers¹³ used the SDS-CE method to characterize the PEGylated interferon α (IFN) conjugates. The authors demonstrated the capability of this methodology to completely resolve PEGylated IFNs of different sizes and to monitor the reaction. Advantages of this technique regarding speed, automation capability and sample consumption were visualized by

the authors when comparing CE with other available techniques.¹³

In PEGylation, the size and structure of the grafted PEG plays a major role in determining the pharmacokinetic and pharmacodynamic properties of the resulting protein conjugates.^{37, 38} The enlargement of the molecular size of the conjugates after PEGylation slows renal elimination and prolongs the residence time of these protein drugs in the bloodstream extending dosage times.³⁷ Therefore, the current PEGylation technology frequently uses high molecular weight PEG reagents to obtain favorable pharmacokinetic profiles.³⁸ SDS-CGE has been used to separate mono-PEGylated IFNs modified with different high molecular weight PEGs with branched and trimeric structures. The SDS-CGE method showed high separation capacity by differentiating PEGylated IFN conjugates with small differences in molecular size and structure, while useful for checking the purity of each mono-PEGylated molecule.³⁸

Semi-aqueous capillary electrophoresis has been used to separate and characterize PEGylated ribonuclease and lysozyme molecules. The use of a semi-aqueous acetonitrile-water buffer (1:1 v/v) resulted in the enhancement on the resolution of PEGylated proteins, while the total analysis time was reduced compared with other reported methods.³⁹

Lee and Na⁴⁰ used both capillary zone electrophoresis (CZE) and SDS-CGE methods for the analysis of PEGylated granulocyte colony stimulation factor (G-CSF) produced by a reaction with aldehyde activated PEG (5 and 20 kDa). The separation methods were also compared with High-Performance Size Exclusion Chromatography (HP-SEC). In this work, CE methods showed a better separation capacity than HP-SEC for the resolution of different PEGylated G-CSF conjugates. The CZE method could separate successfully both the 5 and the 20 kDa conjugates. In the other hand, SDS-CGE method was useful just for the separation of the low molecular weight PEGylated G-CSFs. This was attributed by the authors to the long migration times and low peak efficiency achieved using SDS-CGE. The sensitivity of the CZE method was higher than the one in SDS-CGE.⁴⁰

Other proteins, such as the PEGylated human parathyroid hormone 1–34 (PTH), have been characterized and separated using this technique.¹² CE was used to optimize the PEGylation

of PTH through control of the reaction pH and the molar ratio of reactants. Additionally, it is possible to determine the extent of positional isomers present in the mono-PEG-PTH by quantifying PEGylated fragments in the same CE electropherogram.¹²

CE has not been extensively exploited for the recuperation and purification of PEGylated proteins. However, it has been possible to analyze, characterize and often separate PEGylated proteins, with results that are comparable to those obtained with chromatographic techniques. The experimental conditions of this technique are specific for each protein and there is not enough information to generate an empirical correlation to facilitate the selection of parameter values for optimal resolution of PEGylated conjugates. Nevertheless, the versatility of CE may allow the extension of this technique for the fractionation and purification of different PEGylated proteins and peptides in the nearby future.

Future trends and conclusions

It is clear that the increment of PEGylated proteins with therapeutic properties in clinical trials and the presence of PEGylated drugs already in the market are proofs of the potential of this promising chemical modification on the pharmaceutical industry. However, although the chemical modification of proteins and other bioactives via PEGylation has been optimized during the past two decades, improvements on the purification of PEGylated conjugates are still needed. Therefore, a better characterization of the overall structural properties of the conjugated protein complexes is needed for the development of robust non-chromatographic techniques.⁴¹

It is believed that the membrane separations area will continue the characterization of novel membrane modifications towards the separation of specific proteins. Besides, promising filtration techniques such as Tangential Flow Filtration and Centrifugal Diafiltration, which were not used before for the separation of PEGylated products, are currently being studied for the separation of PEGylated nanoparticles.^{42, 43} On the other hand, it is necessary to fully characterize the behavior of protein-polymer conjugates in ATPS based operations, either for batch or continuous processing. In this context, a promising alternative to establish a continuous system for PEGylated derivates is using countercurrent distribution. *In situ* product recovery through continuous operation of ATPS will be one of the main focuses in the future. PAGE,

which has been used mainly for the analysis of the PEGylated products during and after reaction, is probably the most widely used non-chromatographic technique in this moment. Although advances have been made regarding the characterization of PEGylated derivates using this technique,^{14, 15, 44, 45} it becomes necessary to explore alternative staining techniques to distinguish free PEG and PEGylated proteins in one step. On the other hand, no reports have been found that indicate the use of electrophoresis in two dimensions for the analysis and characterization of PEGylated products, so it may be interesting to carry out research in this area in order to determine the potential of this technique for the present application. CE is a very versatile technique with promising results in the purification and analysis of PEGylated derivates. Nevertheless, further investigation is needed to fully characterize the effect of process parameters such as length and diameter of capillary tube, chemical nature and number of grafted PEG chains, and solvent chemical identity, among others, on the separation of modified bioactives of commercial interest.

The on-line coupling of analytical techniques such as MS and CD to biotechnological processes is increasingly becoming of great importance in areas such as manufacturing and quality control.^{46, 47} Technological advances are allowing their use for the optimization of different manufacturing process parameters as product yields and heterogeneity giving great advantages over time-consuming chromatographic techniques.⁴⁶ This becomes important when fast process-related decisions must be made when working on greater production scales. Even though there is no current industrial PEGylation process where these techniques are used routinely, their importance on laboratory scale analysis of PEG–protein conjugates indicates the need to consider their implementation on both chromatographic and non-chromatographic separation approaches of such molecules.

As discussed, the main challenge in the separation of PEGylated proteins is to establish compact analysis, fractionation and purification technologies with low operation costs without compromising efficiency. It is believed that the non-chromatographic techniques described herein offer the characteristics needed for this purpose as different investigation groups have seen their potential and are currently directing their efforts to fully characterize and optimize them.

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Legends of figures

Figure 3.1 Simplify representation of a comparison between chromatographic and non-chromatographic separation processes of PEGylated proteins. The chromatographic approach is presented in a) while the non-chromatographic approach is shown in b). It can be noted that several techniques or a mixture of these can be used in a non-chromatographic separation strategy. Secondary steps refer to additional unitary operations needed for the final release of the PEGylated products. For the analysis of these proteins in both cases techniques such as mass spectrometry, circular dichroism or electrophoresis or a mixture of these can be used.

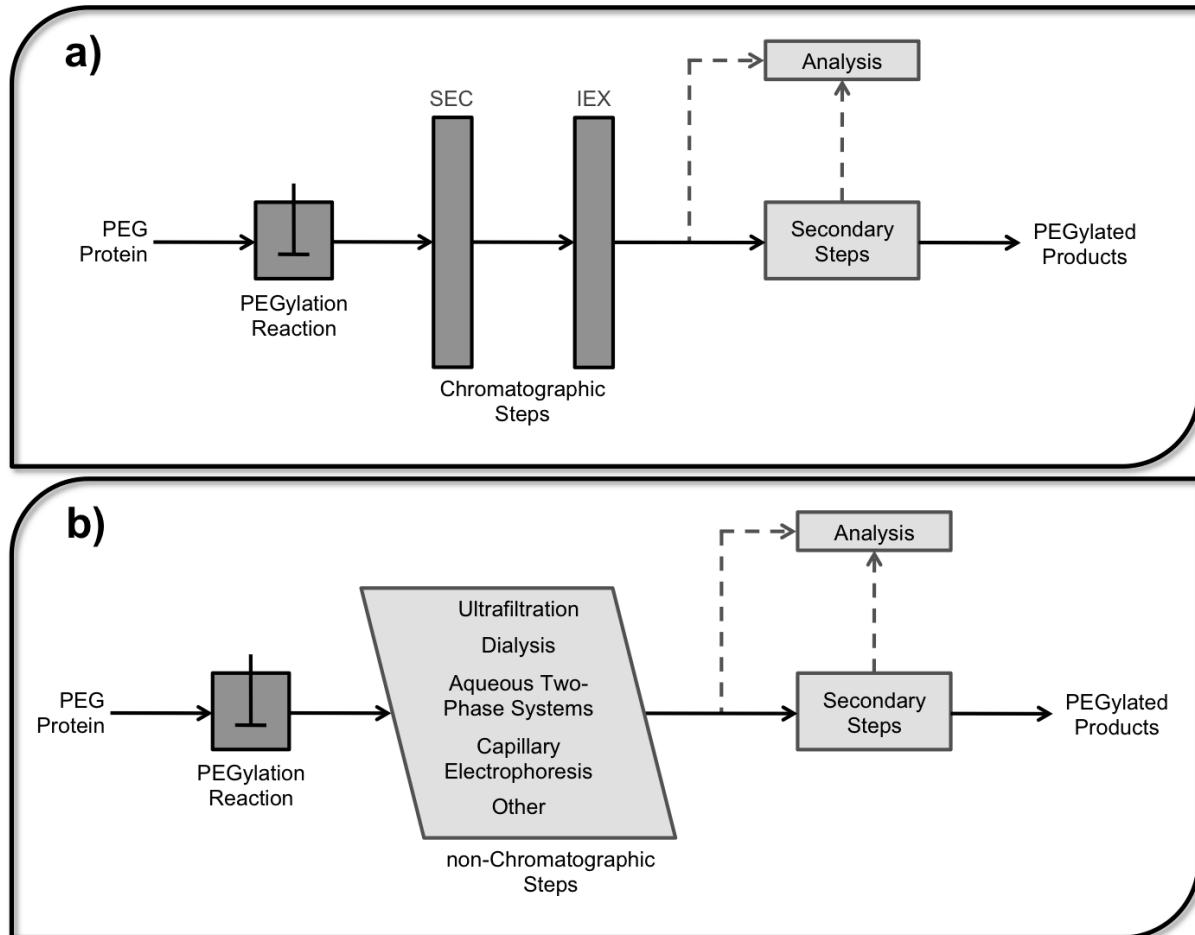


Figure 3.1

Table 3.1 Selected reports exploiting the use of membrane unit for PEGylated proteins separations.

Protein (MW, kDa)	PEG MW (kDa)	Process	Conditions	Goals	Reference
α -Lactalbumin (14.2) Ovalbumin (42.8) BSA (69.3)	5, 10, 30	Ultrafiltration	30 kDa membranes	Evaluate the effects of the number and size of PEG chains	¹⁷
α -Lactalbumin (14.2)	20	Ultrafiltration Diafiltration	30 and 100 kDa membranes	Purification of a single PEGylated specie	¹⁰
α -Lactalbumin (14.2)	5, 10, 20	Ultrafiltration	30, 100 and 300 kDa membranes	Evaluate the influence of the grafted PEG properties upon sieving	¹⁸
α -Lactalbumin (14.2)	2, 5, 10, 20, 30	Ultrafiltration	100 kDa unmodified and charged membranes	Evaluate the effect of electrostatic interactions on protein retention	¹⁹

MW, molecular weight; BSA, bovine serum albumin.

Table 3.2 Reports of PEGylated protein processing using Aqueous Two-Phase Systems (ATPS).

Protein	PEGylated Protein MW (kDa)	Degree of modification (n)	Goals	Reference
GM-CSF	14.4	0-2.5	Correlation of $\log K$ vs n and purification of protein-conjugates from unreacted protein and PEG	²⁵
BSA IgG	66.2 160.0	0-46 0-43	Use of NaCl enriched ATPS for the quantification and purification of protein-conjugates	²⁶
α -Lactalbumin RNase A	13.6 14.1	0-2 0-2	Fractionation of conjugates depending on their n and separation from unreacted protein	¹¹

MW, molecular weight; GM-CSF, granulocyte macrophage colony-stimulating factor; BSA, bovine serum albumin; IgG, Immunoglobulin G; RNase A, ribonuclease A.

Table 3.3 Recent advances in the analysis of PEGylated proteins using Polyacrylamide Gel Electrophoresis (PAGE).

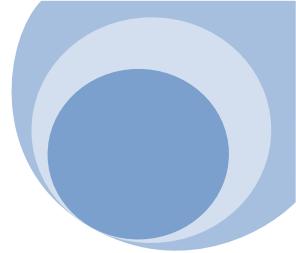
Protein	Conditions	Goal	Reference
hG-CSF	SDS-PAGE and silver stain	Molecular weight determination	44
Recombinant protein	SDS-PAGE and silver stain	Characterization	34
Horseradish peroxidase	SDS-PAGE and Commassie blue stain	Evaluation of the efficiency of PEG conjugation	31
Laccase	SDS-PAGE, Commassie Blue and silver stain	Molecular weight determination	45
Insulin	Non-denaturized PAGE, Commassie blue stain	Observation of differences between mono- and di-PEGylated proteins	30
Interferon α 2b	Imidazol-SDS-zinc reverse staining, Commassie Blue and I_2 stain	Detection of PEGylated proteins with minimal risk of modification	32
rhG-CSF	SDS-PAGE, silver stain for protein and barium iodine method for PEGs	Characterization and modification extent of protein	15
Human Serum Albumin	SDS-PAGE, and Commassie Blue stain	Characterization of PEGylated products	14
Staphylokinase	SDS-PAGE, and Commassie Blue stain	Characterization of PEGylated products	14

MW, molecular weight; PEG, poly (ethylene glycol); rhG-CSF, recombinant human granulocyte colony-stimulating factor

Table 3.4 Current selected research using Capillary Electrophoresis (CE) for the characterization and separation of PEG-modified proteins.

Protein	Process	Goal	Reference
Ribonuclease A and Lysozyme	Semi-aqueous Capillary Electrophoresis	Characterization of PEGylated derivates	39
Interferon α	SDS-CGE	Characterization of PEGylated IFN alpha	13
Human parathyroid hormone	Capillary Electrophoresis	Simultaneous analysis of the PEGylation site and the extent of positional isomers in the mono-PEG-PTH	12
Interferon α -2a	SDS-CGE	Separation and purification of mono-PEGylated IFN	38
G-CSF	CZE and SDS-CGE	Optimization of the PEGylation reaction, as well as purity and stability tests	40

MW, molecular weight; SDS-CGE, sodium dodecyl sulfate-capillary gel electrophoresis; G-CSF, granulocyte-colony stimulating factor, CZE; capillary zone electrophoresis.



Capítulo 4.

Separation of PEGylated from Unmodified Ribonuclease A using Sephadex Media

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4

Separation of PEGylated from unmodified Ribonuclease A using sepharose media

Abstract

PEGylation, used to mitigate some of the problems that affect the effectiveness of therapeutic proteins, often results in a heterogeneous population of conjugated species and unmodified protein that presents a protein separations challenge. This study presents the use of a mildly amphiphilic support, Tris-capped CH Sepharose 4B as an alternative for separating PEGylated proteins from their unmodified counterparts. The effects of parameters such as pH, salt type and salt concentration upon the chromatographic behavior of native, mono-PEGylated and di-PEGylated ribonuclease A on this media were characterized. The separation of the native protein from the PEGylated species was achieved using a gradient elution between a high ionic strength mobile phase (3 M ammonium sulfate in 25 mM potassium phosphate, pH 7.0 or 2 M potassium phosphate, pH 7.0) and a low ionic strength phase (25 mM potassium phosphate, pH 7.0). The pH of the mobile phases as well as the addition of PEG600 (as a potential mobile phase modifier) to the low ionic strength phase had no significant influence on chromatographic behavior of the species. This media provides a simple and practical chromatographic method for the separation of unmodified proteins from their corresponding PEG conjugates.

Key words: aqueous two phase systems, chromatography media development, hydrophobic interaction chromatography, ribonuclease A, PEGylated proteins .

Introduction

The covalent attachment of polyethylene glycol (PEG) molecules to pharmaceutical proteins – a reaction process known as “PEGylation”- can mitigate factors that adversely affect therapeutic effectiveness, including susceptibility to enzymatic degradation, short circulation time, low solubility and immunogenicity [1,2]. However, this can present a challenging separation problem due to the fact that PEGylation reactions often result in a population of conjugate species in addition to the residual unmodified protein. The resulting conjugate species can differ in terms of the number of attached PEG chains and their locations and can differ in biological activity [3]. Chromatographic operations are often used to resolve PEGylation reaction mixtures, with size-based size exclusion and charge-based ion exchange modes most frequently used [4]; comparatively little work has been done to explore other modes such as the hydrophobic interaction mode [5,6].

Hydrophobic interaction chromatography (HIC) is intriguing with respect to the separation of PEGylated protein reaction mixtures as PEG itself exhibits lower critical solution temperature behavior: it can adopt collapsed configurations at higher temperatures and/or salt concentrations that are comparatively more hydrophobic than the relatively extended configurations that occur at lower temperatures and/or salt concentrations [7]. Very little work has been done on exploiting hydrophobic interactions for the separation of PEGylated proteins [4]. HIC consists of injecting a protein sample in a column packed with a hydrophobic media under conditions of high salt concentration to drive hydrophobic interactions between the proteins and the media, analogous to a salting-out process. Bound proteins are eluted by lowering the salt concentration. In the case of a strongly bound protein, it can be eluted by the use of a chaotropic agent or an organic modifier [8]. The most common hydrophobic ligands used in HIC are linear chain alkanes or simple aromatic groups. However, these hydrophobic ligands may promote strong hydrophobic interactions that sometimes result in irreversible adsorption of the proteins or denaturation during adsorption and subsequent elution with harsh mobile phases. Ligands with milder hydrophobic characters can be attractive as they can provide moderate binding strength and bound species can be eluted by simply decreasing the salt concentration of the eluent [9].

In this work, we were able to completely separate PEGylated ribonuclease A (RNase A)

species from unmodified RNase A using a Tris-capped CH Sepharose 4B media with salt gradient elution. RNase A was chosen as a model protein as it is well-characterized, has potential therapeutic application as an aspermatogenic and antitumor agent and studies have shown an improvement of the therapeutic effect when RNase A is conjugated with PEG [10]. RNase A was PEGylated with an aldehyde-activated PEG of 20 kDa nominal molecular weight. The behavior of unmodified RNase A and mono- and di-PEGylated species previously separated using size exclusion chromatography (SEC) was characterized on the proposed Sepharose-HIC separation system as were protein PEGylation reaction mixtures. pH, salt type and salt concentration were the parameters selected to define conditions under which the separation of the unmodified protein from the PEGylated species can be achieved. Free PEG with a nominal molecular weight of 600 Da was also examined as a potential mild mobile phase modifier.

Materials and Methods

Materials

Bovine pancreatic ribonuclease A (cat. No. R5000, Lot 093K0765) was purchased from Sigma Aldrich (St. Louis, MO). Methoxy-poly(ethylene glycol)-butyraldehyde with a nominal molecular weight of 20 kDa came from Nektar Therapeutics (Huntsville, AB). Sodium cyanoborohydride was purchased from ICN Biomedicals Inc. (Costa Mesa, CA). Activated CH sepharose 4B (cat. No. 17-0490-01 Lot 307571) and the chromatography column, a Tricorn 5/100, came from Amersham Biosciences (now GE Healthcare, Uppsala, Sweden). Conjugation and purification buffers were prepared from sodium phosphate monobasic and dibasic salts (Fisher Scientific, Pittsburgh, PA), and potassium chloride (Sigma-Aldrich Company, St. Louis, MO). All HPLC grade reagents were obtained from Fisher Scientific. Other salts and solvents used in this research were of reagent grade. All reagents were used as received.

Preparation of PEGylated protein

PEGylated RNase A was prepared according to the procedure of Daly and co-workers [11]. Briefly, a solution of RNase A (2.0 mL) at 3.0 mg/mL in a pH 5.1, 100 mM sodium phosphate buffer with 20 mM sodium cyanoborohydride was added to a vial containing 30 mg of

the nominal 20 kDa methoxy poly(ethylene glycol) butyraldehyde. The reaction mixture was stirred rapidly for 17 – 19 h at 4 °C. Experiments were conducted both with the final reaction mixture and with species purified using size exclusion chromatography (SEC).

Analysis and separation of PEGylated protein mixture by Size Exclusion Chromatography (SEC) and Mass Spectrometry

The reaction mixture (2.0 mL) was analyzed via size-exclusion chromatography with an Amersham Pharmacia Akta Explorer system (now GE Healthcare, Uppsala, Sweden) using a Sephadex S-300 column (1.6 cm inner diameter, 60 cm length, Amersham Pharmacia, Uppsala, Sweden) with an isocratic mobile phase of 10 mM sodium phosphate buffer, pH 7.2, containing 150 mM potassium chloride at a flow rate of 0.5 mL/min. The column was pre-equilibrated with one-half column volume of distilled water and two column volumes of mobile phase. Fractions having an absorbance at 280 nm were collected and concentrated in an Amicon (Beverly, MA) stirred cell fitted with a YM 10 membrane (molecular weight cutoff 10 kDa) and then diafiltered with 5 volumes of 25 mM sodium phosphate buffer, pH 7.2.

Each SEC peak was analyzed by mass spectrometry (Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry, MALDI-TOF/MS). Prior to analysis, the samples were desalted using centrifugal microconcentrators (Microcon YM-3, Millipore). MALDI-TOF/MS was performed with a PerSeptive Voyager STR mass spectrometer fitted with a standard 337 nm nitrogen laser. The spectra were recorded with the analyzer in the positive-ion linear mode of detection. The ion accelerating potential was 25 kV. A saturated solution of α-3,5-dimethoxy-4-hydroxycinnamic acid (Fluka, Buchs, Switzerland) in 50% acetonitrile was used as a matrix solution. The sample crystals were prepared by mixing 1.0 μL of sample with 1.0 μL of matrix. The product peaks were identified as diPEGylated, monoPEGylated and unmodified RNase A species.

Preparation of chromatography media

Sepharose was used as the base matrix for the construction of a mild amphiphilic support.

Activated CH Sepharose 4B (Amersham Biosciences, Uppsala, Sweden Lot: 307571) was modified with a capping reaction using tris(hydroxyethyl)aminomethane (Tris) as shown in Figure 4.1. The matrix was prepared according to supplier instructions (Amersham Biosciences). About 1.3 g of activated Sepharose were washed with about 250 mL of 1 mM HCl and the washed medium was transferred to 0.1 M Tris-HCl buffer, pH 8. After 1 hour, the modified media was washed with 3 cycles of alternating pH (0.1 M acetate buffer, pH 4.0 containing 0.5 M NaCl followed by 0.1 M Tris-HCl buffer, pH 8 containing 0.5 M NaCl). Based on the activated media product literature, 15 µmol/mL media is an upper bound on the Tris capping group density.

Separation of PEGylated species from unmodified RNase A

Chromatographic experiments were carried out with the Akta Explorer 100 integrated chromatography system operating at room temperature. Protein samples collected from SEC experiments or from the final PEGylation reaction mixture were applied to a Tricorn 5/100 column (Amersham Biosciences, 4.6 x 150 mm) packed with about 3.5 mL of the modified Sepharose media. The column had about 65 theoretical plates, as determined by elution peak width analysis of injections of 1% v/v acetone pulses at 25°C. Gradient elutions were carried out for protein separations at a flow rate of 1 mL/min with a solvent A (3 M ammonium sulfate in 25 mM potassium phosphate pH 7.0, or 2 M potassium phosphate, pH 7.0 or 3 M ammonium sulfate in 25 mM acetate buffer, pH 5.0) and a solvent B (25 mM potassium phosphate, pH 7.0 or 25 mM acetate buffer, pH 5). The following gradient profile was selected: 0% B for 6.5 mL, 0 – 100% B over 29.5 mL, 100% B for 15 mL. Peak elution was monitored via the UV absorption at 280 nm. 100 µL of sample were injected at room temperature. The concentrations were 3, 1.5 and 0.97 mg/mL for unmodified, monoPEGylated and diPEGylated RNase A respectively. The flow rate was 1 mL/min.

Results and Discussion

In order to study the retention behavior of the proteins using the capped Sepharose media, unmodified RNase A and the SEC-purified monoPEGylated and diPEGylated species were applied to the column with an initial concentration of 3 M ammonium sulfate in the 25 mM

phosphate buffer; the corresponding chromatograms are shown in Figure 4.2. The elution profiles of the purified species are shown under stronger loading conditions (3 M ammonium sulfate) and with a steeper gradient (about -0.10 M salt/mL). The unmodified protein elutes about a third of the way through the salt gradient at roughly 220 mS/cm 1.5 M ammonium sulfate. There may be some hysteresis in the adsorption-desorption behavior of the unmodified protein: loading under more strongly binding conditions may require more stringent elution conditions. Both the monoPEGylated and diPEGylated species elute much later at roughly 135 mS/cm and 130 mS/cm, respectively, representing somewhat stronger to the media; again, there may be some binding hysteresis. In this case, there was a slight, but significant difference in the elution behavior of the monoPEGylated and diPEGylated species; this small difference is likely not sufficient for exploitation to resolve PEGylated variants. The elution of the PEGylated species at lower salt concentrations suggests that hydrophobic interactions were the primary mode of interaction with the media; hydrogen bond-based interactions would be disrupted at higher salt concentrations.

Retention in hydrophobic interaction chromatography processes is highly dependent on mobile phase ionic strength and salt type. The separation strategy requires a careful selection of these conditions during both the adsorption and desorption steps [12]. In order to further characterize the behavior of the capped media for the separation, the effect of salt type was evaluated. Figure 4.3 shows the elution behavior of RNase A and its PEG-conjugates using a gradient in the buffer concentration from 2 M to 25 mM potassium phosphate, with a gradient slope of about -0.07 M/mL, to manipulate mobile phase ionic strength. The unmodified protein was more strongly retained relative to the previous ammonium sulfate gradient case, eluting at a conductivity of about 145 mS/cm. The monoPEG and diPEG species eluted at roughly 100 mS/cm and 95 mS/cm, respectively. In the absence of special binding effects, a change in salt concentration or change of salt in the mobile phase to one of greater surface tension increment results in increased retention of proteins by HIC [13]. In fact, for potassium phosphate, a small percentage of proteins was retained on the column after completion of the gradient and this could be due to the low surface tension increment of potassium phosphate with respect to ammonium sulfate. Such behavior is observed because the absorbance obtained in Figure 4.3 does not allow the protein mass balance to close completely.

The pH of mobile phase can also be an important factor that affects the protein retention in HIC. Usually, an increase in the pH value (up to 9–10) decreases the hydrophobic interaction between proteins and the hydrophobic ligands, due to the increased hydrophilicity promoted by the change in the charge of the protein. On the other hand, a decrease in pH results in an apparent increase in hydrophobic interactions. Since each protein will have unique behavior at a given pH relative to its isoelectric point and since the effect of pH on protein retention in HIC is not well defined, this parameter should be used in the optimization of protein separation by HIC [9].

The effect of the pH was tested using the 3 M ammonium sulfate gradient in acetate buffer at pH 5.0. RNase A has a pI (isoelectric point) of 9.3 [14] and it is expected that the conjugate forms, owing to the covalent modification of one or two amino groups, will have slightly lower pIs according to the PEGylation conditions [15]. Under both pH conditions studied, 5.0 and 7.0, the proteins will have a positive net charge. The retention behavior of individual species at low pH is shown in Figure 4.4. The unmodified protein elutes at about 220 mS/cm, roughly equivalent to that at pH 7.0 as shown in Figure 4.2. The monoPEG species, eluting at 140 mS/cm, is slightly less retained than the diPEG species which elutes at about 135 mS/cm. At the resolution of these chromatograms, this retention is the same as the pH 7.0 case. Under these conditions, the pH, in the 5.0 to 7.0 range, has little influence on the retention of the proteins on this media.

The influence of PEG as a potential mobile phase modifier was tested by adding 15 mM polyethylene glycol with a nominal molecular weight of 600 to the low ionic strength mobile phase B (see Figure 4.5). Here, the idea was that free PEG in solution might compete with PEGylated protein for interaction sites on the media and encourage elution. The addition of PEG to the mobile phase B had little effect on the retention of the proteins: unmodified protein eluted at about 225 mS/cm, monoPEGylated protein at 150 mS/cm and diPEGylated protein at 145 mS/cm.

Finally, a PEGylation reaction mixture was applied to the PEG-Sepharose media using a gradient scheme between 3 M ammonium sulfate in 25 mM potassium phosphate, pH 7.0 and 25

mM potassium phosphate, pH 7.0; the corresponding chromatogram is shown in Figure 4.6. As expected from the differing retentions of the unmodified and modified species shown in the previous results, it was possible to separate unmodified protein from the conjugate species. The observed retention differences between the purified monoPEGylated and diPEGylated species under the conditions studied were too small to result in any further resolution of PEGylation variants. No significant differences between the eluting mobile phase conductivities for the reaction mixture and corresponding purifies species, as shown in Figure 4.2, were apparent. There is no apparent interaction between the unmodified and modified proteins during the separation as expected for PEGylated species.

Conclusions

It was demonstrated that mildly amphiphilic chromatographic media can be exploited to separate unmodified proteins from their PEGylated conjugates, using ribonuclease A as model system. High selectivity was found between the unmodified protein and its PEGylated species using a gradient elution between 3 M ammonium sulfate in 25 mM potassium phosphate pH 7.0 and 25 mM potassium phosphate buffer pH 7.0. Variations including the use of 2 M potassium phosphate at pH 7.0 instead of the ammonium sulfate solution or a decrease in pH (from 7.0 to 5.0) had little effect on retention behavior. More stringent loading conditions necessitated more stringent elution conditions; there is hysteresis in the adsorption and desorption behavior of proteins on this media. Hysteretic protein adsorption phenomena have been observed by multiple groups working with HIC-based separations and have been attributed to protein conformation changes in the adsorbed state, lateral interactions between adsorbed proteins and multivalent protein-chromatographic ligand interactions [16,17,18]. Added PEG_{600} did not function as a mobile phase modifier. There were very slight, but consistent observed differences in the retention of monoPEGylated versus diPEGylated species, with diPEGylated species more retained than the monoPEGylated species. These slight differences were not sufficient to result in observable resolution of modified variants from a mixture. To that end, it would also be interesting to evaluate the effect of additional parameters such as ligand density and matrix support type on the partition behavior of the PEG-conjugates species, in order to develop selectivity for PEGylation variants.

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Figure 4.1 Reaction scheme for the modification of activated CH Sepharose 4B by capping with tris(hydroxyethyl)aminomethane.

Figure 4.2 Retention behavior of RNase A and purified monoPEG and diPEG-RNase on the Sepharose Media with strong loading conditions and an ammonium sulfate gradient. Mobile phase A: 3 M ammonium sulfate 25 mM potassium phosphate, pH 7.0; mobile phase B: 25 mM potassium phosphate, pH 7.0. The PEG-conjugate species were separated by SEC and exchanged into a 25 mM potassium phosphate buffer, pH 7.0; 100 µL of sample were injected at room temperature. The flow rate was 1 mL/min.

Figure 4.3 Retention behavior of RNase A and purified monoPEG and diPEG-RNase A on the Sepharose HIC media with a potassium phosphate gradient. Mobile phase A: 2 M potassium phosphate pH 7.0, mobile phase B: 25 mM potassium phosphate pH 7.0. The PEGylated proteins were separated by SEC and put in a 25 mM potassium phosphate buffer pH 7.0. 100 µL of sample were injected at room temperature. The flow rate was 1 mL/min.

Figure 4.4 Retention behavior of RNase A and purified monoPEG and diPEG-RNase A on the Sepharose HIC media with an ammonium sulfate gradient at acidic pH. Mobile phase A: 3 M Ammonium sulfate in 25 mM acetate buffer pH 5.0, mobile phase B: 25 mM acetate buffer pH 5.0. The PEGylated proteins were separated by SEC and put in a 25 mM potassium phosphate buffer pH 7.0. 100 µL of sample were injected at room temperature. The flow rate was 1 mL/min.

Figure 4.5 Effect of PEG₆₀₀ as a mobile phase modifier on the retention behavior of RNase A and its PEG-conjugates. Mobile phase A: 3 M ammonium sulfate in 25 mM potassium phosphate, pH 7.0; mobile phase B: 15mM PEG₆₀₀ in 25 mM potassium phosphate, pH 7.0. The PEG-RNase A proteins were separated by SEC and put in a 25 mM potassium phosphate buffer pH 7.0; 100 µL of sample were applied to the chromatographic system at room temperature. The flow rate was 1 mL/min.

Figure 4.6 Behavior of PEGylation mixture reaction with a gradient elution scheme.

Mobile phase A: 3 M ammonium sulfate 25 mM potassium phosphate, pH 7.0; mobile phase B: 25 mM potassium phosphate, pH 7.0. 100 μ L of sample were injected at room temperature. The flow rate was 1 mL/min. Unmodified RNase A was added to the reaction mixture before injection in order to facilitate its detection.

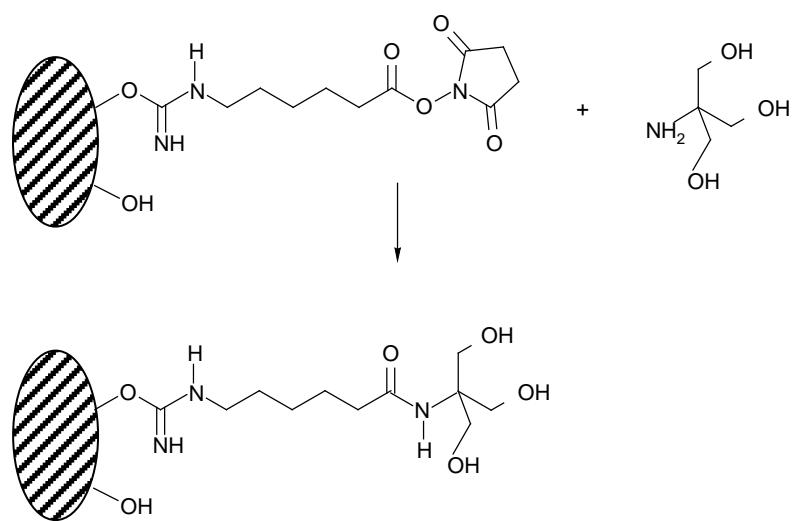


Figure 4.1

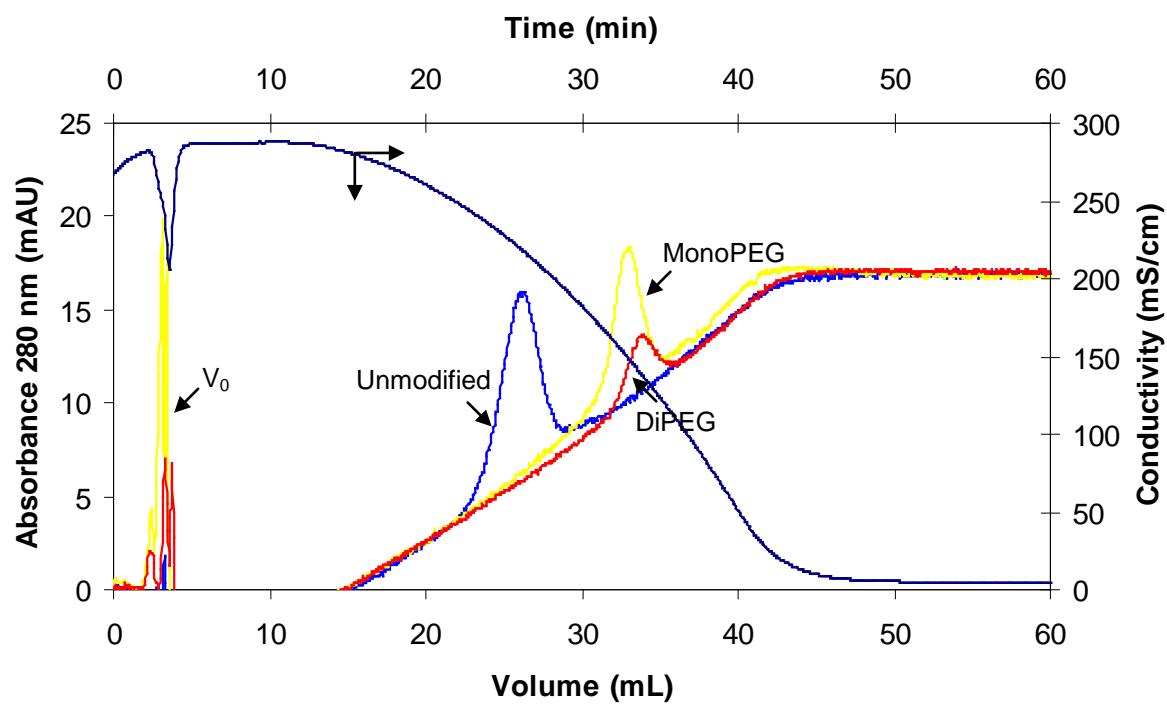


Figure 4.2

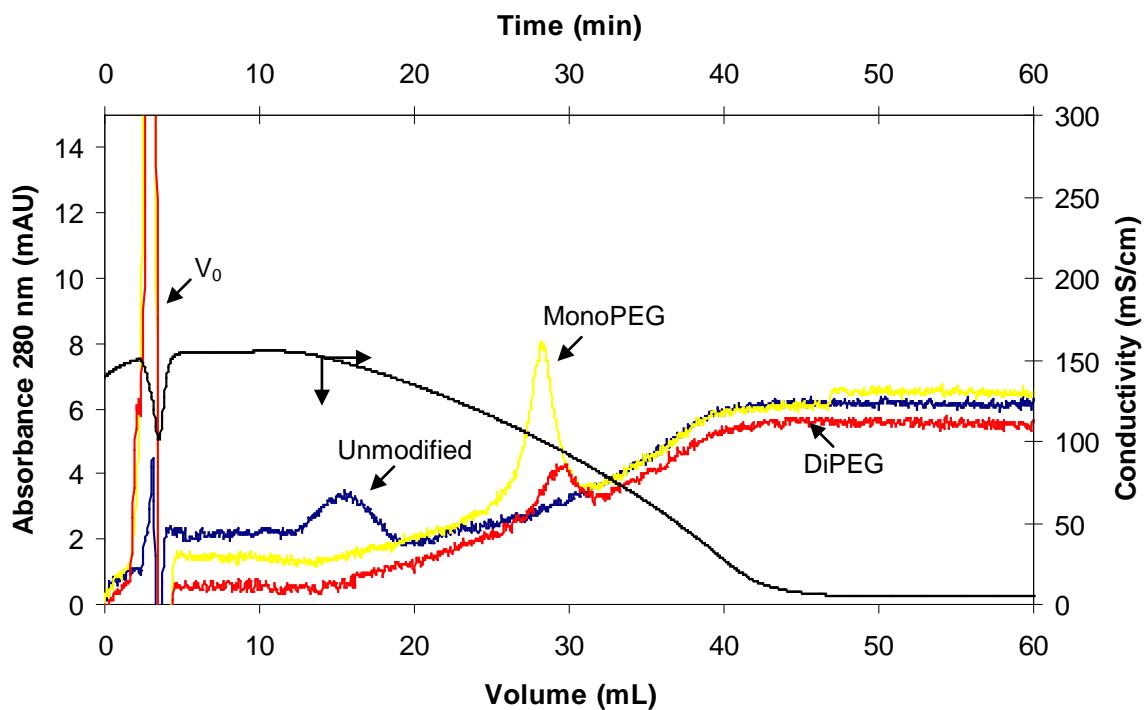


Figure 4.3

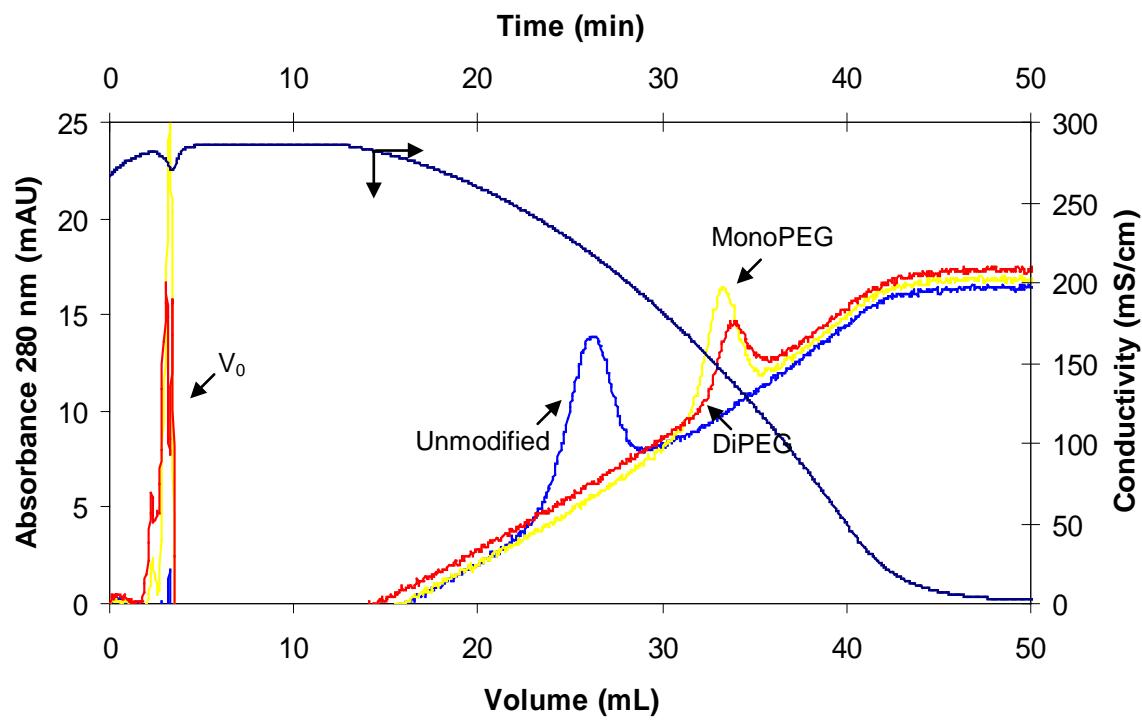


Figure 4.4

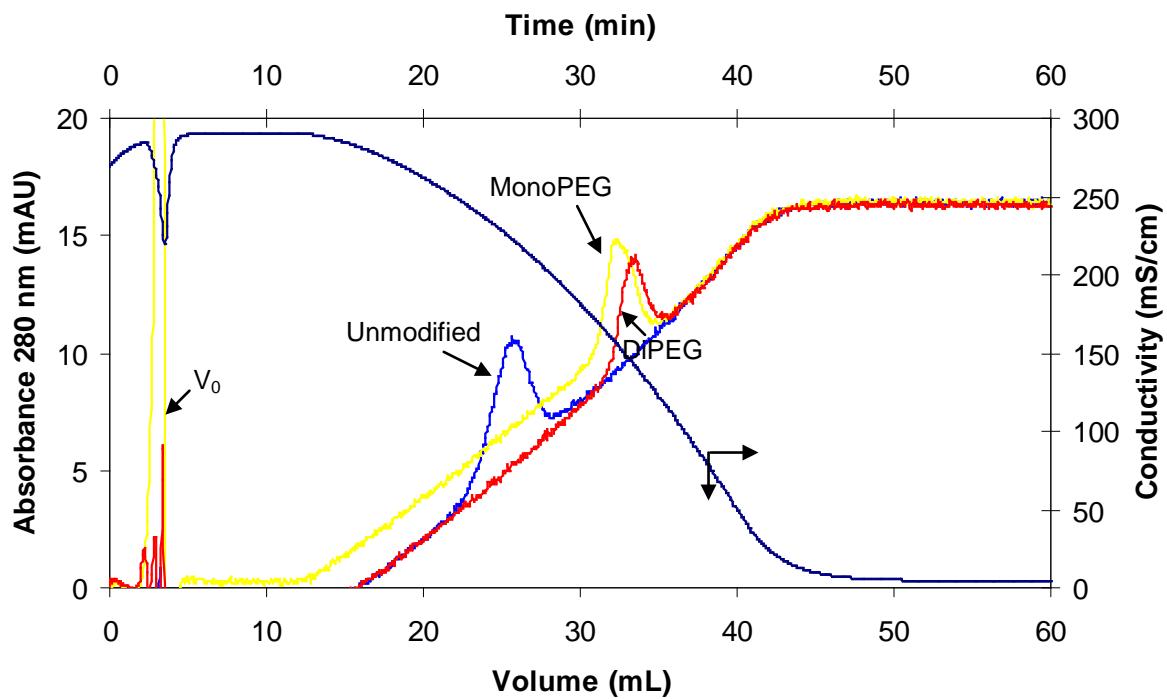


Figure 4.5

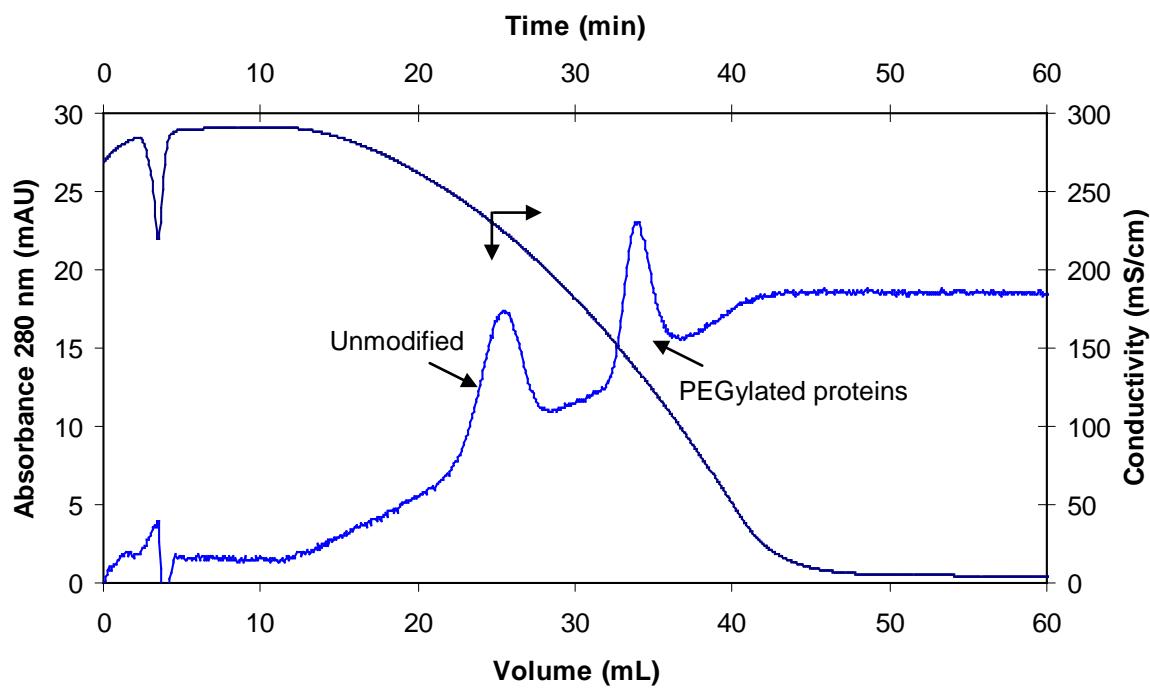
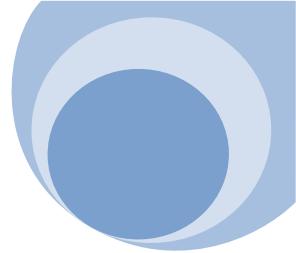


Figure 4.6



Capítulo 5.

Hydrophobic Interaction Chromatography for Purification of monoPEGylated RNase A

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5

Hydrophobic Interaction Chromatography for Purification of monoPEGylated RNase A

Abstract

The chromatographic methods used for the purification of *PEGylated* proteins are mainly Size Exclusion (SEC) and Ion Exchange Chromatography (IEX). Although the *PEGylation* affects the protein hydrophobicity, Hydrophobic Interaction Chromatography (HIC) has not been extensively applied for the separation of these proteins. Purification of *monoPEGylated* Ribonuclease A (RNase A) using HIC is studied in this work. The products of the *PEGylation* reaction of RNase A with 20 kDa methoxy-poly(ethylene glycol) were separated using three resins with different degrees of hydrophobicity: Butyl, Octyl and Phenyl sepharose. The effects of resin type, concentration and salt type (ammonium sulphate or sodium chloride), and gradient length on the separation performance were evaluated. Yield and purity were calculated using the plate model. Under all conditions assayed the native protein was completely separated from *PEGylated* species. The best conditions for the purification of *monoPEGylated* RNase A were: Butyl sepharose, 1 M ammonium sulphate and 35 column volumes (CVs); this resulted in a yield as high as 85% with a purity of 97%. The purity of *monoPEGylated* RNase A is comparable to that obtained when the separation is performed using SEC, but the yield increases from 65% with SEC to ~ 85% with HIC. This process represents a viable alternative for the separation of *PEGylated* proteins.

Key words: *monoPEGylated RNase A, PEGylated proteins, HIC, purification.*

Introduction

PEG-protein conjugates, or *PEGylated* proteins, are an important class of modern therapeutic drugs. However, *PEGylated* proteins must be characterized and purified before use in order to meet the stringent regulatory requirements that demand proven clinical efficacy and safety [1]. The process of *PEGylation* involves formation of a stable covalent bond between activated PEG (polyethylene-glycol) polymers and the polypeptide drug of interest [2]. *PEGylation* changes the physical and chemical properties of the biomedical molecule, such as its conformation, electrostatic binding, and hydrophobicity; resulting in an improvement in the pharmacokinetic behavior of the drug. In general, *PEGylation* improves drug solubility and decreases immunogenicity, increases drug stability and the residence time of the conjugates in blood, and reduces proteolysis and renal excretion, thereby allowing a lower dosing frequency [3].

To prepare a *PEGylated* protein, it is desirable that one PEG molecule is attached to a specific site (site-directed *mono-PEGylation*). However, as the *PEGylation* reaction is not completely understood, it is not easy to adjust or optimize the reaction process [4]. The reaction mixture is complex, from which the desired *PEGylated* protein must be highly purified. Purification of *PEGylated* protein is not a trivial task [5].

PEGylation of proteins creates two basic challenges for purification. The first involves separation of *PEGylated* proteins from other reaction products including non-reacted PEG and protein. The second is the sub-fractionation of *PEGylated* proteins on the basis of their degree of *PEGylation* and positional isomerism [4]. While isolation of the unreacted protein from the *PEGylated* species is relatively easy, separation of the various positional isomers of a *PEGylated* protein mixture remains a significant challenge [6]. Individual and combined chromatographic approaches are currently used to purify *PEGylated* proteins [7]. Chromatographic purification of all proteins implies selection of a mode of chromatography that exploits the differences in physicochemical properties [8]. Fee and Van Alstine [4] reviewed separation methods applied to downstream processes for *PEGylated* proteins. They have reported that Size Exclusion (SEC) and Ion Exchange Chromatography (IEX) are the predominant methods for purification of the

PEGylated products. SEC has been widely used for separation of PEG conjugates as an increase in molecular weight is one of the most evident changes caused by *PEGylation* [9]. IEX enables a single step purification of the target PEG-protein conjugate from *unPEGylated* protein, higher *PEGylated* molecules and unreacted PEG; due to charge differences [9]. *PEGylation* should affect protein surface hydrophobicity, increasing or decreasing it depending on the native proteins hydrophobicity [4] and consequently, Hydrophobic Interaction Chromatography (HIC) can be used as an additional method for separation of PEG modified proteins. Even though HIC is used routinely for production-scale purification of proteins, it has not been highlighted for the separation of *PEGylated* species [1,4,10].

Ribonuclease A (RNase A) is a small model enzyme, with a molecular weight of 13,686 Da [11]. RNase A has proven to have several potential therapeutical applications in vivo as an antitumor agent, mainly in its *PEGylated* form [12]. It has been found that the biological activity exhibited by the *PEGylated* conjugates is related to the number of grafted polymer chains as well as their relative position. For most proteins, an excess in the number of grafted polymer chains generates a steric hindrance for its biological receptor, decreasing their specific activity [13]. It has been reported that the *monoPEGylated* RNase A presents the highest biological activity [12].

The purpose of this work is to establish the conditions for purification of *monoPEGylated* RNase A using HIC. The *PEGylation* reaction used by our group [14,15] is a reaction with the N-terminal amino group of the protein. This reaction generates three products: *monoPEGylated* RNase A (monoPEG), *diPEGylated* RNase A (diPEG), and the unmodified protein. In this case, resin type, salt type (ammonium sulphate or sodium chloride), salt concentration and gradient length were the parameters selected to define conditions under which the purification of the *monoPEGylated* protein can be achieved. Yield and purity were calculated using the plate model [16]. The equations used are described below:

$$\text{yield} = \frac{1}{2} [1 + \text{erf}(V/V_0 - 1/\sqrt{2} * \sigma)] \quad \text{Eq. 1}$$

$$\text{purity} = [y_0(i) * \text{yield}(i)] / [\sum_j y_0(j) * \text{yield}(j)] \quad \text{Eq. 2}$$

where y_0 is the maximum concentration, V_0 is the volume required to elute the maximum

concentration y_0 and $V_0\sigma$ is the standard deviation of the peak [16]. The parameters were determined from the experimental results.

Materials and Methods

Materials.

Bovine pancreatic ribonuclease A (cat. no. R5000, lot 047K1640) and trizma base (cat. no. T6066, lot 076K54521) were purchased from Sigma–Aldrich (St. Louis, MO). MethoxyPEG-propionaldehyde (Lot ZZ004P225) with a nominal molecular weight of 20 kDa came from Jen Kem Technologies (Allen, TX). Sodium cyanoborohydride (cat. no. 71435, lot 414320) was purchased from Fluka (Switzerland). Phenyl sepharose 6FF HS (cat. no. 17-0973-05, lot 286273), Octyl sepharose (cat. no. 17-0946-05, lot 283600) and Butyl sepharose (cat. no. 17-0980-01, lot 28686) were obtained from GE Healthcare (Uppsala, Sweden). Purification buffers were obtained from J.T. Baker (Toluca, México). Water prepared with Milli-Q water cleaning system (Millipore, Bedford, MA) was used in the preparation of the eluents. Other salts and solvents were of reagent grade.

Preparation of PEGylated protein.

PEGylated RNase A was prepared according to the procedure of Daly *et al.* [17]. Briefly, a solution of RNase A (5.5 mL) at 3.0 mg/mL in a pH 5.1, 100 mM sodium phosphate buffer with 20 mM sodium cyanoborohydride was added to a vial containing 75 mg of the nominal weight 20 kDa methoxy poly(ethylene glycol) propionaldehyde. The reaction mixture was stirred vigorously for 17 h at 4 °C. The reaction was stopped by separating the mixture on a size exclusion chromatographic column.

Separation of PEGylated protein mixture by Size Exclusion Chromatography (SEC).

The reaction (5.5 mL) was analyzed by Size Exclusion Chromatography with an Äkta Explorer system (GE Healthcare, Uppsala, Sweden) equipped with a 5 mL injection loop. A Sephadryl S-300 HP column (2.6 cm ID, 60 cm length, GE Healthcare, Uppsala, Sweden) was used with an isocratic mobile phase of 10 mM sodium phosphate buffer pH 7.2, containing 150 mM potassium chloride at a flow rate of 0.5 mL/min. The column was pre-equilibrated with one-

half column volume of distilled water and two column volumes of mobile phase. Fractions that absorbed at 215 nm were pooled and concentrated by ultrafiltration under nitrogen atmosphere using an Amicon ultrafiltration cell (MA, USA) with a 10 kDa Diaflo ultrafiltration membrane (Amicon Inc., MA, USA). Finally, each *PEGylated* protein were lyophilized and stored at -4°C [14]. These lyophilized *PEGylated* proteins obtained by SEC were used to perform the studies of HIC separation.

Separation of PEGylated proteins by Hydrophobic Interaction Chromatography (HIC).

An Äkta Purifier System (GE Healthcare, Uppsala, Sweden) was employed, equipped with a 100 µL injection loop. The chromatographic columns were 1 mL (100 mm x 5 mm ID) packed with Phenyl sepharose 6FF HS, Octyl sepharose or Butyl sepharose 4 FF. The experiments were performed at room temperature, using flow rate equal to 0.8 mL/min and 25, 35 or 45 column volumes (CVs) [18].

Elution was obtained by a lineal decreasing gradient of ammonium sulphate (AS) or sodium chloride. The initial eluent (solution B) utilized was 20 mM TRIS-HCl, pH 7.0 plus different concentrations of salt (AS or sodium chloride). The final buffer (solution A) used was 20 mM TRIS-HCl, pH 7.0. All buffers were filtered through 0.22-µm Millipore filters after preparation, and degassed with helium for 5 min. The column outlet was monitored at 215 nm. The conductivity was also monitored. Yield and purity were calculated using the plate model [16].

Results and Discussion

SEC purification.

Initially, the *PEGylation* reaction was monitored by SEC to obtain the *PEGylated* protein standards required for the HIC analysis. The reaction generates two *PEGylated* species: *monoPEGylated* RNase A and *diPEGylated* RNase A. These products were previously characterized [14]. Figure 5.1 shows the chromatographic profile of SEC, where in addition to the *PEGylated* proteins, unmodified RNase A can be observed. Despite SEC being the most popular route for separation of *PEGylated* species [4], the resolution obtained is low. Plate

model was used to calculate the yield of the separation of monoPEGylated RNase A and its purity , obtaining values of 65% and 98% respectively. Each *PEGylated* specie was collected, concentrated, lyophilized and stored at -4°C.

HIC purification.

For HIC purification, three different hydrophobic resins were tested: Butyl sepharose (weakly hydrophobic), Octyl sepharose (moderately hydrophobic) and Phenyl sepharose (strongly hydrophobic). Each *PEGylated* specie and unmodified RNase A was analyzed separately in HIC. Adsorption of proteins to HIC media is favoured by a high salt concentration, but due to differences in the interaction strength between the adsorbent and different proteins, the concentration of salt needed for adsorption can vary considerably [19]. Based on previous studies [14], high concentrations of ammonium sulphate (AS) were used in the initial experiments with the aim that unmodified RNase A was retained by the resins and desorbed in the gradient elution. As shown in Figure 5.2, applying a linear gradient (25 CV) of ammonium sulphate resulted in completed separation of unmodified protein from mono and *diPEGylated* RNase A with all resins. The *PEGylation* effect changes the hydrophobicity of the protein. However, it is not sufficient to obtain a high resolution separation of the PEGylated species. Although there exist some difference between retention volumes of *PEGylated* proteins when employing Octyl and Phenyl sepharose (Figure 5.1B and 5.1C), *diPEGylated* RNase A is completely included into the *monoPEGylated* RNase A. Namely, a stronger hydrophobic resin shows less resolution in the separation of *PEGylated* proteins. Moreover, it is observed that *PEGylated* proteins are not completely soluble at the concentrations of ammonium sulphate used in Figure 5.2, while the native protein is completely soluble. Müller *et. al.* [10] conducted a detailed study on the solubility of *PEGylated* conjugates of lysozyme, finding that the solubility in ammonium sulphate decreases with increased length of PEG chains linked to the protein. In our case the solubility decreases as the degree of *PEGylation* increases because it is using only a molecular weight of PEG. When Butyl sepharose is used (Figure 5.2A) a slight separation between the *PEGylated* proteins is observed, in fact this is the only profile where it is clear that the *monoPEGylated* protein is not totally pure. For this reason it was decided to refine the parameters of purification using Butyl sepharose.

The effect of AS concentration and gradient length on product separation is depicted in Figure 5.3. It is observed that decreasing the concentration of salt did not result in native protein retention on the resin; however, the separation of *PEGylated* proteins is better. When increasing the column volume the difference was more evident. It is clear that there exists a difference between the hydrophobicity of proteins as a result of the *PEGylation* process, in this case the less hydrophobic protein is the native protein whereas the most hydrophobic is *diPEGylated* RNase A. The best conditions for the separation of *PEGylated* proteins include the use of 1 M AS and gradient elution of 35 CVs (Figure 5.3D), under these conditions the native protein it is not retained by the adsorbent and all proteins are completely soluble in 1 M AS. It is not possible to work at a lower concentration than 1 M ammonium sulphate because all proteins are eluted in the dead time.

As mentioned before, the purity and yield were calculated using the plate model for the chromatograms shown in Figure 5.3. Calculations were performed focusing on the purity of *monoPEGylated* RNase A. Table 5.1 shows that whilst the purity remained practically unchanged, the yield increases with decreasing salt concentration (AS). Figure 5.4 clearly shows the fractionation of *PEGylated* proteins, using 1 M AS and 35 CVs. The use of these conditions resulted in a yield of up to 84.80% with a purity of 97%. Purity of *monoPEGylated* RNase A is comparable to that obtained when the separation is performed using SEC, but the yield increases from 65% with SEC to ~ 85% with HIC. On the other hand, Butyl sepharose is unable to retain the unmodified RNase A using 4M NaCl (data not shown). The *PEGylated* proteins appear immediately at the start of the elution gradient at the same retention volume (~10 mL).

Figure 5.5 shows the effect of sodium chloride concentration on product separation using Phenyl sepharose as adsorbent. The unmodified protein is not retained by the resin; nevertheless, the *PEGylated* proteins are strongly adsorbed with a slight change in their retention volumes (~3 mL Figure 5.5A and ~5.5 mL Figure 5.5B). The order of elution of the proteins remains equal to that obtained with Butyl sepharose - ammonium sulphate. Since the resolution of the peaks is lower, only two concentrations of NaCl were used with the same length of gradient elution (25 CVs). Unlike their behaviour with ammonium sulphate, in this case all proteins were completely soluble at the two concentrations of NaCl used, but that was not enough to achieve a definite separation between the *PEGylated* conjugates. Table 5.2 shows the yield and purity obtained

from the chromatograms of Figure 5.5. With purity similar to that obtained with Butyl sepharose - AS, the yield of *monoPEGylated* RNase A is between 46.9 and 57.5%.

Figure 5.6 shows the results obtained for the separation of the products of the *PEGylation* reaction (without using SEC) after selection of the best conditions for the separation of bioconjugates. The mixture was injected directly into the column packed with Butyl sepharose. This validated the results obtained individually for each product of the reaction.

Previously we reported that the separation of the reaction of *PEGylation* of RNase A using a Tris-capped CH Sepharose 4B media with salt gradient elution, resulted in the separation of only unmodified protein and the *PEGylated* conjugates eluted at virtually the same retention time [14]. Using hydrophobic resins as Butyl and Phenyl sepharose, it is possible to separate *PEGylated* proteins. It is noteworthy that the elution order is the same for the system reported by Cisneros-Ruiz *et. al.* [14] and mentioned here, regardless of the resin and / or salt used.

Müller and collaborators [10] reported the separation of reaction products of *PEGylation* of lysozyme with 5 kDa PEG, using a TSKgel Butyl-NPR column with 1.2 M ammonium sulphate; under these conditions, they achieved complete separation of the *PEGylated* conjugates from the unmodified lysozyme. Furthermore, contrary to our observations, the elution order changes depending on the type of salt. They inferred that the shift in the elution order can be explained by the different solubilities of the *PEGylated* lysozymes in the two different salt solutions (AS and NaCl). One possible explanation for this difference may be due to the length of the PEG and the intrinsic properties of the proteins.

The *PEGylated* protein separation using HIC has been little investigated, especially as a first step in the purification process. It has been suggested that SEC followed by IEX and then HIC may form the basis for a general approach for the purification of PEG-protein conjugates [4,20]. The results shown here suggest that HIC can be used as the first stage in the *PEGylated* protein purification; however, in order to propose the general application it is necessary to optimize the process and analyze the separation of other *monoPEGylated* proteins. The results obtained here can serve as a basis for modelling elution curves of *PEGylated* conjugates and to

facilitate the optimization of the purification process [18].

Conclusions

It is clear that HIC can be used to separate *monoPEGylated* RNase A, *diPEGylated* RNase A and native RNase A. Native RNase A could be separated completely from *PEGylated* proteins under all conditions assayed; demonstrating that the *PEGylation* affected the hydrophobicity of the protein, increasing it with increasing degree of *PEGylation*. The difference between the retention volumes of *PEGylated* proteins is higher when using a weak strength hydrophobic resin such as Butyl sepharose and ammonium sulphate mobile phase. While all proteins were soluble in sodium chloride; the resolution, yield, and purity were very low. The best conditions for the purification of *monoPEGylated* RNase A include the use of Butyl sepharose, 35 CV and 1 M ammonium sulphate; under these conditions, it is possible to obtain *monoPEGylated* RNase A with a yield as high as 85% and 97% purity.

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Figure Captions.

Figure 5.1 Size exclusion chromatography profile of PEGylated reaction mixture. Column: phacryl S-300 HP. Isocratic mobile phase: 10 mM sodium phosphate buffer pH 7.2 + 150 mM KCl. Flow rate: 0.5 mL/min. The vertical lines show where the fractions were collected.

Figure 5.2 Effect of type of resin in the purification of monoPEGylated RNase A on product separation. Buffer A: TRIS-HCl 20 mM pH 7.0 Buffer B: TRIS-HCl 20 mM pH 7.0 containing **2 or 2.5 M $(\text{NH}_4)_2\text{SO}_4$** . Loop: 100 μL , Flow rate: 0.8 mL/min. CV: 25. Gradient lineal from B to A. a) Butyl sepharose, 2.5 M $(\text{NH}_4)_2\text{SO}_4$; b) Octyl sepharose, 2.5 M $(\text{NH}_4)_2\text{SO}_4$; c) Phenyl sepharose, 2 M $(\text{NH}_4)_2\text{SO}_4$. Each PEGylated protein and unmodified RNase A was analyzed separately. The chromatograms were superimposed.

Figure 5.3 Effect of ammonium sulphate concentration and gradient length on product separation. Resin: Butyl sepharose. Buffer A: TRIS-HCl 20 mM pH 7.0 Buffer B: TRIS-HCl 20 mM pH 7.0 containing **2, 1.75, 1.5 or 1 M $(\text{NH}_4)_2\text{SO}_4$** . Loop: 100 μL , Flow rate: 0.8 mL/min. Gradient lineal from B to A. [monoPEG RNase A]: 1.5 mg/mL, [diPEG RNase A]: 0.5 mg/mL, [RNase A]: 0.5mg/mL. A) 2 M, 45 CV; B) 1.75 M, 35 CV; C) 1.5 M, 35 CV; D) 1 M, 35 CV. Each PEGylated protein and unmodified RNase A was analyzed separately. The chromatograms were superimposed.

Figure 5.4 Fractionation of monoPEGylated RNase A highly pure. Resin: Butyl sepharose. Buffer A: TRIS-HCl 20 mM pH 7.0 Buffer B: TRIS-HCl 20 mM pH 7.0 containing 1 M $(\text{NH}_4)_2\text{SO}_4$, 35 CV. Yield: 84.80%, Purity: 97%. Each PEGylated protein and unmodified RNase A was analyzed separately. The chromatograms were superimposed.

Figure 5.5 Effect of sodium chloride concentration on product separation. Resin: Phenyl sepharose. Buffer A: TRIS-HCl 20 mM pH 7.0 Buffer B: TRIS-HCl 20 mM pH 7.0 containing **2 or 2.5 M (NaCl)**. Loop: 100 μ L, flow: 0.8 mL/min. Gradient lineal from B to A. [monPEG RNase A]: 1.5 mg/mL, [diPEG RNase A]: 0.5 mg/mL, [RNase A]: 0.5mg/mL. A) 2 M, 25 CV; B) 2.5 M, 25 CV. Each PEGylated protein and unmodified RNase A was analyzed separately. The chromatograms were superimposed.

Figure 5.6 Separation of products of RNase A PEGylation reaction using HIC. Resin: Butyl sepharose. Buffer A: TRIS-HCl 20 mM pH 7.0 Buffer B: TRIS-HCl 20 mM pH 7.0 containing 1 M $(\text{NH}_4)_2\text{SO}_4$, 35 CV.

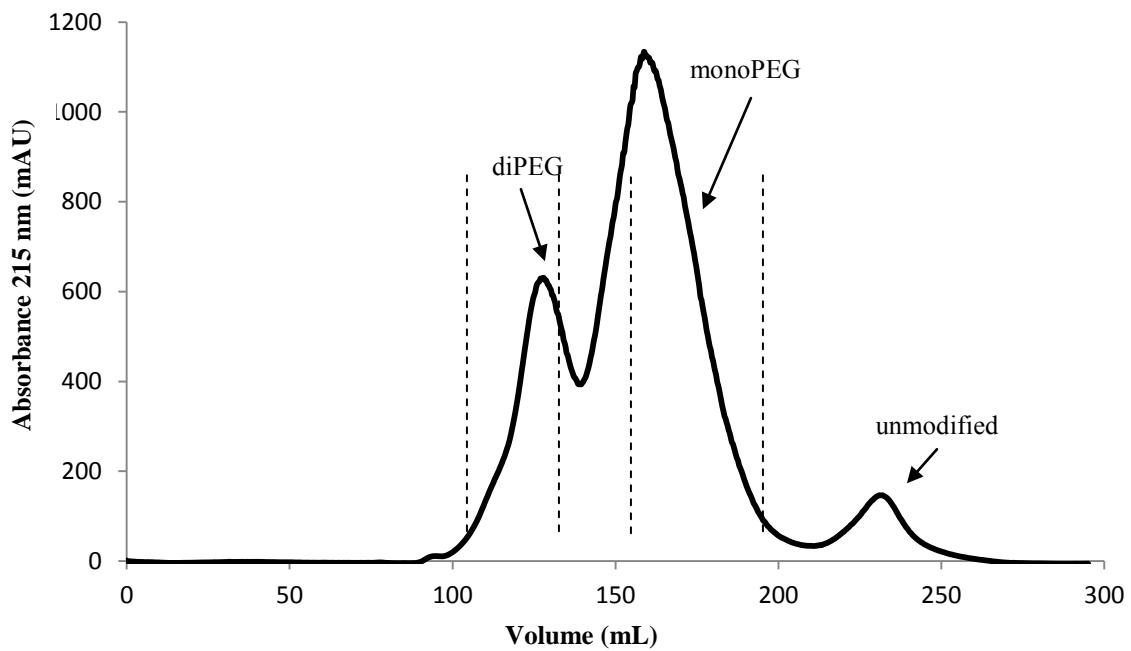
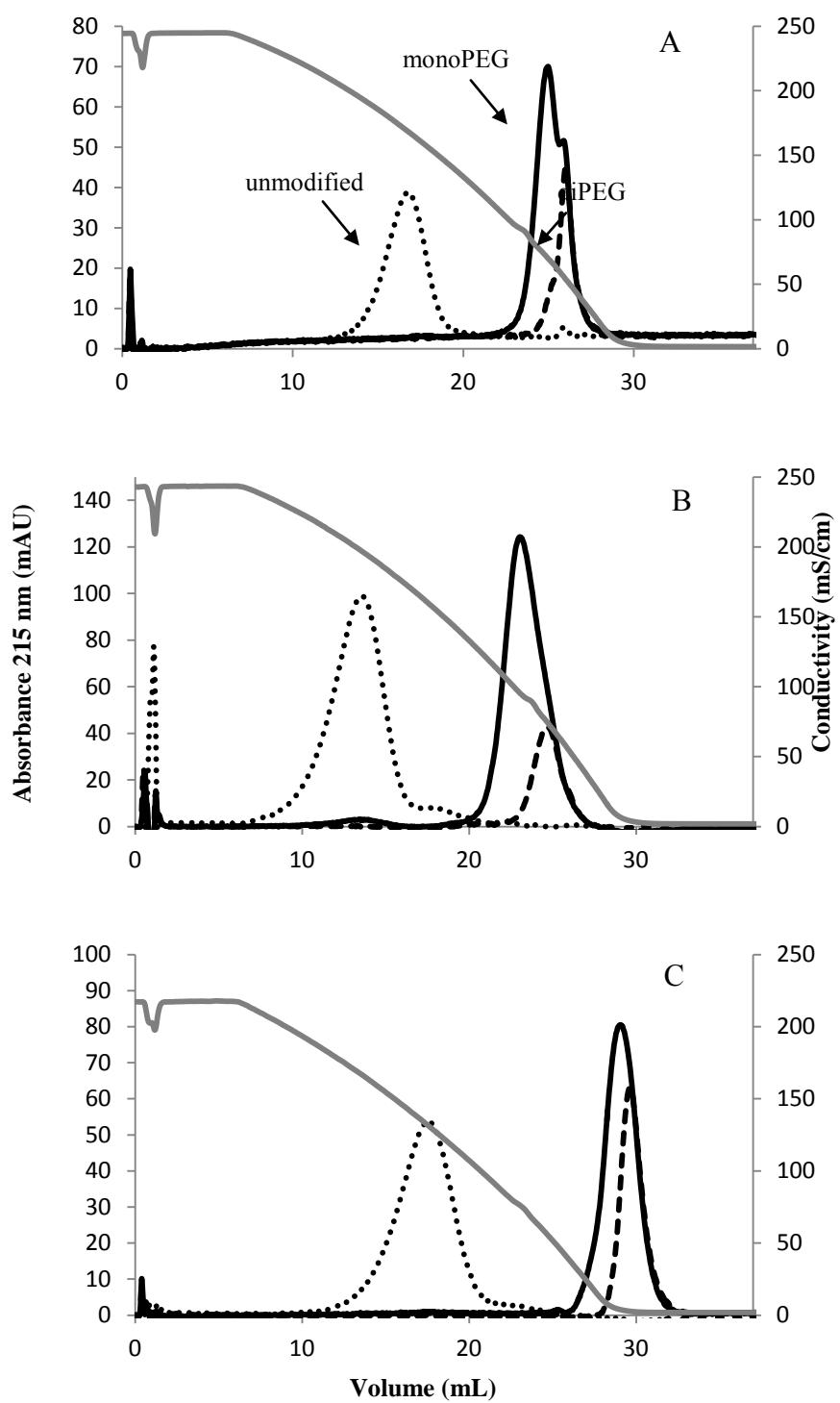


Figure 5.1

**Figure 5.2**

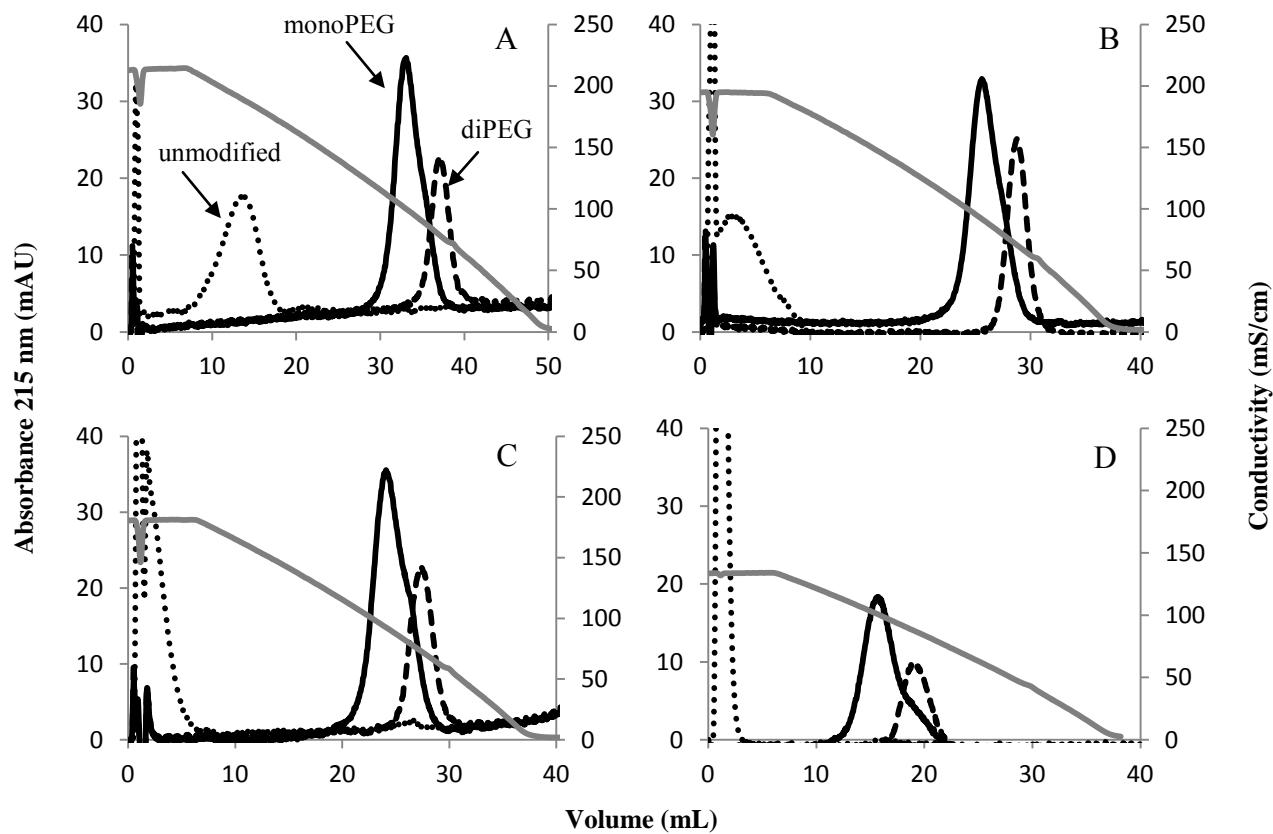


Figure 5.3

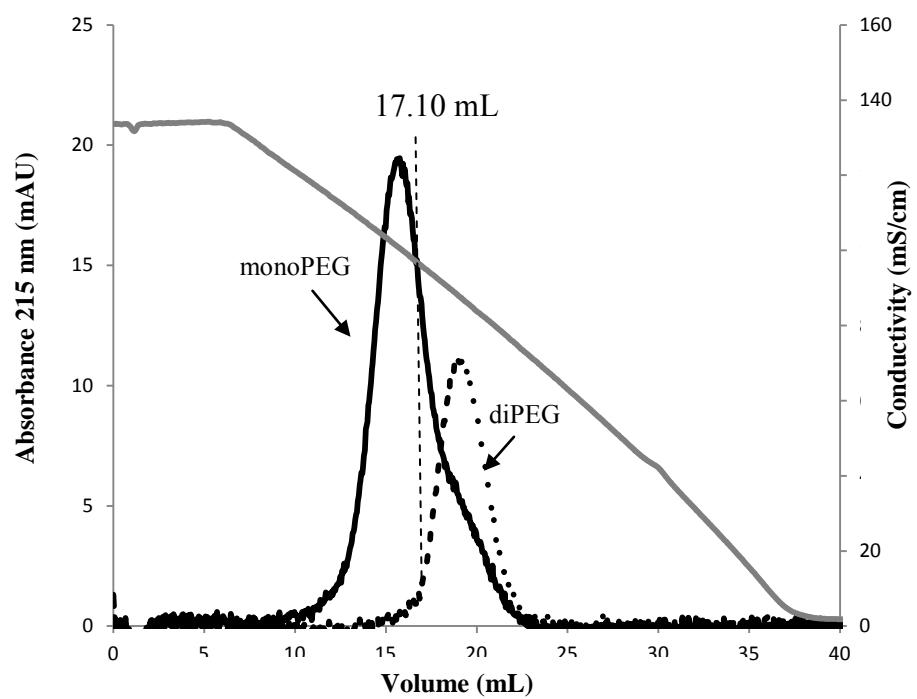


Figure 5.4

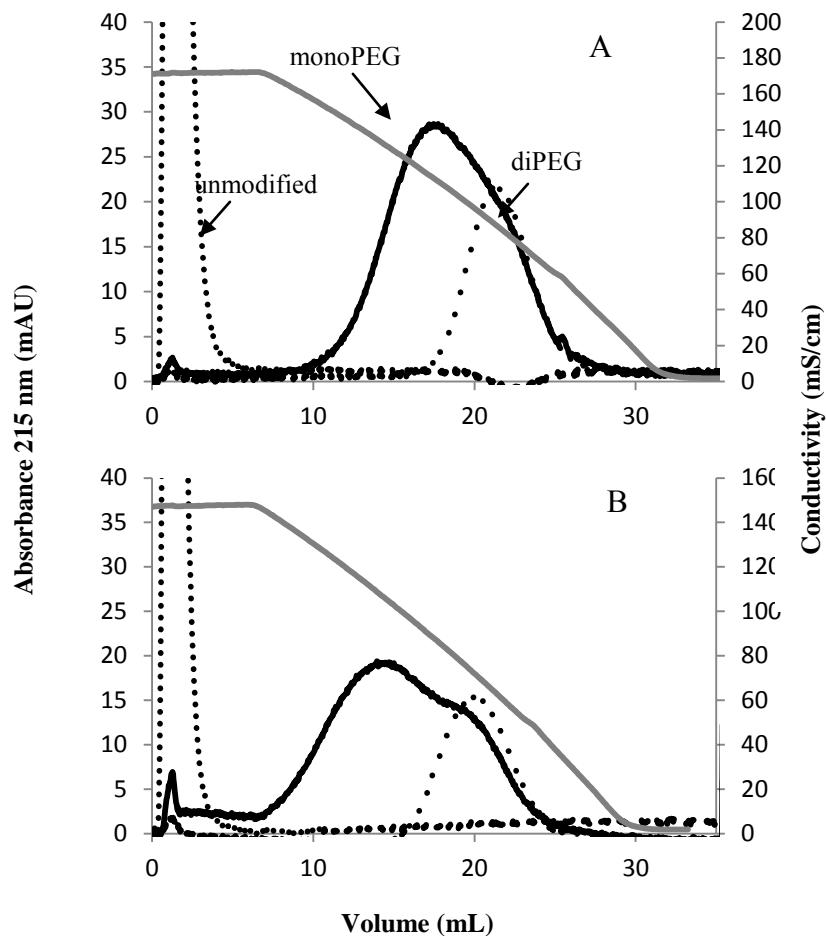


Figure 5.5

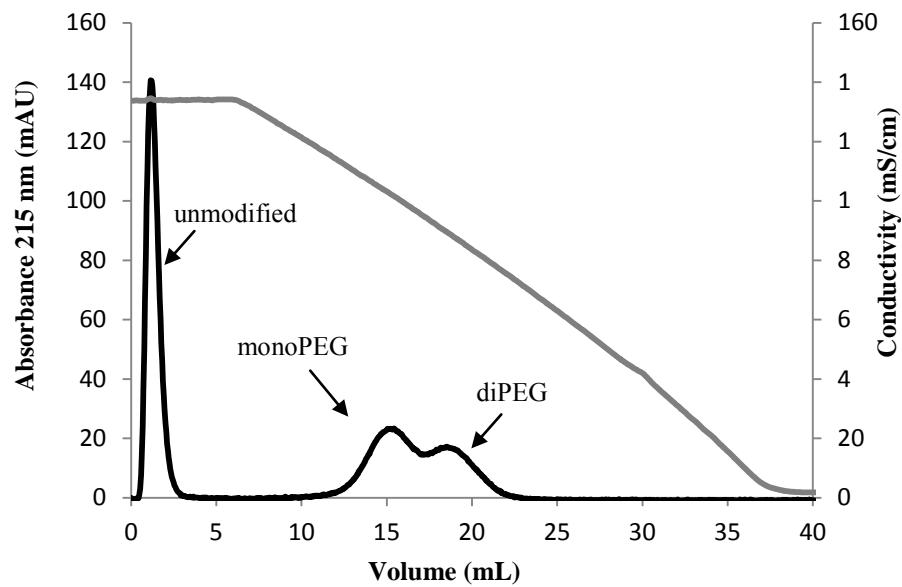


Figure 5.6

Tables

Table 5.1 Recovery and purity of *monoPEGylated* RNase A using Butyl sepharose and different concentrations of ammonium sulphate. Data were calculated from the chromatograms of Figure 5.3.

	AS (M)	CVs	Volume (mL)	Yield (%)¹	Purity (%)¹
Butyl sepharose	2.00	45	33.37	54.66	99.26
	1.75	35	26.80	62.30	98.50
	1.50	35	25.12	66.88	97.80
	1.00	35	17.10	84.80	97.00

AS: ammonium sulphate.

CVs: column volumes.

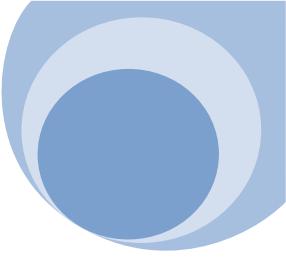
¹ Calculated using the plate model.

Table 5.2 Recovery and purity of *monoPEGylated* RNase A using Phenyl sepharose and sodium chloride. Data were calculated from the chromatograms of Figure 5.5.

			Volume (mL)	Yield (%)¹	Purity (%)¹
	NaCl (M)	CVs			
Phenyl sepharose	2.5	25	17.43	46.9	98.52
	2.0	25	15.06	57.5	97.62

¹Calculated using the plate model.

CVs: column volumes.



Capítulo 6.

Conclusiones y perspectivas

6

Conclusiones y perspectivas

Conclusiones.

La PEGilación es una técnica versátil que permite superar muchas de las limitaciones farmacológicas de las proteínas terapéuticas. Durante el desarrollo de esta técnica han surgido importantes avances en cuanto a la reacción de PEGilación, la generación de más bioconjugados, el entendimiento de su comportamiento y las estrategias para su purificación. Sin embargo, aún se presentan nuevos retos en materia de ingeniería, tanto en la preparación como en la purificación de las moléculas PEGiladas. Los productos aprobados por la FDA son una clara demostración del éxito de la PEGilación tanto en el mejoramiento de las propiedades de las proteínas terapéuticas como de su aplicación en el tratamiento de diversas enfermedades; sin embargo, aún hace falta profundizar sobre la aplicación de proteínas PEGiladas en diversas áreas de la biotecnología.

Los métodos cromatográficos de purificación clásicos (*v.gr.* SEC e IEC) han sido útiles pero no ofrecen una resolución óptima; siguen siendo procesos en varias etapas, de alto costo y con rendimientos bajos, además de que en casos muy específicos no ha sido posible separar los isómeros conformacionales.

Una revisión detallada de la bibliografía mostró que las técnicas no-cromatográficas (ultrafiltración, sistemas de dos fases acuosas, electroforesis, etc.) son muy útiles principalmente como herramientas para la caracterización de los productos de la reacción de PEGilación, sin que hasta ahora puedan competir directamente con los métodos de cromatográficos.

En este trabajo se demostró que la cromatografía de interacción hidrofóbica es una técnica de separación que puede ser implementada en la purificación de proteínas PEGiladas en base a su grado de PEGilación, utilizando como modelo RNasa A. Bajo cualquier condición

experimental (tipo de resina, concentración y tipo de sal, longitud del gradiente, etc.) fue posible separar la proteína nativa del resto de los productos de la reacción de PEGilación.

Cuando se utilizó una resina ligeramente hidrofóbica como la sefarosa 4B modificada fue posible separar únicamente la proteína nativa; mientras que entre las proteínas mono- y di-PEGiladas se apreció solo un ligero cambio en el tiempo de retención, que no es suficiente para separarlas. Los efectos del tipo y concentración de la sal, el pH y la adición de PEG 600 no contribuyeron a la separación de los bioconjungados.

El uso de resinas cromatográficas con ligandos más hidrofóbicos como butil, octil y fenil sefarosa permitió separar completamente la proteína nativa. Los efectos de la concentración de sulfato de amonio y la longitud del gradiente cuando se utiliza butil sefarosa impactaron positivamente en la purificación de RNasa A monoPEGilada; mientras que el efecto del tipo de sal utilizada no afectó el orden de elución de las proteínas. A pesar de la alta solubilidad de las proteínas PEGiladas en cloruro de sodio; la resolución, el rendimiento y la pureza obtenidos fueron bajos.

Las mejores condiciones obtenidas para la purificación de RNasa A monoPEGilada incluyen el uso de butil sefarosa, sulfato de amonio 1M y un gradiente de elución de 35 CVs; bajo dichas condiciones, es posible obtener RNasa A monoPEGilada con un rendimiento del 85% y una pureza del 97%.

Este es el primer trabajo en donde se reporta el uso de sefarosa 4B modificada y de resinas de interacción hidrofóbica para la purificación de RNasa A monoPEGilada, con resultados que pueden competir con la cromatografía de exclusión molecular. Con esto, HIC se convierte en una alternativa viable que puede ser implementada en la purificación de otras proteínas PEGiladas.

Perspectivas.

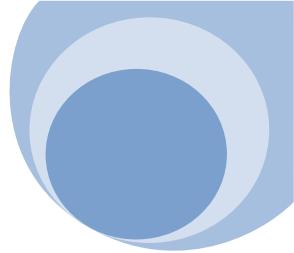
Derivado del análisis de este trabajo, se sugieren tres líneas de investigación que podrían servir como el trabajo de tesis de futuros candidatos doctorales; pues hasta ahora no han sido exploradas.

En primer lugar, los resultados obtenidos en este trabajo pueden ser utilizados directamente para la modelación matemática de las curvas de elución. El uso de modelos matemáticos para la optimización de separaciones cromatográficas puede reducir los costos y el tiempo de experimentación; pues permite simular y/o predecir el comportamiento de elución de una o varias proteínas durante su purificación. De manera que se pueden optimizar las condiciones experimentales como: el tipo de resina, la concentración ideal de la mezcla de proteínas, el tipo de sal, la concentración iónica del buffer de elución, la pendiente del gradiente de elución, etc. El modelo de Balance de Masas (*Rate model*) puede ser utilizado puesto que ya ha sido reportado para la simulación de curvas de elución de proteínas en cromatografía de interacción hidrofóbica. La aplicación de dicho modelo permitirá optimizar no solo el proceso de purificación de RNasa A monoPEGilada, sino el de otras proteínas PEGiladas adecuando las condiciones de operación del proceso.

Por otro lado, en este trabajo se utilizó un PEG lineal de 20 kDa para llevar a cabo la reacción de PEGilación; sin embargo, sería muy interesante analizar el efecto del tamaño y tipo de PEG (lineal o ramificado) sobre los procesos de purificación en HIC. Hasta ahora solo se conocen estudios sobre las implicaciones que el tamaño y el tipo de PEG tienen sobre el peso y el radio hidrodinámico de las proteínas PEGiladas cuando se purifican por SEC. La importancia de realizar un estudio de este tipo es observar si las diferencias en la conformación del PEG confieren cambios más marcados en la hidrofobicidad de las proteínas PEGiladas. Esto tendría implicaciones no solo en el proceso de purificación; sino también en el proceso de producción, pues ya se ha reportado que la PEGilación con PEG's ramificados aumenta aún más la vida media de las proteínas comparada con la PEGilación con PEG's lineales.

Adicionalmente, es necesario entender las interacciones moleculares entre las proteínas,

el PEG y las resinas cromatográficas; para ello es necesario llevar a cabo estudios de modelación de acoplamiento (*docking*) molecular. Este tipo de estudios con proteínas PEGiladas resulta interesante e innovador dado que hasta ahora se han realizado entre proteínas y un ligando pequeño, además de que no se conocen estudios de acoplamiento molecular entre proteínas PEGiladas y matrices cromatográficas. Para ello debe obtenerse primero la estructura tridimensional de las proteínas modelo y un archivo de ligandos potenciales (tanto de las resinas como el mismo PEG). El siguiente paso, deberá consistir en un algoritmo de computadora que tome cada uno de los ligandos de la base datos y lo coloque dentro del sitio de unión de la proteína en diferentes orientaciones. Finalmente, el programa de cómputo ordenará los diferentes ligandos probados de acuerdo al puntaje de su orientación óptima. Con respecto al software, pueden utilizarse los siguientes programas: *AUTODOCK*, que sirve para determinar la ubicación espacial adecuada de los ligandos dentro de los sitios de unión de la proteína; *AMBER* (*Assisted Model Building with Energy Refinement*), utilizado para la simulación de biomoléculas a través de un conjunto de campos de fuerza mecánica molecular; *GRAMM*, que puede servir como una herramienta complementaria para evaluar la interacción de las moléculas y *PyDock*, que podrá utilizarse para refinar los acoplamientos. El análisis computacional de las formas de unión entre los ligandos y las proteínas permitirá no solo adecuar los procesos de purificación de la proteína PEGilada de interés, sino también establecer nuevas reacciones de PEGilación.



Anexo 1.

Artículos publicados





PROTEÍNAS PEGILADAS: PRODUCCIÓN, PURIFICACIÓN Y APLICACIONES

PEGYLATED PROTEINS: PRODUCTION, PURIFICATION, AND APPLICATIONS

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Resumen

La PEGilación es la conjugación de una proteína y/o péptido con una o más moléculas de poli(etilen glicol). El poli(etilen glicol) es un polímero no tóxico, no inmunogénico y está aprobado por la FDA (Food and Drug Administration, USA). En los últimos años, la PEGilación ha sido utilizada para mejorar las propiedades fisicoquímicas de proteínas y drogas terapéuticas, por lo que esta tecnología ha impactado fuertemente a la industria bio-farmacéutica. La PEGilación permite prolongar el tiempo de residencia en el cuerpo, mejorar la estabilidad, aumentar la solubilidad, disminuir la proteólisis y excreción renal. Desde el surgimiento de esta tecnología, diferentes proteínas han sido PEGiladas para el tratamiento de enfermedades como: hepatitis C, leucemia, artritis reumatoide, etc. Este artículo de revisión presenta una descripción del desarrollo de la PEGilación en los últimos años, así como de los procedimientos usados para la producción de bio-conjugados. Además, se revisan las estrategias de purificación utilizadas para la recuperación de proteínas PEGiladas, siendo este uno de los grandes retos en el proceso debido a que la reacción de PEGilación puede generar bio-conjugados con diferentes grados de PEGilación. Por último, se presentan las aplicaciones de dichos bio-conjugados y los retos futuros que se identifican para su aplicación genérica.

Palabras clave: PEG, PEGilación, proteínas terapéuticas, bio-conjugados.

Abstract

PEGylation is the covalent attachment of protein and/or peptide to poly(ethylene glycol). The poly(ethylene glycol) is a polymer, non toxic, non immunogenic, and FDA (Food and Drug Administration, USA) approved. In the last years, PEGylation has been used to improve the physicochemical properties of some proteins and therapeutic drugs; this technology has impacted heavily on the bio-pharmaceutical industry. PEGylation prolongs the body-residence time and stability, decreases the proteolysis and renal excretion. Since the emergence of this technology, some proteins have been PEGylated for the treatment of diseases including hepatitis C, leukemia, rheumatoid arthritis, etc. This review presents a description of the PEGylation development in the last years and the chemical procedures used to obtain some bio-conjugated products. Strategies of purification used to obtain PEGylated proteins are reviewed; purification is one of the major problems to establish suitable processes due to the fact that the reaction can generate bio-conjugates with different degree of PEGylation. Finally the applications of PEGylated proteins and the future challenges that are identified for generic application are presented.

Keywords: PEG, PEGylation, therapeutic proteins, bio-conjugates.

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1. Introducción

La revolución biotecnológica y nanotecnológica ha producido novedosos péptidos y proteínas que están siendo utilizados como nuevas drogas para el tratamiento del cáncer y de diversas enfermedades (Harris y Chess, 2003; Parveen y Sahoo, 2006). Algunas técnicas han sido desarrolladas para mejorar las propiedades terapéuticas de dichas macromoléculas, éstas incluyen la alteración de la secuencia de aminoácidos para reducir su degradación o la fusión de péptidos con immunoglobulina o albúmina para incrementar la vida media. Hasta ahora, la técnica más exitosa ha sido la conjugación de péptidos y/o proteínas a una o varias cadenas de poli(etilen glicol) (PEG), llamada PEGilación (Ryan *y col.*, 2008).

El término PEGilación ha sido utilizado desde 1977 después de que Abuchowsky y colaboradores describieran por primera vez un método para adherir covalentemente una o varias moléculas de PEG a una proteína (Abuchowsky *y col.*, 1977). El PEG es un poliéter lineal o ramificado con un grupo hidroxilo en cada extremo (Fig. 1), este polímero es altamente soluble en agua así como en varios solventes orgánicos y está aprobado por la FDA para su administración en seres humanos (Morar *y col.*, 2006; Wattendorf y Merkle, 2008). Muchos de los beneficios de la PEGilación de proteínas están ligados a las propiedades del PEG. El PEG es inerte, no tóxico y no immunogénico, además es fácilmente desechado por el cuerpo a través del riñón (pesos moleculares del polímero menores a 20 kDa), o del hígado (pesos moleculares arriba de 20 kDa) (Morar *y col.*, 2006).

La conjugación de PEG con una proteína generalmente mejora sus propiedades debido a que aumenta su vida media, causa una reducción del reconocimiento de la proteína por el sistema inmune, aumenta su resistencia al ataque proteolítico, aumenta su solubilidad y estabilidad (Fig. 2). La mayoría de estos fenómenos pueden ser explicados debido a la expansión del radio hidrodinámico del conjugado proteína-PEG como un resultado de la capacidad del PEG de coordinar numerosas moléculas de agua y de la alta flexibilidad de la cadena polimérica (Gaberc-Porekar *y col.*, 2008).

Existen varios métodos químicos y enzimáticos para llevar a cabo la PEGilación (Veronese y Pasut, 2005). El primer paso en el proceso es la “activación” de la molécula de PEG, la modificación de PEG más utilizada es el metoxi-PEG (mPEG) (Fig. 1) (Hamidi *y col.*, 2006). El PEG activado puede ser ligado a un sitio específico de las proteínas, frecuentemente sobre un grupo amino,

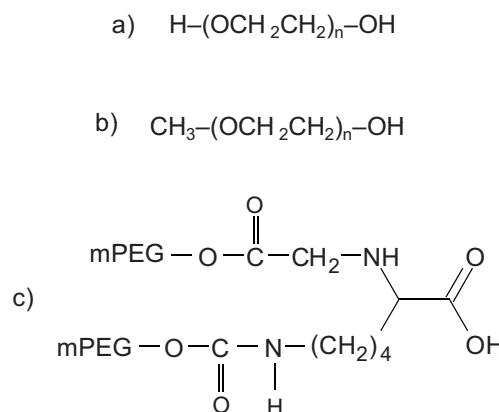


Fig. 1: Fórmulas estructurales del poli(etilen)glicol (PEG). a) PEG, b) metoxi-poli(etilen glicol) lineal (mPEG) y c) mPEG ramificado (tomado de Hamidi *y col.*, 2006).

sulfidrilo u otro grupo nucleofílico. En muchos casos, el sitio preferido para la modificación es el grupo amino de la lisina o el grupo amino N-terminal de la cadena polipeptídica (Veronese y Pasut, 2005; Hamidi *y col.*, 2006). Sin embargo, la PEGilación del grupo amino genera un alto número de isómeros lo que dificulta en gran medida el siguiente paso en el proceso de PEGilación, la purificación de los conjugados (Veronese y Pasut, 2005). Debido a los costos extremadamente altos de los procesos de producción de proteínas terapéuticas, uno de los retos en la ingeniería de la reacción de PEGilación es generar reacciones sitio-específicas lo más eficientes posibles, que produzcan un solo conjugado sin alterar las propiedades fisicoquímicas de la proteína de interés.

La purificación de proteínas PEGiladas envuelve la remoción de todas las especies moleculares que no sean parte del producto de interés, que pueden incluir a la proteína no modificada y a la proteína con diferentes grados de PEGilación (mono-, di-, tri-, etc.). Actualmente los procesos de purificación de proteínas PEGiladas están dominados por la cromatografía de exclusión molecular e intercambio iónico. Otros métodos también han sido utilizados aunque con menor frecuencia, ejemplos de ellos son la cromatografía en fase reversa y la cromatografía de interacción hidrofóbica, ultrafiltración, electroforesis, electroforesis capilar, diáisisis, sistemas de dos fases acuosas, etc. (Delgado *y col.*, 1997; Fee y Van Alstine, 2006).

Desde el surgimiento de la PEGilación, un gran número de proteínas han sido PEGiladas en ellas se incluyen: factores de crecimiento, adenosin des-

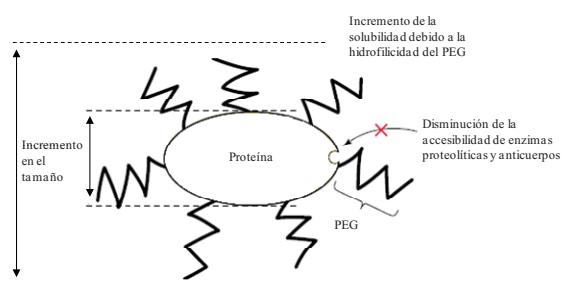


Fig. 2: Ventajas de la PEGilación. La figura representa la conjugación de una proteína con varias moléculas de PEG (modificado de Veronese y Pasut, 2005).

aminasa, asparaginasa, interferones, ribonucleasa A, albúmina de suero bovino, α -lactoalbúmina, entre otras. Muchas de ellas están siendo utilizadas en el tratamiento de enfermedades como: leucemia, artritis reumatoide, hepatitis C, acromegalía, etc. (Veronese y Pasut, 2005; Gaberc-Porekar *y col.*, 2008).

A pesar de que en los últimos años los trabajos en el desarrollo de los procesos de PEGilación se han intensificado notablemente, aún no se han encontrado métodos eficientes que eleven el rendimiento en la recuperación de los productos bio-conjugados y disminuyan los altos costos de producción. La ingeniería química y bioquímica juegan un papel fundamental en el diseño de reacciones en donde se controle el sitio de PEGilación de manera que se evite la generación de proteínas multiPEGiladas. Otra área de oportunidad es la ingeniería de bioseparaciones de proteínas PEGiladas, puesto que aún no se han encontrado procesos que puedan ser aplicados de manera genérica en la etapa de purificación.

El objetivo de este artículo es el de presentar un panorama general del estado del arte en cuanto a la reacción de PEGilación, las estrategias de purificación utilizadas para la recuperación de proteínas PEGiladas, las aplicaciones y los retos futuros que se identifican en el desarrollo de dichos conjugados proteína-polímero.

2. Reacción de PEGilación: producción de proteínas PEGiladas

Para llevar a cabo la reacción de PEGilación, se deben tomar en cuenta varios factores que incluyen el objetivo por el cual una proteína debe de ser PEGilada. La estructura y el tamaño del

PEG son dos variables que limitan el proceso; por ejemplo, PEGs ramificados incrementan el peso molecular de la proteína mono-PEGilada, pero también pueden limitar la disponibilidad estérica del sitio de PEGilación. Además, otros factores como el tiempo de reacción, pH, temperatura, concentración de PEG y proteína deben ser tomados en cuenta (Gaberc-Porekar *y col.*, 2008).

En una reacción típica, un PEG activado se hace reaccionar con uno o más residuos de lisina o con el grupo amino N-terminal. La PEGilación de otros sitios nucleofílicos tales como cisteína, histidina, arginina o tirosina también son posibles. Por otro lado, es posible utilizar enzimas que se encarguen de la conjugación de la proteína con el PEG.

Al llevar a cabo la reacción, la solución de proteína es mezclada con el PEG activado bajo condiciones de pH, temperatura y agitación controladas. Las moléculas de proteína mono-PEGiladas con los sitios más reactivos son las primeras en formarse, los sitios menos reactivos forman las especies di-PEGiladas y así sucesivamente (Morar *y col.*, 2006).

2.1. Modificación del grupo amino

La modificación química más común para llevar a cabo la reacción de PEGilación se da en el grupo ϵ -amino de los residuos de lisina, a través de alquilación o acilación (Fee y Van Alstine, 2006; Veronese y Mero, 2008). La alquilación mantiene la carga positiva del grupo amino, mientras que la acilación genera una pérdida de la carga debido a la formación de una amida (Veronese y Mero, 2008). En una proteína típica las lisinas constituyen el 10 % del total de los aminoácidos, su disponibilidad hace que la conjugación sea sencilla; sin embargo, el gran número de sitios presentes para la conjugación dificulta la posibilidad de obtener un número específico de aductos por lo que es muy común que se generen mezclas de PEGámeros. Una forma de controlar la reacción es cambiar el pH, a valores altos de pH (arriba de 8.0) se favorece la conjugación con los grupos ϵ -amino de las lisinas presentes, mientras que una reacción a pH ácido favorece el enlace con el grupo amino N-terminal (Gaberc-Porekar *y col.*, 2008). Los agentes para la modificación de grupos amino incluyen: mPEG-diclorotriazina, mPEG-tresilato, mPEG-succimidil carbonato, mPEG-N-hidroxisuccimida, mPEG-propilaldehído, mPEG-p-nitrofenil-carbonato, etc. (Roberts *y col.*, 2002; Veronese y Mero, 2008). En la Fig. 3, se muestra una de las reacciones más utilizadas para llevar a

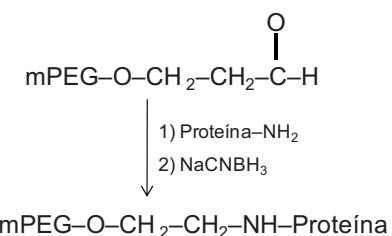


Fig. 3: Modificación del grupo amino que mantiene la carga positiva del residuo de la proteína (tomada de Veronese y Mero, 2008).

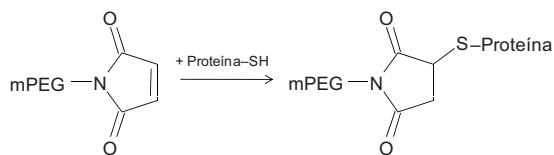


Fig. 4: Modificación sitio-específica de cisteína utilizando mPEG-maleimido (tomada de Veronese y Mero, 2008).

cabo la modificación del grupo amino N-terminal, en dicha reacción el aldehído interacciona con la amina para producir una base de Schiff que finalmente es reducida a una amina secundaria estable (Veronese y Mero, 2008).

2.2. Modificación de residuos de cisteínas

Los polímeros utilizados para la modificación de cisteínas incluyen a: mPEG-maleimido (Fig. 4), mPEG-iodoacetato, mPEG-tiol, mPEG-vinilsulfona y mPEG-piridildisulfido. La PEGilación sitio-específica de residuos de cisteína rara vez se lleva a cabo, debido a que este aminoácido, cuando está presente, se encuentra participando en los enlaces disulfuro o es requerido para la actividad biológica (Veronese y Mero, 2008). En ausencia de cisteínas libres en la proteína nativa, una o más cisteínas pueden ser insertadas por ingeniería genética; sin embargo, en ocasiones se pueden generar puentes disulfuro incorrectos y por lo tanto la dimerización de la proteína (Roberts *y col.*, 2002; Veronese y Mero 2008).

2.3. PEGilación específica utilizando enzimas

La conjugación específica de PEG al grupo amino de una glutamina o al grupo hidroxilo de las serinas y treoninas es solo posible utilizando enzimas. Existen enzimas que reconocen a la glutam-

ina como sustrato, llamadas transglutaminasas (Veronese y Pasut, 2005). Sato (2002) reportó que la glutamina de las proteínas puede ser el sustrato de la enzima transglutaminasa, si un PEG-amino es usado como donador nucleofílico, por lo que el PEG puede ser ligado a la proteína a través de un residuo de glutamina.

La reacción para la producción de proteínas PEGiladas juega un papel fundamental en el proceso de PEGilación, los productos que se obtienen son a) la proteína en sus diferentes grados de PEGilación, b) el PEG en exceso y c) la proteína que no logró reaccionar. El conocimiento de la estructura primaria de la proteína es fundamental, el uso de herramientas como la bioinformática pueden ayudar a predecir los sitios de PEGilación y las posibles consecuencias sobre la estructura tridimensional, además de facilitar el proceso de purificación. A pesar de que la PEGilación de cisteínas genera mezclas menos complejas, sigue siendo más utilizada la PEGilación de los grupos amino debido a que las cisteínas son aminoácidos que generalmente participan en el sitio activo o en la conformación de la estructura tridimensional, lo que en muchas ocasiones afecta negativamente a la proteína. El diseño de la reacción debe de ser específico para la proteína de interés, dependiendo de sus propiedades fisicoquímicas y de su aplicación.

3. Estrategias de purificación de proteínas PEGiladas

La purificación de proteínas PEGiladas consiste en remover todas las especies que no formen parte del producto de interés, lo que involucra dos retos principalmente: 1) la separación de las proteínas PEGiladas del resto de los productos de la reacción y 2) el sub-fraccionamiento de las proteínas PEGiladas en base al grado de PEGilación y a los isómeros posicionales o PEGámeros. La purificación se complica debido a que no solo se deben tomar en cuenta las características de la proteína, también la naturaleza amfipática del PEG afecta fuertemente al proceso de separación (Fee y Van Alstine, 2006). En la Tabla 1 se muestran algunos de los métodos utilizados para la separación de proteínas PEGiladas. En esta Tabla 1 es evidente que PEG de distintos pesos moleculares han sido utilizados para la obtención de productos PEGilados. Adicionalmente, se ha documentado que para la recuperación de las proteínas PEGiladas, métodos cromatográficos y no-cromatográficos han demostrado su factibilidad.

Tabla 1. Métodos de separación utilizados para la purificación de proteínas PEGiladas.

Proteína	PEG (PM, kDa)	Método(s) de purificación	Referencia
Factor de crecimiento epidérmico	3.4	Exclusión molecular , fase reversa y ultrafiltración	Lee y Park, (2002)
Factor de crecimiento epidérmico	2.5	Diálisis y liofilización	Kim y col., (2002)
α -interferon 2b PEG Intron®(Shering-Plough)	12	Intercambio catiónico y exclusión molecular	Wang y col., (2002)
α -interferon 2a PEG-asys®(Hoffman-La Roche Inc.)	40	Intercambio catiónico	Reddy y col., (2002)
β -interferon	20	Intercambio catiónico, ultrafiltración y exclusión molecular	Arduini y col., (2004)
Insulina	750 Da	Exclusión molecular	Calctei y col., (2004)
α -lactoalbúmina	2,5	Exclusión molecular	Fee y Van Alstine (2004)
β -lactoglobulina	10		
Albúmina de suero bovino	20, 40		
Hemoglobina	5	Exclusión molecular y fase reversa	Li y col., (2006)
Insulina	2 y 5	Diálisis, intercarbio catiónico y fase reversa	Dou y col., (2007)
Ribonucleasa A	20	Interacción hidrofóbica	Cisneros-Ruiz y col., (2009)
Factor estimulador de colonias de granulocitos	10, 20, 30	Exclusión molecular, fase reversa e intercambio iónico.	Zhai y col., (2009)

PM, peso molecular

3.1. Métodos cromatográficos

Históricamente, la cromatografía de exclusión molecular (*Size Exclusion Chromatography*, SEC) ha sido ampliamente usada para la separación de productos PEGilados debido al significativo incremento del radio hidrodinámico de los conjugados comparado con las especies nativas. El poder de resolución de la cromatografía de exclusión para diferentes especies PEGiladas no es muy alto (Fig. 5). Esta técnica es inherentemente inadecuada para resolver mezclas de isómeros que tienen el mismo número de cadenas de PEG ligadas a la proteína, pero en diferentes sitios (Garberc-Porekar y col., 2008).

La cromatografía de intercambio iónico (*Ion-Exchange Chromatography*, IEC) ofrece la posibilidad de efectuar la separación del PEG, la proteína nativa y las especies PEGiladas en un solo paso. Debido a esto, IEC es comúnmente utilizada para separar proteínas PEGiladas; sin embargo, el método requiere ser optimizado. Para ello, debe tomarse en cuenta que el PEG es un polímero neutral pero puede afectar la carga de las proteínas

en tres maneras diferentes. Primero, la presencia del PEG conjugado puede proteger la carga superficial de la proteína y de este modo debilitar el enlace con la resina de intercambio iónico. Segundo, la conjugación a residuos de aminoácidos que altera la carga neta de la proteína o cambia a ciertos valores de pH altera la carga potencial y por lo tanto el punto isoeléctrico (pI). Tercero, la superficie de la proteína en donde se localiza el PEG puede formar puentes de hidrógeno (Fee y Van Alstine, 2006).

La cromatografía de intercambio catiónico es especialmente ventajosa y parece ser el método más fino para separar mezclas de PEGilados (Kinstler y col., 2002; Fee y Van Alstine, 2006; Garberc-Porekar y col., 2008). En la PEGilación aleatoria (básicamente grupos amino), el orden usual es que eluyen primero las especies altamente PEGiladas, después las di-PEGiladas seguidas de las mono-PEGiladas, la proteína no PEGilada eluye al final; sin embargo, el mismo orden de elución se puede obtener cuando se requiere separar proteínas PEGiladas sobre un residuo de cisteína

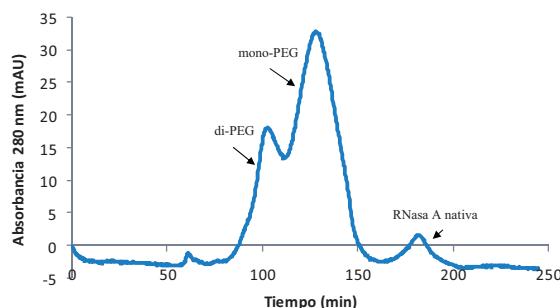


Fig. 5: Separación de Ribonucleasa A nativa de sus formas mono- y di-PEGiladas utilizando Cromatografía de Exclusión Molecular (tomado de Cisneros-Ruiz, 2006).

(Seely *y col.*, 2005). Aunque la cromatografía de intercambio catiónico es altamente efectiva para resolver mezclas complejas de proteínas PEGiladas, cargar demasiado la columna puede disminuir la resolución. La mayoría de los espacios de la columna están ocupados por el PEG resultando en una baja capacidad del medio en términos de masa de proteína por volumen de resina. El tiempo de vida útil del medio cromatográfico usado es relativamente corto, por lo que se requiere empacar la columna en repetidas ocasiones. Todos estos factores contribuyen a generar altos costos de separación, a pesar de el uso de resinas de intercambio iónico relativamente económicas (Garberc-Porekar *y col.*, 2008).

La cromatografía de fase reversa (*Reverse Phase Chromatography*, RPC) es una opción atractiva para la resolución de conjugados proteína-polímero debido a que la cadena del PEG sobresale del conjugado actuando como un sitio hidrofóbico y de esa manera dominar la interacción con la superficie hidrofóbica (Daly *y col.*, 2005; Cisneros-Ruiz, 2006). Sin embargo, RPC puede estar limitada por diferentes factores, incluyendo cambios estructurales en los productos proteicos de interés, además de bajos niveles de recuperación debido a la desnaturización causada por el uso de solventes orgánicos (Cisneros-Ruiz, 2006).

La cromatografía de interacción hidrofóbica (*Hydrophobic Interaction Chromatography*, HIC) ha sido aplicada con menos frecuencia para la separación de proteínas PEGiladas. Esta técnica generalmente trabaja pobemente debido a que el PEG por sí mismo también se liga al medio, lo que interviene en la separación (Fee y Van Alstine, 2006; Garberc-Porekar *y col.*, 2008). Cisneros-Ruiz y colaboradores (2009) reportaron que bajo ciertas condiciones es posible separar ribonucleasa A nativa de sus especies PEGiladas; sin embargo, no

es posible separar las proteína mono-PEGilada de la di-PEGilada. Algunos autores consideran que esta técnica no ha sido debidamente explotada, por lo que es necesario realizar más investigación al respecto (Fee y Van Alstine, 2006).

En términos generales, las técnicas cromatográficas clásicas para la separación de proteínas, no ofrecen de manera individual un desempeño óptimo para la purificación de conjugados proteína-polímero. En muchas ocasiones es necesario utilizar un conjunto de las técnicas antes mencionadas, algunos autores han sugerido que la cromatografía de exclusión molecular seguida por intercambio iónico e interacción hidrofóbica podrían ser la mejor propuesta de una aplicación genérica para la purificación de proteínas PEGiladas (Fee y Van Alstine, 2006; Garberc-Porekar *y col.*, 2008).

3.2. Métodos no cromatográficos

El incremento en tamaño de los conjugados proteína-PEG ha sido explotado para llevar a cabo su separación haciendo uso de membranas de ultrafiltración. Con mayor frecuencia, dicha técnica ha sido empleada para remover el agua y la solución buffer del resto de los componentes de la reacción de PEGilación; sin embargo, también es posible separar la proteína nativa de las moléculas PEGiladas así como retirar el PEG remanente (Lee y Park, 2002; Pabst *y col.*, 2007; Molek y Zydny, 2007), además ha sido examinado su uso potencial para la separación de los conjugados de manera cuantitativa. El diseño y optimización para la aplicación de la ultrafiltración en la recuperación de proteínas PEGiladas requiere de la adecuada selección del tamaño de poro de la membrana, el pH, fuerza iónica y el flujo del filtrado (Molek y Zydny, 2007).

Sistemas de dos fases acuosas (SDFA) también han sido utilizados para la separación de proteínas PEGiladas; sin embargo, reportes que documenten la caracterización de proteínas PEGiladas en estos sistemas no son muy comunes. SDFA es un método de separación líquido-líquido en donde la separación está basada en la diferencia de partición de los solutos entre las fases (Rito-Palomares, 2004), que se visualiza como una alternativa atractiva para la recuperación de proteínas PEGiladas. En este contexto, estudios previos mostraron que los conjugados PEG-proteína de las proteínas albúmina, factor de estimulación de colonias de granulocitos y macrofágos e inmunoglobulina G, se comportan diferente que sus equivalentes proteínas nativas en sistemas que utilizan PEG y dextrano

como formadores de fases. Los resultados muestran que el coeficiente de partición (K) incrementa con el número de moléculas de PEG ligadas a la proteína (Delgado *y col.*, 1994; Delgado *y col.*, 1997). Si bien estos estudios demostraron el potencial de utilizar S DFA para la recuperación de conjugados proteína-PEG, la falta de una caracterización extensa del comportamiento de partición es evidente. Alternativamente, Sookkumnerd y Hsu (2000) explotaron la distribución a contracorriente en sistemas de dos fases acuosas (PEG-fosfatos) como técnica para purificar conjugados PEG-lisozima. Los resultados de esta investigación mostraron que a través de esta técnica es posible separar cada una de las especies PEGiladas y la proteína nativa. A pesar de los estudios realizados, una extensa caracterización de los conjugados proteína-polímero utilizando estrategias que explotan los mecanismos de partición en dos fases acuosas es necesaria.

Como se ha podido apreciar, se han realizado diversos esfuerzos por mejorar los procesos de purificación de proteínas PEGiladas, la mayoría de ellos utilizando métodos cromatográficos. Sin embargo, los procesos siguen llevándose a cabo en varias etapas, lo que prolonga el tiempo de recuperación de los bio-conjugados e impacta negativamente en el rendimiento del proceso de PEGilación. Es en este punto donde herramientas de ingeniería química y bioquímica puede ser de gran impacto en el desarrollo de métodos de separación más rápidos y efectivos. Lo ideal sería el diseño de procesos con dos etapas, una de recuperación primaria (utilizando métodos como ultrafiltración, diálisis o sistemas de dos fases acuosas) en donde se separen el PEG y la proteína que no reaccionaron y una segunda etapa (cromatográfica) que permita la separación de los diferentes bio-conjugados (mono-PEGilados, di-PEGilados, etc.).

Aunque los procesos cromatográficos son los más utilizados en la separación de bio-conjugados, hasta ahora no se conocen modelos matemáticos que ayuden a predecir el comportamiento de las proteínas PEGiladas en el proceso de purificación, lo cual sería de gran ayuda en la optimización e intensificación de los métodos de separación.

4. Aplicaciones, tendencias y retos futuros

Diferentes clases de drogas proteicas, como enzimas, citoquinas y anticuerpos han sido significativamente mejoradas debido al proceso de PEGi-

lación. La Tabla 2 compila los ejemplos más importantes de bio-conjugados aprobados por la FDA, que explotando las ventajas de la PEGilación, han sido utilizados en la terapia de diversas enfermedades (Veronese y Pasut, 2005; Fishburn, 2008). PEG-amadasa bovina (PEG-adenosin deaminasa, Adagen®, Enzon Inc.) fue la primera proteína PEGilada en ser comercializada satisfactoriamente. Fue aprobada por la FDA en 1990 para tratar la enfermedad de inmunodeficiencia combinada severa (*Severe Combined Immunodeficiency*, SCID). Adenosin desaminasa fue PEGilada aleatoriamente con PEG 5 kDa para extender el tiempo que permanece en el plasma y reducir su immunogenicidad. Este fue un paso predominante en el desarrollo de la PEGilación porque se demostró por primera vez la viabilidad de esta tecnología (Veronese y Mero, 2008).

La segunda proteína biológicamente activa en ser conjugada con PEG fue L-asparaginasa (PEGaspargasa; Oncaspar®, Enzon Inc.). PEGaspargasa fue aprobada por la FDA en 1994 para pacientes en los cuales la proteína no modificada (o nativa) provocaba una reacción alérgica. La conjugación de la proteína con múltiples cadenas de PEG 5 kDa incrementó el tiempo de eliminación tres veces más comparado con la proteína nativa. El producto PEGilado fue tan efectivo como la droga nativa en el tratamiento de pacientes con leucemia linfoblástica aguda, además mostró un bajo grado de immunogenicidad (Graham, 2003).

El factor de crecimiento de colonias de granulocitos (*Granulocyte Colony-Stimulating Factor*, G-CSF) es el mayor regulador de la granulopoyesis in vivo. Su tiempo de vida media es relativamente corto (3.5-3.8 h) por lo que a diario se requieren de múltiples dosis. El conjugado PEG-G-CSF, pegfilgrastim (Neulasta®, Amgen Inc.), fue producido por la unión de una molécula de PEG 20 kDa a el grupo α -amino del residuo N-terminal de metionina. Pegfilgrastim permanece en el plasma el tiempo suficiente para permitir una simple inyección subcutánea para tratamientos de quimioterapia (Kinstler *y col.*, 2002; Veronese y Mero, 2008).

Los interferones combinados con ribavirina son los tratamientos más usados para tratar infecciones virales, en su forma nativa, tienen un tiempo de vida muy corto (4-5 h). PEG-interferón α 2a (Pega-sys®, Hoffman La Roche Inc.) fue obtenido por el acoplamiento covalente de PEG-N-hidroxisuccimida 40 kDa a un residuo de lisina. Dicha reacción produce una mezcla de cuatro isómeros mono-PEGilados en Lys³¹, Lys²¹, Lys¹³¹ y Lys¹³⁴. Recientemente, los isómeros de PEG-in-

Tabla 2. Conjugados proteína-polímero aprobados por la FDA.

Nombre comercial	Conjugado PEG - proteína	PM PEG (kDa)	Indicación	Año de aprobación
Andagen®	PEG-adenosin deaminasa	5	SCID	1990
Oncaspar®	PEG-asparaginasasa	5	Leucemia	1994
PEG-Intron®	PEG-interferon α 2b	12	Hepatitis C	2001
Pegasys®	PEG-interferon α 2a	40	Hepatitis C	2002
Neulasta®	PEG-G-CSF	20	Neutropenia	2002
Somavert®	PEG-GH (antagonista)	4-5 x 5	Acromegalia	2003
Mircera®	PEG-eritropoyetina	40	Anemia	2007
Cimzia®	PEG-TNF	40	Artritis reumatoide y enfermedad de Crohn	2008

FDA, Food and Drug Administration (USA); PM, peso molecular; SCID, enfermedad de inmunodeficiencia combinada severa; G-CSF, factor de estimulación de colonias de granulocitos; GH, hormona del crecimiento. (Hamidi *y col.*, 2006; Fishburn, 2008; Veronese y Mero, 2008).

terferón α 2a fueron separados cromatográficamente y su actividad fue evaluada; los isómeros Lys³¹ y Lys¹³⁴, fueron los más activos. Lo que demuestra que es posible diseñar nuevos PEG-interferones que retengan mayor actividad biológica que la proteína nativa, además de que se evidencia que aún hace falta investigación sobre estrategias de purificación más eficientes (Veronese y Mero, 2008).

Las ventajas de la PEGilación no están limitadas a su aplicación en proteínas terapéuticas, también han sido utilizadas para mejorar la estabilidad en solventes orgánicos y la eficiencia catalítica de proteínas como la lacasa, utilizada en procesos de biorremediación debido a su capacidad de oxidar un amplio rango de compuestos fenólicos y poliaromáticos (Vandertol-Vanier *y col.*, 2002; López-Cruz *y col.*, 2006). Otra proteína utilizada también como biocatalizador en reacciones de oxidación de compuestos de estructura química diversa y que representan un problema de contaminación ambiental, es el citocromo C. El citocromo C ha sido modificado químicamente mediante PEGilación, lo que ha permitido aumentar su estabilidad térmica a temperaturas mayores a 100°C (García-Arellano *y col.*, 2002). La PEGilación también podría ser utilizada para mejorar la estabilidad de proteínas de afinidad (v.gr. proteína A) utilizadas en lechos cromatográficos para la separación de anticuerpos.

Además de proteínas y péptidos, otras moléculas como: cofactores, oligonucleótidos, lípidos sacáridos y bio-materiales están siendo PEGilados, lo que representa un área de oportunidad para el desarrollo de diversas investigaciones en el área.

Hasta ahora, el desarrollo de la PEGilación de

proteínas ha estado enfocado en su aplicación terapéutica, que sin duda ha sido de gran impacto para el desarrollo de nuevas drogas. Sin embargo, esta técnica puede mejorar la estabilidad de prácticamente cualquier proteína, de ahí la necesidad de profundizar la investigación en este campo pues aún existen diversos retos por superar.

En cuanto a la producción (reacción) de bioconjugados, es necesario diseñar reacciones de PEG-ilación sitio-específicas que eviten la formación de PEGámeros sin dañar el sitio catalítico; además de conocer a detalle las características intramoleculares de las especies PEGiladas que ayuden a entender su comportamiento bajo diferentes condiciones. La optimización de la reacción de PEGilación es crucial, puesto que el exceso tanto del PEG como de la proteína que no reaccionan aumenta la viscosidad de la solución, lo que complica el proceso de purificación. Sería ideal contar con procesos compuestos por una etapa de recuperación primaria (v.gr. ultrafiltración, fases acuosas, etc.) y una de purificación (utilizando métodos cromatográficos). Otra alternativa es llevar a cabo la reacción de PEGilación y la separación en un solo paso utilizando cromatografía de exclusión molecular, esta es una técnica que ha sido muy poco explorada por lo cual tiene que ser afinada, pero ofrece la posibilidad de realizar tanto el proceso de producción como el de purificación a través de una misma etapa con la ayuda de sistemas cromatográficos.

Conclusiones

La PEGilación es una técnica versátil que permite superar muchas de las limitaciones farma-

cológicas de las proteínas terapéuticas. Durante el desarrollo de esta técnica han surgido importantes avances en cuanto a la reacción de PEGilación, la generación de más bio-conjugados, el entendimiento de su comportamiento y las estrategias para su purificación. Sin embargo, aún se presentan nuevos retos en materia de ingeniería, tanto en la preparación como en la purificación de las moléculas PEGiladas. Los métodos clásicos de separación han sido útiles pero no ofrecen una resolución óptima; siguen siendo procesos en varias etapas, de alto costo y con rendimientos bajos, además de que en casos muy específicos no ha sido posible separar los isómeros conformacionales. De manera que es necesario profundizar en la investigación de métodos no convencionales de separación que puedan ser aplicados en la purificación de proteínas PEGiladas. Los productos aprobados por la FDA son una clara demostración del éxito de la PEGilación tanto en el mejoramiento de las propiedades de las proteínas terapéuticas como de su aplicación en el tratamiento de diversas enfermedades; sin embargo, aún hace falta profundizar sobre la aplicación de proteínas PEGiladas en diversas áreas de la biotecnología.

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Current advances in the non-chromatographic fractionation and characterization of PEGylated proteins

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Abstract

For more than 30 years, PEGylation has been used to improve the physicochemical properties of several proteins and therapeutic drugs having a major impact in the biopharmaceutical industry. The purification of PEGylated proteins usually involves two basic challenges: (1) the separation of PEG-proteins from other reaction products; and (2) the sub-fractionation of PEG-proteins on the basis of their degree of PEGylation and positional isomerism. Currently, most PEGylated protein purification processes are based on chromatographic techniques, especially size exclusion chromatography (SEC) and ion exchange chromatography (IEX). Nonetheless, other less frequently used strategies based on non-chromatographic techniques such as ultrafiltration, electrophoresis, capillary electrophoresis, and aqueous two-phase systems have been developed in order to fractionate and analyze PEGylated derivates. This review presents current advances in some of the most widely used non-chromatographic strategies for the fractionation and analysis of PEG-protein conjugates.

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Keywords: PEGylation; PEGylated proteins; fractionation and analysis; non-chromatographic methods

INTRODUCTION

The use of peptides and proteins in the pharmaceutical industry has increased significantly during the last few years. Therefore, the development of strategies oriented towards the improvement of the pharmacological function and kinetics of therapeutic bioactives once inside the human body has drawn attention from research groups in industry and academia. In this context, PEGylation has been playing a major role in the new proposed strategies. PEGylation consists in the modification of a molecule or protein attaching at least one chain of polyethylene glycol (PEG).¹ The PEGylation of proteins or certain groups in the membranes of different organelles and cells has been reported, increasing the application range of this novel technique.^{2,3}

After the reaction, the coupled PEG chains improve the properties of the modified molecules, and once administered, slow down their degradation by proteolytic enzymes, reduce their clearance by the kidneys, improving their circulation times, lower the production of antibodies towards them and increase their solubility.⁴ However, during the PEGylation reaction the formation of several conjugated forms varying in the number of grafted chains, their length and their grafting site usually occurs.¹ It must be noted that during the early years of PEGylation, optimization efforts were focused on the directed grafting of PEG chains mainly using residual amino groups as objective sites. Nevertheless, problems exist due to the large number of derivates produced as a consequence of the PEGylation reaction. This results in a need to purify these mixtures for their commercial approval.¹

The purification of PEGylated conjugates consists in removing from the obtained mixture, all species that because of their characteristics are not desired (contaminants). This implies two

main challenges: (1) the separation of the PEGylated proteins from the rest of the reaction products; and (2) the subfractionation of these proteins according to their degree of PEGylation and their positional isomerism.⁵ These challenges are not trivial due to the nature of PEG polymers and the fact that PEGylated molecules are hybrid and have properties that do not necessarily imitate the properties of the protein or the PEG from which they come. Fractionation and characterization of the positional isomers becomes of great importance due to the fact that usually only one or very few species exhibit optimal biological properties.^{6,7}

Individual and combined chromatographic approaches are currently used to separate PEGylated proteins. Because of the increment in hydrodynamic radius, size exclusion chromatography (SEC) separates these molecules reducing their accessibility through the porous support and altering their elution times. However, the differences between the sizes of species are not enough to achieve positional isomer separation.⁸ For its part, ion exchange chromatography (IEX) is capable of separating these proteins because for each PEG molecule grafted to an amino group a PEGylated protein has one less positive charge. However,

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PEG chains sterically interfere in the interaction of the charged residues in the polypeptide and the ionic exchange support or can produce a masking effect of the charges.⁹ Together, both SEC and IEX are the most commonly used chromatographic techniques for the separation of polymer conjugates.⁵ Hydrophobic interaction chromatography (HIC) and reversed phase chromatography (RPC) have also been studied, although not extensively, for this purpose. These methods are interesting because PEGylation may also affect hydrophobicity in the protein. For RPC low resolutions have been reported while trying to separate conjugates and it has been proved that this technique can produce structural changes in the separated species. HIC has low capacity and poor resolution between adjacent peaks.⁹

To date, it is not possible to conclude which type of chromatography or which combination of techniques has better separation yields or analytical resolutions. Furthermore, there is no generalized chromatographic protocol to study the different PEGylated molecules making it necessary to describe empirically the separation strategies on a case-by-case basis. An extensive analysis of chromatographic methods for separation and purification of PEGylated proteins has been reported before.⁵ Additionally, non-chromatographic methods have been used and developed in order to fractionate and analyze PEGylated conjugates. These methodologies represent an attractive alternative to chromatographic strategies for economic and technical reasons. However, many of these non-chromatographic strategies have not been fully characterized for the fractionation and analysis of PEGylated conjugates.

Figure 1 presents a simplified diagram depicting the main differences between a fully chromatographic and a non-chromatographic approach to fractionate PEGylated proteins. It is evident that in the chromatographic strategy at least two different types of chromatography must be used while the more robust non-chromatographic techniques offer the potential to reduce or even eliminate these time consuming steps. For example, exploiting the differences in molecular size, membrane separations (i.e. ultrafiltration, diafiltration, dialysis) have been used to separate different PEGylated species from their unmodified precursors.^{5,10} New advances have also been reported in the use of aqueous two-phase systems (ATPS) to separate native from PEGylated proteins. Regarding this liquid–liquid fractionation strategy it has been reported that one single grafted chain is enough to promote a selective fractionation towards a specific system phase.¹¹ Also, the use of polyacrylamide gel electrophoresis (PAGE) and capillary electrophoresis (CE) has been reported for the analysis of PEGylated proteins.^{12–15} PAGE has been widely used for the characterization of the PEGylation reaction. CE has applications that allow monitoring and analysis that facilitate the optimization of the PEGylation reaction. In addition to PAGE and CE, analysis techniques such as mass spectrometry (MS) and circular dichroism (CD) are used to evaluate the impact of PEGylation on the secondary and tertiary structure of proteins.

The aim of this review is to present current advances and future trends available on the non-chromatographic fractionation and analysis strategies for PEGylated conjugates, including membrane operations, aqueous two-phase systems, electrophoresis and capillary electrophoresis. The advantages, disadvantages and the impact of the selected strategies on the efficiency of PEGylated conjugate purification processes are discussed.

MEMBRANE SEPARATIONS

Membrane separation techniques are the simplest non-chromatographic methods currently used for PEGylated conjugate preparative or large-scale fractionation and processing based on their molecular weight and hydrodynamic radius. It was reported by Fee and Van Alstine⁵ that unit operations such as ultrafiltration, diafiltration, and dialysis may be used for the removal of low molecular weight species, buffer exchange and concentration.⁵ Additionally, the authors also mention that in some instances the use of ultrafiltration may generate unacceptable product losses, particularly when using membranes with pores considerably smaller than the PEGylated protein hydrodynamic radius.⁵ This problem specifically refers to a report regarding the concentration of mono-PEGylated tumor necrosis factor receptor type I (~85 kDa) using 10 kDa molecular weight cut-off membranes.¹⁶ However, current research regarding the different uses and characteristics of ultrafiltration membranes and PEGylated proteins performed mainly by the research group directed by Dr Andrew L. Zydny^{10,17–19} confirms that such technique can be used to separate the native from the PEGylated proteins in a reaction mixture (see Table 1) and explain the reasons why this was not achieved previously.^{10,17–19} For example, PEGylated α -Lactalbumin (α -Lac) was separated from its precursor and other byproducts using a two-stage ultrafiltration strategy that exploits the differences in molecular size and electrical charge between the conjugates and the other molecules in the reaction mixture. This was achieved using a regenerated cellulose membrane to remove the native protein followed by a negatively charged membrane to remove PEG.¹⁰ Additionally, their reports indicate that the hydrodynamic volume cannot be used to predict the sieving coefficients for PEGylated proteins, since these values depend not only on the total molecular weight of the species but also on the number and length of the grafted polymer branches.¹⁷ Furthermore, it has been proved that due to the presence of the grafted PEG chains the conjugates exhibit a significant flexibility that cannot be described using rigid sphere models and that this flexibility is responsible for molecular elongation during ultrafiltration processes.¹⁸ In fact, this elongation increases as filtrate flux and grafted PEG chain length increases, indicating a higher hydrodynamic drag through the membrane pores, as can be deduced from the increments in the sieving coefficients.¹⁸ At low filtration fluxes PEGylated proteins behave as spherical molecules and therefore present lower sieving through different membranes.¹⁸ The effects of using surface-modified membranes to achieve a larger retention of PEGylated products has been also explained with models that take into consideration the increments in the effective protein size, the increase of the net negative charge and the alterations in the electrostatic potential field of a PEGylated protein.¹⁹ The simple use of charged composite regenerated cellulose membranes gives a reduction in the sieving coefficients. However, a higher reduction in sieving coefficients is observed when decreasing the ionic strength of the ultrafiltration buffer. This behavior is attributed to the increment in the effective size of the protein and the effect of strong electrostatic interactions due to the displacement of the effective protein charge to the outer surface of the PEGylated species.¹⁹ Overall results show that PEGylated species can be fractionated and recovered efficiently using ultrafiltration and diafiltration achieving high yields and purification factors.¹⁰ Even though membrane separation techniques cannot separate conjugates according to their positional isomerism, these unit operations seem to present certain advantages over SEC and IEX where proteins of the same molecular size but different polymer

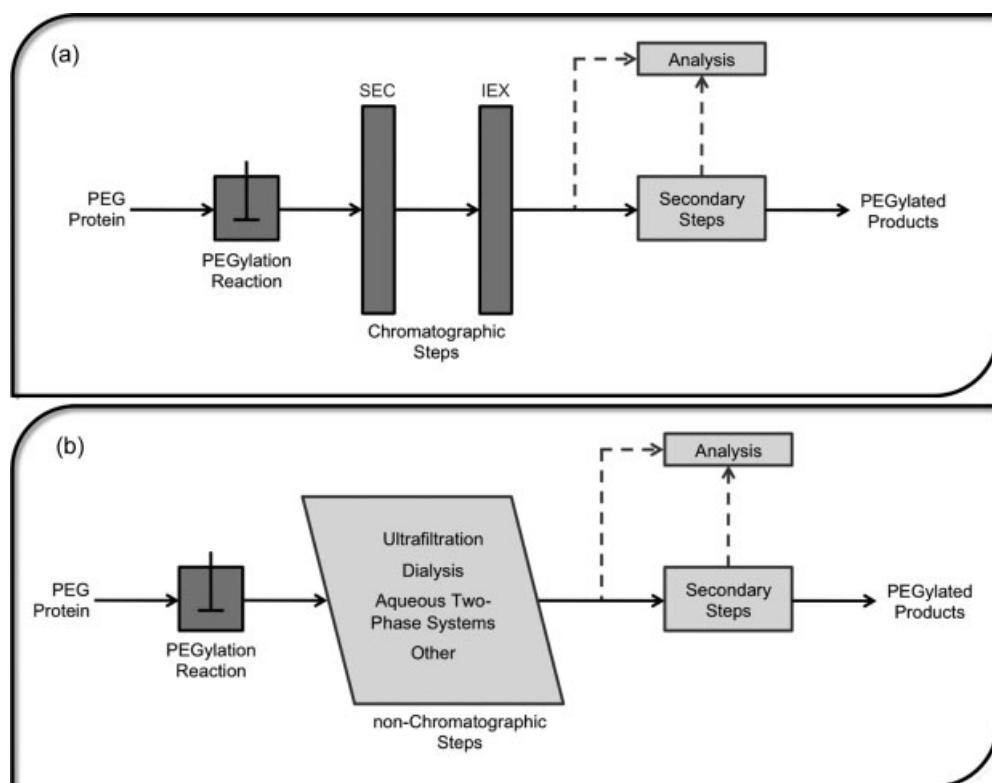


Figure 1. Simplified representation of a comparison between chromatographic and non-chromatographic separation processes of PEGylated proteins. The chromatographic approach is presented in (a) while the non-chromatographic approach is shown in (b). It can be noted that several techniques or a mixture of these can be used in a non-chromatographic separation strategy. Secondary steps refer to subsequent unitary operations needed for the final purification of the PEGylated products, considering that such steps may include chromatographic step. For the analysis of these proteins in both cases techniques such as mass spectrometry, circular dichroism or electrophoresis or a mixture of these can be used.

Table 1. Selected reports exploiting the use of membrane unit for PEGylated proteins separations

Protein (MW, kDa)	PEG MW (kDa)	Process	Conditions	Goals	Reference
α -Lactalbumin (14.2)	5, 10, 30	Ultrafiltration	30 kDa membranes	Evaluate the effects of the number and size of PEG chains	17
Ovalbumin (42.8) BSA (69.3)					
α -Lactalbumin (14.2)	20	Ultrafiltration Diafiltration	30 and 100 kDa membranes	Purification of a single PEGylated species	10
α -Lactalbumin (14.2)	5, 10, 20	Ultrafiltration	30, 100 and 300 kDa membranes	Evaluate the influence of the grafted PEG properties upon sieving	18
α -Lactalbumin (14.2)	2, 5, 10, 20, 30	Ultrafiltration	100 kDa unmodified and charged membranes	Evaluate the effect of electrostatic interactions on protein retention	19

MW, molecular weight; BSA, bovine serum albumin.

lengths and number of grafting sites cannot be separated or fully resolved¹⁸ and the grafted PEG layer apparently shields or blocks the electrostatic binding interactions,¹⁹ respectively. A summary of recent scientific reports addressing the fractionation of protein PEGylated conjugates using membrane unit operations is presented in Table 1. No current work known by the authors to separate protein–polymer conjugates using dialysis has been reported. As mentioned earlier, Fee and Van Alstine reported different projects that used this technique to stop the PEGylation reaction by buffer

exchange, concentration and removal of byproducts rather than analytical separations.⁵

AQUEOUS TWO-PHASE SYSTEMS

Aqueous two-phase systems (ATPS) is a well-known and useful technique for the fractionation, recovery and partial purification of several biological products, particularly proteins.^{5,20–23} Chemical compounds such as salts (phosphates, sulfates, citrates, etc.)

Table 2. Reports of PEGylated protein processing using aqueous two-phase systems (ATPS)

Protein	PEGylated Protein MW (kDa)	Degree of modification (<i>n</i>)	Goals	Reference
GM-CSF	14.4	0–2.5	Correlation of log <i>K</i> vs <i>n</i> and purification of protein-conjugates from unreacted protein and PEG	25
BSA	66.2	0–46	Use of NaCl enriched ATPS for the quantification and purification of protein-conjugates	26
IgG	160.0	0–43		
α -Lactalbumin	13.6	0–2	Fractionation of conjugates depending on their <i>n</i> and separation from unreacted protein	11
RNase A	14.1	0–2		

MW, molecular weight; GM-CSF, granulocyte macrophage colony-stimulating factor; BSA, bovine serum albumin; IgG, Immunoglobulin G; RNase A, Ribonuclease A.

and polymers (PEG, dextran and other copolymers) are used for the construction of ATPS. In this liquid–liquid fractionation technique two aqueous immiscible phases are formed due to the electrostatic repulsion and hydrophobicity differences between the phase forming chemicals.^{5,21} Biomolecules fractionate due to the differences in their physicochemical properties such as molecular weight, superficial electrochemical charge and relative hydrophobicity. ATPS provide favorable conditions to manipulate fractionation in a cost effective and non-denaturing manner due the high contents of water in the system.²⁴ Additionally, the use of ATPS may service as a preliminary purification strategy of PEGylated proteins in a reaction mixture.⁵ Table 2 presents information regarding reports addressing the fractionation, recovery and partial purification of PEGylated conjugates using ATPS.

Delgado *et al.*²⁵ used PEG/dextran ATPS as a feasible technique for the characterization of PEG in PEGylated bovine serum albumin (PEG-BSA) and PEGylated granulocyte-macrophage colony stimulating factor (PEG-GM-CSF) in mixtures of PEG-protein conjugates. They reported a progressive increase in partition coefficient (*K*, defined as the ratio of the concentration of a target molecule at the top and bottom phase)²⁰ with the increase in concentration of the grafted PEG molecule due to the increase in the degrees of substitution of the species present in the resulting mixtures. However, at high grafted polymer concentrations a precipitation of a fraction of the conjugates to the bottom phase was observed, resulting in a decrease in log *K*. This is attributed to the exclusion of bulky molecules from the PEG rich phase. The authors determined a correlation between the partition behavior of the protein derivates and the degree of modification (*n*). As a result, evidence supporting the proposal that ATPS could be used for the quantitative measure for the degree of modification of PEG-protein conjugates at low concentrations and in mixtures with carrier proteins was obtained.

A similar approach to conjugate purification using ATPS was also used by Delgado *et al.*²⁶ who identified a simple correlation between the number of grafted polymer chains and the partition coefficient in three different proteins: granulocyte-macrophage colony stimulator factor (GM-CSF), bovine serum albumin (BSA) and immunoglobulin G (IgG) (see Table 2). They used NaCl-enriched PEG-dextran ATPS to assess a minimal electrostatic chemical potential and to increase sensitivity to polymer grafting. Despite using slightly different polymers, it was proved that the interfacial

region of the conjugates would be favored to be polymer-enriched as the polymer-grafting ratio increased. Furthermore, it was stated that log *K* varies directly with *n* as a function of the interfacial properties of the conjugate. However, this relationship reached a plateau at high *n* values (>20). The authors concluded that further studies were needed to obtain specific data regarding the influence of the protein molecular weight, phase system composition and system parameters to develop an accurate model of PEG-protein partition.

In this context, González-Valdez *et al.*¹¹ evaluated the effect of PEG molecular weight (PEG MW), tie-line length (TLL) and volume ratio (*V_R*) of PEG-phosphate ATPS on the partition of native RNase A and native α -Lactalbumin (α -Lac) and their PEGylated conjugates to establish the optimal conditions for their fractionation in a single stage. The use of ATPS constructed with high PEG molecular weight (8000 g mol⁻¹), tie-line lengths of 25 and 35% w/w, and *V_R* values of 1 and 3 allowed single step selective fractionation of native RNase A and α -Lactalbumin, respectively, from their PEGylated conjugates on opposite phases. Such conditions resulted in an RNase A bottom phase recovery of 99%, while a top phase recovery greater than 88% was achieved for both conjugates. For its part, α -Lac had a bottom phase recovery of 92% while its mono and di-PEGylated conjugates were recovered at the top phase with yields superior to 76%. The results presented by the authors demonstrate the potential application of ATPS for the fractionation of PEGylated conjugates from their unreacted precursors.

The extended application of ATPS for PEGylated protein separation was reported by Sookkumnerd and Hsu.²⁷ This report documented the successful separation of unmodified, mono and di-PEGylated lysozymes using counter current distribution aqueous two-phase systems (CCD-ATPS). CCD-ATPS is a technique that allows the separation of proteins that like PEGylated conjugates, present minor differences in their partition behaviors or are structurally closely related.²⁸ Their experiments were conducted manually using PEG–potassium phosphate systems and as a result three peaks corresponding to each of the species were clearly identified. However, no complete separation between the native and the mono-PEGylated lysozyme was achieved. Furthermore, the authors suggested the use of high-speed countercurrent chromatography or thin layer countercurrent distribution to achieve higher purities of each conjugate at preparative scale.²⁷

Table 3. Recent advances in the analysis of PEGylated proteins using polyacrylamide gel electrophoresis (PAGE)

Protein	Conditions	Goal	Reference
hG-CSF	SDS-PAGE and silver stain	Molecular weight determination	44
Recombinant protein	SDS-PAGE and silver stain	Characterization	34
Horseradish peroxidase	SDS-PAGE and blue stain	Evaluation of the efficiency of PEG conjugation	31
Laccase	SDS-PAGE, Blue and silver stain	Molecular weight determination	45
Insulin	Non-denaturized PAGE, blue stain	Observation of differences between mono- and di-PEGylated proteins	30
Interferon α 2b	Imidazol-SDS-zinc reverse staining, Blue and I_2 stain	Detection of PEGylated proteins with minimal risk of modification	32
rhG-CSF	SDS-PAGE, silver stain for protein and barium iodine method for PEGs	Characterization and modification extent of protein	15
Human serum albumin	SDS-PAGE, and Blue stain	Characterization of PEGylated products	14
Staphylokinase	SDS-PAGE, and Blue stain	Characterization of PEGylated products	14

MW, molecular weight; PEG, polyethylene glycol; rhG-CSF, recombinant human granulocyte colony-stimulating factor

Despite the studies presented above, it is not possible to create a generic separation process for the purification of PEGylated conjugates using ATPS fractionation. A better understanding of the mechanisms related to the partition of protein conjugates is needed to optimize this technique. Therefore, even when the great potential of ATPS to separate, recover and partially purify PEGylated proteins according to the number of grafting sites has been demonstrated, more experimental studies must be conducted in order to generate partition behavior data to elucidate a robust predictive model for the fractionation of PEGylated conjugates on ATPS.

ELECTROPHORESIS

Polyacrylamide gel electrophoresis (PAGE) is widely applied for the analysis and characterization of PEGylated proteins²⁹ (see Table 3). However, unlike the case of native proteins where standard protein ladders can be used to make quantitative determinations of molecular weight, no current correlation exists to determine PEGylated protein molecular weight by this method. Since the migration rates of PEGylated proteins through porous gels are slowed by the large, heavily hydrated and uncharged PEG chains attached to the proteins, their molecular masses cannot be determined from their positions relative to standard protein ladders.²⁹ On the other hand, PAGE has been used to analyze the extent of PEG conjugation, to characterize the PEGylated products and to indirectly estimate the molecular weight of some PEGylated proteins.^{14,15,30,31}

It was reported by Na *et al.* that PEG moieties in PEGylated conjugates may be specifically stained with a barium iodide solution facilitating the visualization of free PEG and PEGylated derivates on SDS-PAGE.¹³ Jeng *et al.* reported the use of SDS-PAGE to evaluate the efficiency of conjugation in the PEGylation reaction of horseradish peroxidase (HRP).³² The authors followed the PEG conjugation of HRP with stepwise addition of activated PEG in order to determine the molar ratio leading to an optimal conversion of HRP to PEGylated HRP. In this case, conversion was assayed using SDS-PAGE (12% polyacrylamide gel stained with Blue) and quantification was carried out in a densitometer.³¹

As mentioned before, protein-bound and free PEG in PAGE may be visualized with barium iodine staining, whereas the protein component either free or conjugated is stained with Blue. Unfortunately, the barium iodine and Blue stains are not suitable to isolate specific conjugate species from gels for subsequent experimentation. Therefore, different techniques for the detection of PEGylated proteins in polyacrylamide gels have been developed. For example, it has been shown that a reverse staining technique using zinc and imidazol salts is applicable for detecting gel-separated PEGylated proteins with minimal risk of modification.³² Hardy and coworkers demonstrated that PEGylated proteins (i.e. interferon α 2b) could be straightforwardly reverse stained in analytical (<1 mm) gels. Furthermore, they additionally proved that the properties of PEG-interferon conjugates recovered from gels remain unchanged.³² SDS-PAGE has been used to analyze and characterize the PEGylation reaction of recombinant human granulocyte-colony stimulating factor (rhG-CSF) and its purification with size exclusion and cation exchange chromatography. In this case, Zhai *et al.* silver stained the proteins and used barium iodine for PEGs. Their results were consistent with the results obtained with chromatographic techniques.¹⁵

Recently, microchip-based electrophoresis, has successfully demonstrated its capabilities as a high-performance analytical technique for PEGylated proteins. This technique provides several advantages, including high speed (10 samples within 25 min), small sample volumes (only 4 μ L of protein sample required) and reduced reagent consumption.³³ In 2006, Yu and collaborators reported the application of microchip electrophoresis for measuring the extent of PEGylation of proteins.³⁴ Later, Park and Na used the microchip electrophoretic method to monitor and characterize the PEGylation reaction of α -Lac and BSA with several grafted PEG chains of different molecular weights. They demonstrated that this method is indeed a very useful tool for protein PEGylation studies that involve reaction monitoring, purity checks, and characterization of polymer conjugates.³³

As seen, SDS-PAGE has been widely used in the analysis and characterization of PEGylated proteins. The versatility of this technique allows the visualization of both the reaction products and the ungrafted PEG in several reaction mixtures through

Table 4. Current selected research using capillary electrophoresis (CE) for the characterization and separation of PEG-modified proteins

Protein	Process	Goal	Reference
Ribonuclease A and Lysozyme	Semi-aqueous capillary electrophoresis	Characterization of PEGylated derivates	39
Interferon α	SDS-CGE	Characterization of PEGylated IFN alpha	13
Human parathyroid hormone	Capillary electrophoresis	Simultaneous analysis of the PEGylation site and the extent of positional isomers in the mono-PEG-PTH	12
Interferon α -2a	SDS-CGE	Separation and purification of mono-PEGylated IFN	38
G-CSF	CZE and SDS-CGE	Optimization of the PEGylation reaction, as well as purity and stability tests	40

MW, molecular weight; SDS-CGE, sodium dodecyl sulfate-capillary gel electrophoresis; G-CSF, granulocyte-colony stimulating factor, CZE; capillary zone electrophoresis.

different stains. Additionally, it has proved to be extremely useful for the separation of PEGylated conjugates for subsequent mass spectrometry (MS) analysis.

CAPILLARY ELECTROPHORESIS

Capillary electrophoresis (CE) has been used in the analysis and small-scale separation of PEGylated proteins. CE has proved to be a powerful technique for the high resolution separation of biomolecules such as peptides and proteins.³⁵ The sodium dodecyl sulfate-capillary gel electrophoresis (SDS-SCE) method using a hydrophilic replaceable polymer network matrix, which combines the principles of SDS-PAGE with the instrumentation and small-diameter capillaries of CE, provides faster and more efficient separations than SDS-PAGE in the slab-gel format³⁶ allowing its use mainly in polymer conjugate analysis and characterization. Table 4 shows recent studies for the separation and analysis of PEGylated proteins using CE.

Na and coworkers¹³ used the SDS-CE method to characterize the PEGylated interferon α (IFN) conjugates. The authors demonstrated the capability of this methodology to completely resolve PEGylated IFNs of different sizes and to monitor the reaction. Advantages of this technique regarding speed, automation capability and sample consumption were visualized by the authors when comparing CE with other available techniques.¹³

In PEGylation, the size and structure of the grafted PEG plays a major role in determining the pharmacokinetic and pharmacodynamic properties of the resulting protein conjugates.^{37,38} The enlargement of the molecular size of the conjugates after PEGylation slows renal elimination and prolongs the residence time of these protein drugs in the bloodstream extending dosage times.³⁷ Therefore, the current PEGylation technology frequently uses high molecular weight PEG reagents to obtain favorable pharmacokinetic profiles.³⁸ SDS-CGE has been used to separate mono-PEGylated IFNs modified with different high molecular weight PEGs with branched and trimeric structures. The SDS-CGE method showed high separation capacity by differentiating PEGylated IFN conjugates with small differences in molecular size and structure, while useful for checking the purity of each mono-PEGylated molecule.³⁸

Semi-aqueous capillary electrophoresis has been used to separate and characterize PEGylated ribonuclease and lysozyme molecules. The use of a semi-aqueous acetonitrile–water buffer (1:1 v/v) resulted in enhancement of the resolution of PEGylated

proteins, while the total analysis time was reduced compared with other reported methods.³⁹

Lee and Na⁴⁰ used both capillary zone electrophoresis (CZE) and SDS-CGE methods for the analysis of PEGylated granulocyte-colony stimulating factor (G-CSF) produced by a reaction with aldehyde activated PEG (5 and 20 kDa). The separation methods were also compared with high-performance size exclusion chromatography (HP-SEC). In this work, CE methods showed a better separation capacity than HP-SEC for the resolution of different PEGylated G-CSF conjugates. The CZE method separated successfully both the 5 and the 20 kDa conjugates. On the other hand, the SDS-CGE method was useful just for the separation of the low molecular weight PEGylated G-CSFs. The authors attributed this to the long migration times and low peak efficiency achieved using SDS-CGE. The sensitivity of the CZE method was higher than that in SDS-CGE.⁴⁰

Other proteins, such as the PEGylated human parathyroid hormone 1–34 (PTH), have been characterized and separated using this technique.¹² CE was used to optimize the PEGylation of PTH through control of the reaction pH and the molar ratio of reactants. Additionally, it is possible to determine the extent of positional isomers present in the mono-PEG-PTH by quantifying PEGylated fragments in the same CE electropherogram.¹²

CE has also been exploited for the small-scale recovery and purification of PEGylated protein while its industrial implementation has not been exploited due to technical scale-up incapability. It has been possible to analyze, characterize and often separate PEGylated proteins by CE, with results that are comparable with those obtained with chromatographic techniques. The experimental conditions of this technique are specific for each protein and there is not enough information to generate an empirical correlation to facilitate the selection of parameter values for optimal resolution of PEGylated conjugates. Nevertheless, the versatility of CE may allow the extension of this technique to the fractionation and purification of different PEGylated proteins and peptides in the nearby future.

FUTURE TRENDS AND CONCLUSIONS

It is clear that the increase in PEGylated proteins with therapeutic properties in clinical trials and the presence of PEGylated drugs already in the market are proofs of the potential of this promising chemical modification in the pharmaceutical industry. However, although the chemical modification of proteins and

other bioactives via PEGylation has been optimized during the past two decades, improvements on the purification of PEGylated conjugates are still needed. Therefore, a better characterization of the overall structural properties of the conjugated protein complexes is needed for the development of robust non-chromatographic techniques.⁴¹

It is believed that the membrane separations area will continue the characterization of novel membrane modifications towards the separation of specific proteins. Besides, promising filtration techniques such as tangential flow filtration and centrifugal diafiltration, which were not used before for the separation of PEGylated products, are currently being studied for the separation of PEGylated nanoparticles.^{42,43} On the other hand, it is necessary to fully characterize the behavior of protein–polymer conjugates in ATPS based operations, either for batch or continuous processing. In this context, a promising alternative to establish a continuous system for PEGylated derivates is using countercurrent distribution. *In situ* product recovery through continuous operation of ATPS will be one of the main focuses in the future. PAGE, which has been used mainly for the analysis of the PEGylated products during and after reaction, is probably the most widely used non-chromatographic technique at this time. Although advances have been made regarding the characterization of PEGylated derivates using this technique,^{14,15,44,45} it is necessary to explore alternative staining techniques to distinguish free PEG and PEGylated proteins in one step. On the other hand, no reports have been found that indicate the use of electrophoresis in two dimensions for the analysis and characterization of PEGylated products, so it may be interesting to carry out research in this area in order to determine the potential of this technique for the present application. CE is a very versatile technique with promising results in the analysis and small-scale purification of PEGylated derivates. Nevertheless, further investigation is needed to fully characterize the effect of process parameters such as length and diameter of capillary tube, chemical nature and number of grafted PEG chains, and solvent chemical identity, among others, on the separation of modified bioactives of commercial interest.

The on-line coupling of analytical techniques such as MS and CD to biotechnological processes is increasingly becoming of great importance in areas such as manufacturing and quality control.^{46,47} Technological advances are allowing their use for the optimization of different manufacturing process parameters as product yields and heterogeneity giving great advantages over time-consuming chromatographic techniques.⁴⁶ This becomes relevant when fast process-related decisions must be made when working on greater production scales. Even though there is no current industrial PEGylation process where these techniques are used routinely, their importance at laboratory scale analysis of PEG–protein conjugates indicates the need to consider their implementation on both chromatographic and non-chromatographic separation approaches of such molecules.

As discussed, the main challenge in the separation of PEGylated proteins is to establish compact analysis, fractionation and purification technologies with low operating costs without compromising efficiency. It is believed that the non-chromatographic techniques described herein offer the characteristics needed for this purpose as different investigation groups have seen their potential and are currently directing their efforts to fully characterize and optimize them.

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Separation of PEGylated from unmodified ribonuclease A using sepharose media

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ABSTRACT

PEGylation, used to mitigate some of the problems that affect the effectiveness of therapeutic proteins, often results in a heterogeneous population of conjugated species and unmodified protein that presents a protein separations challenge. This study presents the use of a mildly amphiphilic support, Tris-capped CH Sepharose 4B as an alternative for separating PEGylated proteins from their unmodified counterparts. The effects of parameters such as pH, salt type and salt concentration upon the chromatographic behavior of native, mono-PEGylated and di-PEGylated ribonuclease A on this media were characterized. The separation of the native protein from the PEGylated species was achieved using a gradient elution between a high ionic strength mobile phase (3 M ammonium sulfate in 25 mM potassium phosphate, pH 7.0 or 2 M potassium phosphate, pH 7.0) and a low ionic strength phase (25 mM potassium phosphate, pH 7.0). The pH of the mobile phases as well as the addition of PEG₆₀₀ (as a potential mobile phase modifier) to the low ionic strength phase had no significant influence on chromatographic behavior of the species. This media provides a simple and practical chromatographic method for the separation of unmodified proteins from their corresponding PEG conjugates.

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1. Introduction

The covalent attachment of polyethylene glycol (PEG) molecules to pharmaceutical proteins – a reaction process known as “PEGylation” – can mitigate factors that adversely affect therapeutic effectiveness, including susceptibility to enzymatic degradation, short circulation time, low solubility and immunogenicity [1,2]. However, this can present a challenging separation problem due to the fact that PEGylation reactions often result in a population of conjugate species in addition to the residual unmodified protein. The resulting conjugate species can differ in terms of the number of attached PEG chains and their locations and can differ in biological activity [3]. Chromatographic operations are often used to resolve PEGylation reaction mixtures, with size-based size exclusion and charge-based ion exchange modes most frequently used [4]; comparatively little work has been done to explore other modes such as the hydrophobic interaction mode [5,6].

Hydrophobic interaction chromatography (HIC) is intriguing with respect to the separation of PEGylated protein reaction mixtures as PEG itself exhibits lower critical solution temperature behavior: it can adopt collapsed configurations at higher tem-

peratures and/or salt concentrations that are comparatively more hydrophobic than the relatively extended configurations that occur at lower temperatures and/or salt concentrations [7]. Very little work has been done on exploiting hydrophobic interactions for the separation of PEGylated proteins [4]. HIC consists of injecting a protein sample in a column packed with a hydrophobic media under conditions of high salt concentration to drive hydrophobic interactions between the proteins and the media, analogous to a salting-out process. Bound proteins are eluted by lowering the salt concentration. In the case of a strongly bound protein, it can be eluted by the use of a chaotropic agent or an organic modifier [8]. The most common hydrophobic ligands used in HIC are linear chain alkanes or simple aromatic groups. However, these hydrophobic ligands may promote strong hydrophobic interactions that sometimes result in irreversible adsorption of the proteins or denaturation during adsorption and subsequent elution with harsh mobile phases. Ligands with milder hydrophobic characters can be attractive as they can provide moderate binding strength and bound species can be eluted by simply decreasing the salt concentration of the eluent [9].

In this work, we were able to completely separate PEGylated ribonuclease A (RNase A) species from unmodified RNase A using a Tris-capped CH Sepharose 4B media with salt gradient elution. RNase A was chosen as a model protein as it is well-characterized, has potential therapeutic application as an aspermatogenic and

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antitumor agent and studies have shown an improvement of the therapeutic effect when RNase A is conjugated with PEG [10]. RNase A was PEGylated with an aldehyde-activated PEG of 20 kDa nominal molecular weight. The behavior of unmodified RNase A and mono- and di-PEGylated species previously separated using size exclusion chromatography (SEC) was characterized on the proposed Sepharose-HIC separation system as were protein PEGylation reaction mixtures. pH, salt type and salt concentration were the parameters selected to define conditions under which the separation of the unmodified protein from the PEGylated species can be achieved. Free PEG with a nominal molecular weight of 600 Da was also examined as a potential mild mobile phase modifier.

2. Materials and methods

2.1. Materials

Bovine pancreatic ribonuclease A (cat. no. R5000, Lot 093K0765) was purchased from Sigma-Aldrich (St. Louis, MO). Methoxy-poly(ethylene glycol)-butyraldehyde with a nominal molecular weight of 20 kDa came from Nektar Therapeutics (Huntsville, AB). Sodium cyanoborohydride was purchased from ICN Biomedicals Inc. (Costa Mesa, CA). Activated CH sepharose 4B (cat. no. 17-0490-01 Lot 307571) and the chromatography column, a Tricorn 5/100, came from Amersham Biosciences (now GE Healthcare, Uppsala, Sweden). Conjugation and purification buffers were prepared from sodium phosphate monobasic and dibasic salts (Fisher Scientific, Pittsburgh, PA), and potassium chloride (Sigma-Aldrich Company, St. Louis, MO). All HPLC grade reagents were obtained from Fisher Scientific. Other salts and solvents used in this research were of reagent grade. All reagents were used as received.

2.2. Preparation of PEGylated protein

PEGylated RNase A was prepared according to the procedure of Daly et al. [11]. Briefly, a solution of RNase A (2.0 mL) at 3.0 mg/mL in a pH 5.1, 100 mM sodium phosphate buffer with 20 mM sodium cyanoborohydride was added to a vial containing 30 mg of the nominal 20 kDa methoxy poly(ethylene glycol) butyraldehyde. The reaction mixture was stirred rapidly for 17–19 h at 4 °C. Experiments were conducted both with the final reaction mixture and with species purified using size exclusion chromatography (SEC).

2.3. Analysis and separation of PEGylated protein mixture by size exclusion chromatography (SEC) and mass spectrometry

The reaction mixture (2.0 mL) was analyzed via size exclusion chromatography with an Amersham Pharmacia Akta Explorer system (now GE Healthcare, Uppsala, Sweden) using a Sephadryl S-300 column (1.6 cm inner diameter, 60 cm length, Amersham Pharmacia, Uppsala, Sweden) with an isocratic mobile phase of 10 mM sodium phosphate buffer, pH 7.2, containing 150 mM potassium chloride at a flow rate of 0.5 mL/min. The column was pre-equilibrated with one-half column volume of distilled water and two column volumes of mobile phase. Fractions having an absorbance at 280 nm were collected and concentrated in an Amicon (Beverly, MA) stirred cell fitted with a YM 10 membrane (molecular weight cutoff 10 kDa) and then diafiltrated with 5 volumes of 25 mM sodium phosphate buffer, pH 7.2.

Each SEC peak was analyzed by mass spectrometry (Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry, MALDI-TOF/MS). Prior to analysis, the samples were desalting using centrifugal microconcentrators (Microcon YM-3, Millipore). MALDI-TOF/MS was performed with a PerSeptive Voyager STR mass spectrometer fitted with a standard 337 nm

nitrogen laser. The spectra were recorded with the analyzer in the positive-ion linear mode of detection. The ion accelerating potential was 25 kV. A saturated solution of α-3,5-dimethoxy-4-hydroxycinnamic acid (Fluka, Buchs, Switzerland) in 50% acetonitrile was used as a matrix solution. The sample crystals were prepared by mixing 1.0 μL of sample with 1.0 μL of matrix. The product peaks were identified as diPEGylated, monoPEGylated and unmodified RNase A species.

2.4. Preparation of chromatography media

Sepharose was used as the base matrix for the construction of a mild amphiphilic support. Activated CH Sepharose 4B (Amersham Biosciences, Uppsala, Sweden, Lot: 307571) was modified with a capping reaction using tris(hydroxyethyl)aminomethane (Tris) as shown in Fig. 1. The matrix was prepared according to supplier instructions (Amersham Biosciences). About 1.3 g of activated Sepharose were washed with about 250 mL of 1 mM HCl and the washed medium was transferred to 0.1 M Tris-HCl buffer, pH 8. After 1 h, the modified media was washed with three cycles of alternating pH (0.1 M acetate buffer, pH 4.0 containing 0.5 M NaCl followed by 0.1 M Tris-HCl buffer, pH 8 containing 0.5 M NaCl). Based on the activated media product literature, 15 μmol/mL media is an upper bound on the Tris capping group density.

2.5. Separation of PEGylated species from unmodified RNase A

Chromatographic experiments were carried out with the Akta Explorer 100 integrated chromatography system operating at room temperature. Protein samples collected from SEC experiments or from the final PEGylation reaction mixture were applied to a Tricorn 5/100 column (Amersham Biosciences, 4.6 mm × 150 mm) packed with about 3.5 mL of the modified Sepharose media. The column had about 65 theoretical plates, as determined by elution peak width analysis of injections of 1% (v/v) acetone pulses at 25 °C. Gradient elutions were carried out for protein separations at a flow rate of 1 mL/min with a solvent A (3 M ammonium sulfate in 25 mM potassium phosphate pH 7.0, or 2 M potassium phosphate, pH 7.0 or 3 M ammonium sulfate in 25 mM acetate buffer, pH 5.0) and a solvent B (25 mM potassium phosphate, pH 7.0 or 25 mM acetate buffer, pH 5). The following gradient profile was selected: 0% B for 6.5 mL, 0–100% B over 29.5 mL, and 100% B for 15 mL. Peak elution was monitored via the UV absorption at 280 nm. 100 μL of sample was injected at room temperature. The concentrations were 3, 1.5 and 0.97 mg/mL for unmodified, monoPEGylated and diPEGylated RNase A, respectively. The flow rate was 1 mL/min.

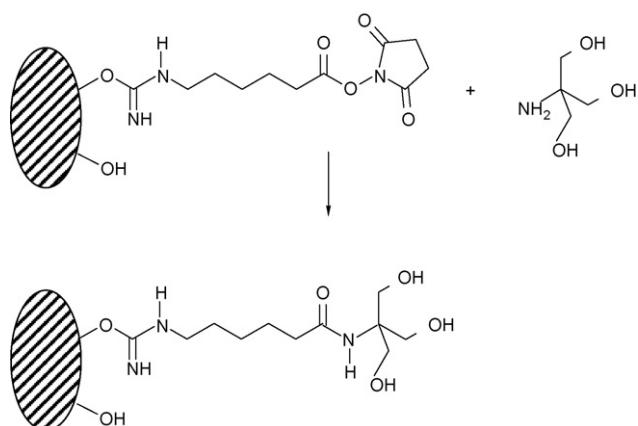


Fig. 1. Reaction scheme for the modification of activated CH Sepharose 4B by capping with tris(hydroxyethyl)aminomethane.

3. Results and discussion

In order to study the retention behavior of the proteins using the capped Sepharose media, unmodified RNase A and the SEC-purified monoPEGylated and diPEGylated species were applied to the column with an initial concentration of 3 M ammonium sulfate in the 25 mM phosphate buffer; the corresponding chromatograms are shown in Fig. 2. The elution profiles of the purified species are shown under stronger loading conditions (3 M ammonium sulfate) and with a steeper gradient (about -0.10 M salt/mL). The unmodified protein elutes about a third of the way through the salt gradient at roughly 220 mS/cm 1.5 M ammonium sulfate. There may be some hysteresis in the adsorption–desorption behavior of the unmodified protein: loading under more strongly binding conditions may require more stringent elution conditions. Both the monoPEGylated and diPEGylated species elute much later at roughly 135 mS/cm and 130 mS/cm, respectively, representing somewhat stronger to the media; again, there may be some binding hysteresis. In this case, there was a slight, but significant difference in the elution behavior of the monoPEGylated and diPEGylated species; this small difference is likely not sufficient for exploitation to resolve PEGylated variants. The elution of the PEGylated species at lower salt concentrations suggests that hydrophobic interactions were the primary mode of interaction with the media; hydrogen bond-based interactions would be disrupted at higher salt concentrations.

Retention in hydrophobic interaction chromatography processes is highly dependent on mobile phase ionic strength and salt type. The separation strategy requires a careful selection of these conditions during both the adsorption and desorption steps [12]. In order to further characterize the behavior of the capped media for the separation, the effect of salt type was evaluated. Fig. 3 shows the elution behavior of RNase A and its PEG-conjugates using a gradient in the buffer concentration from 2 M to 25 mM potassium phosphate, with a gradient slope of about -0.07 M/mL, to manipulate mobile phase ionic strength. The unmodified protein was more strongly retained relative to the previous ammonium sulfate gradient case, eluting at a conductivity of about 145 mS/cm. The monoPEG and diPEG species eluted at roughly 100 mS/cm and 95 mS/cm, respectively. In the absence of special binding effects, a change in salt concentration or change of salt in the mobile phase to one of greater surface tension increment results in increased retention of proteins by HIC [13]. In fact, for potassium phosphate, a small percentage of proteins was retained on the column after completion of the gradient and this could be due to the low surface tension

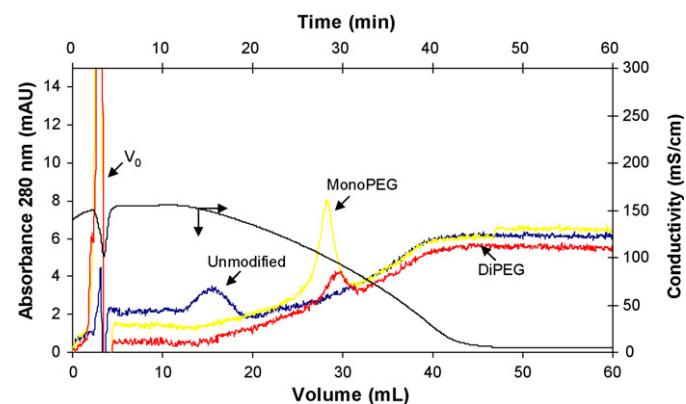


Fig. 3. Retention behavior of RNase A and purified monoPEG and diPEG-RNase A on the Sepharose HIC media with a potassium phosphate gradient. Mobile phase A: 2 M potassium phosphate pH 7.0, mobile phase B: 25 mM potassium phosphate pH 7.0. The PEGylated proteins were separated by SEC and put in a 25 mM potassium phosphate buffer pH 7.0. 100 μ L of sample were injected at room temperature. The flow rate was 1 mL/min.

increment of potassium phosphate with respect to ammonium sulfate. Such behavior is observed because the absorbance obtained in Fig. 3 does not allow the protein mass balance to close completely.

The pH of mobile phase can also be an important factor that affects the protein retention in HIC. Usually, an increase in the pH value (up to 9–10) decreases the hydrophobic interaction between proteins and the hydrophobic ligands, due to the increased hydrophilicity promoted by the change in the charge of the protein. On the other hand, a decrease in pH results in an apparent increase in hydrophobic interactions. Since each protein will have unique behavior at a given pH relative to its isoelectric point and since the effect of pH on protein retention in HIC is not well defined, this parameter should be used in the optimization of protein separation by HIC [9].

The effect of the pH was tested using the 3 M ammonium sulfate gradient in acetate buffer at pH 5.0. RNase A has a pI (isoelectric point) of 9.3 [14] and it is expected that the conjugate forms, owing to the covalent modification of one or two amino groups, will have slightly lower pIs according to the PEGylation conditions [15]. Under both pH conditions studied, 5.0 and 7.0, the proteins will have a positive net charge. The retention behavior of individual

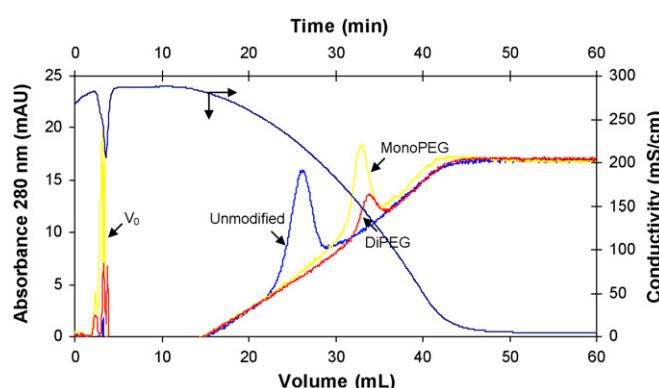


Fig. 2. Retention behavior of RNase A and purified monoPEG and diPEG-RNase on the Sepharose Media with strong loading conditions and an ammonium sulfate gradient. Mobile phase A: 3 M ammonium sulfate 25 mM potassium phosphate, pH 7.0; mobile phase B: 25 mM potassium phosphate, pH 7.0. The PEG-conjugate species were separated by SEC and exchanged into a 25 mM potassium phosphate buffer, pH 7.0; 100 μ L of sample was injected at room temperature. The flow rate was 1 mL/min.

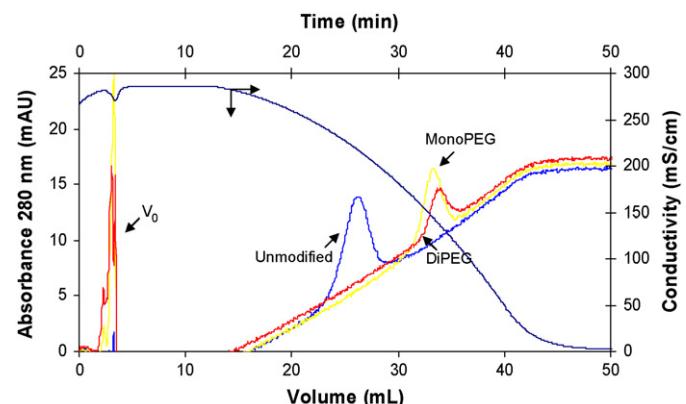


Fig. 4. Retention behavior of RNase A and purified monoPEG and diPEG-RNase A on the Sepharose HIC media with an ammonium sulfate gradient at acidic pH. Mobile phase A: 3 M Ammonium sulfate in 25 mM acetate buffer pH 5.0, mobile phase B: 25 mM acetate buffer pH 5.0. The PEGylated proteins were separated by SEC and put in a 25 mM potassium phosphate buffer pH 7.0. 100 μ L of sample was injected at room temperature. The flow rate was 1 mL/min.

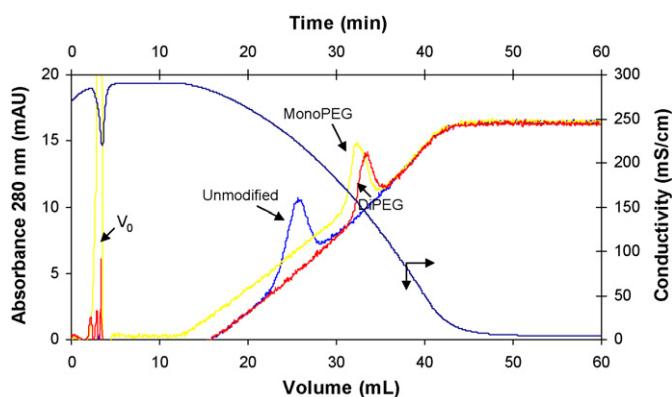


Fig. 5. Effect of PEG₆₀₀ as a mobile phase modifier on the retention behavior of RNase A and its PEG-conjugates. Mobile phase A: 3 M ammonium sulfate in 25 mM potassium phosphate, pH 7.0; mobile phase B: 15 mM PEG₆₀₀ in 25 mM potassium phosphate, pH 7.0. The PEG-RNase A proteins were separated by SEC and put in a 25 mM potassium phosphate buffer pH 7.0; 100 μL of sample were applied to the chromatographic system at room temperature. The flow rate was 1 mL/min.

species at low pH is shown in Fig. 4. The unmodified protein elutes at about 220 mS/cm, roughly equivalent to that at pH 7.0 as shown in Fig. 2. The monoPEG species, eluting at 140 mS/cm, is slightly less retained than the diPEG species which elutes at about 135 mS/cm. At the resolution of these chromatograms, this retention is the same as the pH 7.0 case. Under these conditions, the pH, in the 5.0–7.0 range, has little influence on the retention of the proteins on this media.

The influence of PEG as a potential mobile phase modifier was tested by adding 15 mM polyethylene glycol with a nominal molecular weight of 600 to the low ionic strength mobile phase B (see Fig. 5). Here, the idea was that free PEG in solution might compete with PEGylated protein for interaction sites on the media and encourage elution. The addition of PEG to the mobile phase B had little effect on the retention of the proteins: unmodified protein eluted at about 225 mS/cm, monoPEGylated protein at 150 mS/cm and diPEGylated protein at 145 mS/cm.

Finally, a PEGylation reaction mixture was applied to the PEG-Sepharose media using a gradient scheme between 3 M ammonium sulfate in 25 mM potassium phosphate, pH 7.0 and 25 mM potassium phosphate, pH 7.0; the corresponding chromatogram is shown in Fig. 6. As expected from the differing retentions of the unmodified and modified species shown in the

previous results, it was possible to separate unmodified protein from the conjugate species. The observed retention differences between the purified monoPEGylated and diPEGylated species under the conditions studied were too small to result in any further resolution of PEGylation variants. No significant differences between the eluting mobile phase conductivities for the reaction mixture and corresponding purifies species, as shown in Fig. 2, were apparent. There is no interaction apparent between the unmodified and modified proteins during the separation as expected for PEGylated species.

4. Conclusions

It was demonstrated that mildly amphiphilic chromatographic media can be exploited to separate unmodified proteins from their PEGylated conjugates, using ribonuclease A as model system. High selectivity was found between the unmodified protein and its PEGylated species using a gradient elution between 3 M ammonium sulfate in 25 mM potassium phosphate pH 7.0 and 25 mM potassium phosphate buffer pH 7.0. Variations including the use of 2 M potassium phosphate at pH 7.0 instead of the ammonium sulfate solution or a decrease in pH (from 7.0 to 5.0) had little effect on retention behavior. More stringent loading conditions necessitated more stringent elution conditions; there is hysteresis in the adsorption and desorption behavior of proteins on this media. Hysteretic protein adsorption phenomena have been observed by multiple groups working with HIC-based separations and have been attributed to protein conformation changes in the adsorbed state, lateral interactions between adsorbed proteins and multivalent protein–chromatographic ligand interactions [16–18]. Added PEG₆₀₀ did not function as a mobile phase modifier. There were very slight, but consistent observed differences in the retention of monoPEGylated versus diPEGylated species, with diPEGylated species more retained than the monoPEGylated species. These slight differences were not sufficient to result in observable resolution of modified variants from a mixture. To that end, it would also be interesting to evaluate the effect of additional parameters such as ligand density and matrix support type on the partition behavior of the PEG-conjugates species, in order to develop selectivity for PEGylation variants.

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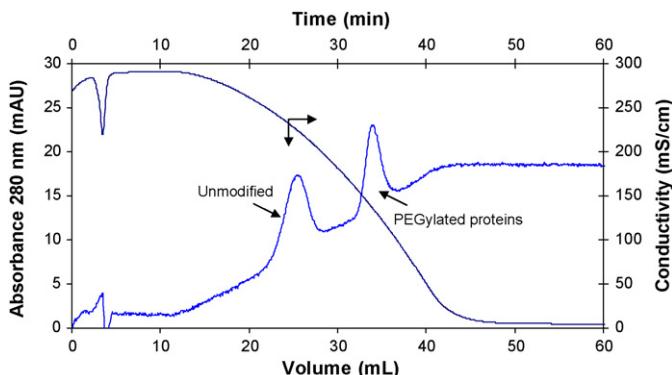


Fig. 6. Behavior of PEGylation mixture reaction with a gradient elution scheme. Mobile phase A: 3 M ammonium sulfate 25 mM potassium phosphate, pH 7.0; mobile phase B: 25 mM potassium phosphate, pH 7.0. 100 μL of sample was injected at room temperature. The flow rate was 1 mL/min. Unmodified RNase A was added to the reaction mixture before injection in order to facilitate its detection.

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Hydrophobic interaction chromatography for purification of *monoPEGylated* RNase A[☆]

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ABSTRACT

The chromatographic methods used for the purification of PEGylated proteins are mainly Size Exclusion (SEC) and Ion Exchange Chromatography (IEX). Although the PEGylation affects the protein hydrophobicity, Hydrophobic Interaction Chromatography (HIC) has not been extensively applied for the separation of these proteins. Purification of *monoPEGylated* Ribonuclease A (RNase A) using HIC is studied in this work. The products of the PEGylation reaction of RNase A with 20 kDa methoxy-poly(ethylene glycol) were separated using three resins with different degrees of hydrophobicity: Butyl, Octyl and Phenyl sepharose. The effects of resin type, concentration and salt type (ammonium sulphate or sodium chloride), and gradient length on the separation performance were evaluated. Yield and purity were calculated using the plate model. Under all conditions assayed the native protein was completely separated from PEGylated species. The best conditions for the purification of *monoPEGylated* RNase A were: Butyl sepharose, 1 M ammonium sulphate and 35 column volumes (CVs); this resulted in a yield as high as 85% with a purity of 97%. The purity of *monoPEGylated* RNase A is comparable to that obtained when the separation is performed using SEC, but the yield increases from 65% with SEC to ~85% with HIC. This process represents a viable alternative for the separation of PEGylated proteins.

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1. Introduction

PEG-protein conjugates, or PEGylated proteins, are an important class of modern therapeutic drugs. However, PEGylated proteins must be characterized and purified before use in order to meet the stringent regulatory requirements that demand proven clinical efficacy and safety [1]. The process of PEGylation involves formation of a stable covalent bond between activated PEG (polyethylene-glycol) polymers and the polypeptide drug of interest [2]. PEGylation changes the physical and chemical properties of the biomedical molecule, such as its conformation, electrostatic binding, and hydrophobicity; resulting in an improvement in the pharmacokinetic behavior of the drug. In general, PEGylation improves drug solubility and decreases immunogenicity, increases drug stability and the residence time of the conjugates in blood, and reduces

proteolysis and renal excretion, thereby allowing a lower dosing frequency [3].

To prepare a PEGylated protein, it is desirable that one PEG molecule is attached to a specific site (site-directed *mono-PEGylation*). However, as the PEGylation reaction is not completely understood, it is not easy to adjust or optimize the reaction process [4]. The reaction mixture is complex, from which the desired PEGylated protein must be highly purified. Purification of PEGylated protein is not a trivial task [5].

PEGylation of proteins creates two basic challenges for purification. The first involves separation of PEGylated proteins from other reaction products including non-reacted PEG and protein. The second is the sub-fractionation of PEGylated proteins on the basis of their degree of PEGylation and positional isomerism [4]. While isolation of the unreacted protein from the PEGylated species is relatively easy, separation of the various positional isomers of a PEGylated protein mixture remains a significant challenge [6]. Individual and combined chromatographic approaches are currently used to purify PEGylated proteins [7]. Chromatographic purification of all proteins implies selection of a mode of chromatography that exploits the differences in physicochemical properties [8]. Fee and Van Alstine [4] reviewed separation methods applied to

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downstream processes for *PEGylated* proteins. They have reported that Size Exclusion (SEC) and Ion Exchange Chromatography (IEX) are the predominant methods for purification of the *PEGylated* products. SEC has been widely used for separation of PEG conjugates as an increase in molecular weight is one of the most evident changes caused by *PEGylation* [9]. IEX enables a single step purification of the target PEG-protein conjugate from *unPEGylated* protein, higher *PEGylated* molecules and unreacted PEG; due to charge differences [9]. *PEGylation* should affect protein surface hydrophobicity, increasing or decreasing it depending on the native proteins hydrophobicity [4] and consequently, Hydrophobic Interaction Chromatography (HIC) can be used as an additional method for separation of PEG modified proteins. Even though HIC is used routinely for production-scale purification of proteins, it has not been highlighted for the separation of *PEGylated* species [1,4,10].

Ribonuclease A (RNase A) is a small model enzyme, with a molecular weight of 13,686 Da [11]. RNase A has proven to have several potential therapeutical applications *in vivo* as an antitumor agent, mainly in its *PEGylated* form [12]. It has been found that the biological activity exhibited by the *PEGylated* conjugates is related to the number of grafted polymer chains as well as their relative position. For most proteins, an excess in the number of grafted polymer chains generates a steric hindrance for its biological receptor, decreasing their specific activity [13]. It has been reported that the *monoPEGylated* RNase A presents the highest biological activity [12].

The purpose of this work is to establish the conditions for purification of *monoPEGylated* RNase A using HIC. The *PEGylation* reaction used by our group [14,15] is a reaction with the N-terminal amino group of the protein. This reaction generates three products: *monoPEGylated* RNase A (monoPEG), *diPEGylated* RNase A (diPEG), and the unmodified protein. In this case, resin type, salt type (ammonium sulphate or sodium chloride), salt concentration and gradient length were the parameters selected to define conditions under which the purification of the *monoPEGylated* protein can be achieved. Yield and purity were calculated using the plate model [16]. The equations used are described below:

$$\text{yield} = \frac{1}{2} \left[1 + \operatorname{erf} \left(\frac{V}{V_0} - \frac{1}{\sqrt{2}} \sigma \right) \right] \quad (1)$$

$$\text{purity} = \frac{[y_0(i)_* \text{yield}(i)]}{[\sum_j y_0(j)_* \text{yield}(j)]} \quad (2)$$

where y_0 is the maximum concentration, V_0 is the volume required to elute the maximum concentration y_0 and $V_0\sigma$ is the standard deviation of the peak [16]. The parameters were determined from the experimental results.

2. Materials and methods

2.1. Materials

Bovine pancreatic ribonuclease A (cat. no. R5000, lot 047K1640) and trizma base (cat. no. T6066, lot 076K54521) were purchased from Sigma-Aldrich (St. Louis, MO). MethoxyPEG-propionaldehyde (Lot ZZ004P225) with a nominal molecular weight of 20 kDa came from Jen Kem Technologies (Allen, TX). Sodium cyanoborohydride (cat. no. 71435, lot 414320) was purchased from Fluka (Switzerland). Phenyl sepharose 6FF HS (cat. no. 17-0973-05, lot 286273), Octyl sepharose (cat. no. 17-0946-05, lot 283600) and Butyl sepharose (cat. no. 17-0980-01, lot 28686) were obtained from GE Healthcare (Uppsala, Sweden). Purification buffers were obtained from J.T. Baker (Toluca, México). Water prepared with Milli-Q water cleaning system (Millipore, Bedford, MA) was used

in the preparation of the eluents. Other salts and solvents were of reagent grade.

2.2. Preparation of *PEGylated* protein

PEGylated RNase A was prepared according to the procedure of Daly et al. [17]. Briefly, a solution of RNase A (5.5 mL) at 3.0 mg/mL in a pH 5.1, 100 mM sodium phosphate buffer with 20 mM sodium cyanoborohydride was added to a vial containing 75 mg of the nominal weight 20 kDa methoxy poly(ethylene glycol) propionaldehyde. The reaction mixture was stirred vigorously for 17 h at 4 °C. The reaction was stopped by separating the mixture on a size exclusion chromatographic column.

2.3. Separation of *PEGylated* protein mixture by size exclusion chromatography (SEC)

The reaction (5.5 mL) was analyzed by Size Exclusion Chromatography with an Äkta Explorer system (GE Healthcare, Uppsala, Sweden) equipped with a 5 mL injection loop. A Sephadex S-300 HP column (2.6 cm ID, 60 cm length, GE Healthcare, Uppsala, Sweden) was used with an isocratic mobile phase of 10 mM sodium phosphate buffer pH 7.2, containing 150 mM potassium chloride at a flow rate of 0.5 mL/min. The column was pre-equilibrated with one-half column volume of distilled water and two column volumes of mobile phase. Fractions that absorbed at 215 nm were pooled and concentrated by ultrafiltration under nitrogen atmosphere using an Amicon ultrafiltration cell (MA, USA) with a 10 kDa Diaflo ultrafiltration membrane (Amicon Inc., MA, USA). Finally, each *PEGylated* protein were lyophilized and stored at –4 °C [14]. These lyophilized *PEGylated* proteins obtained by SEC were used to perform the studies of HIC separation.

2.4. Separation of *PEGylated* proteins by hydrophobic interaction chromatography (HIC)

An Äkta Purifier System (GE Healthcare, Uppsala, Sweden) was employed, equipped with a 100 μL injection loop. The chromatographic columns were 1 mL (100 mm × 5 mm ID) packed with Phenyl sepharose 6FF HS, Octyl sepharose or Butyl sepharose 4 FF. The experiments were performed at room temperature, using a flow rate equal to 0.8 mL/min and 25, 35 or 45 column volumes (CVs) [18].

Elution was obtained by a linear decreasing gradient of ammonium sulphate (AS) or sodium chloride. The initial eluent (solution B) utilized was 20 mM TRIS-HCl, pH 7.0 plus different concentrations of salt (AS or sodium chloride). The final buffer (solution A) used was 20 mM TRIS-HCl, pH 7.0. All buffers were filtered through 0.22-μm Millipore filters after preparation, and degassed with helium for 5 min. The column outlet was monitored at 215 nm. The conductivity was also monitored. Yield and purity were calculated using the plate model [16].

3. Results and discussion

3.1. SEC purification

Initially, the *PEGylation* reaction was monitored by SEC to obtain the *PEGylated* protein standards required for the HIC analysis. The reaction generates two *PEGylated* species: *monoPEGylated* RNase A and *diPEGylated* RNase A. These products were previously characterized [14]. Fig. 1 shows the chromatographic profile of SEC, where in addition to the *PEGylated* proteins, unmodified RNase A can be observed. Despite SEC being the most popular route for separation of *PEGylated* species [4], the resolution obtained is low. The plate

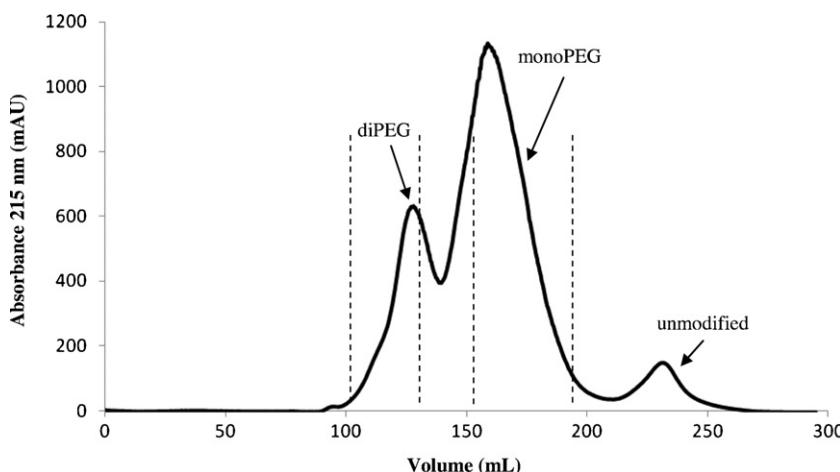


Fig. 1. Size exclusion chromatography profile of *PEGylated* reaction mixture. Column: Sephadryl S-300 HP. Isocratic mobile phase: 10 mM sodium phosphate buffer pH 7.2 + 150 mM KCl. Flow rate: 0.5 mL/min. The vertical lines show where the fractions were collected.

model was used to calculate the yield of the separation of monoPEGylated RNase A and its purity, obtaining values of 65% and 98% respectively. Each *PEGylated* specie was collected, concentrated, lyophilized and stored at -4°C .

3.2. HIC purification

For HIC purification, three different hydrophobic resins were tested: Butyl sepharose (weakly hydrophobic), Octyl sepharose (moderately hydrophobic) and Phenyl sepharose (strongly hydrophobic). Each *PEGylated* specie and unmodified RNase A was analyzed separately in HIC. Adsorption of proteins to HIC media is favoured by a high salt concentration, but due to differences in the interaction strength between the adsorbent and different proteins, the concentration of salt needed for adsorption can vary considerably [19]. Based on previous studies [14], high concentrations of ammonium sulphate (AS) were used in the initial experiments with the aim that unmodified RNase A was retained by the resins and desorbed in the gradient elution. As shown in Fig. 2, applying a linear gradient (25 CV) of ammonium sulphate resulted in complete separation of unmodified protein from mono and *diPEGylated* RNase A with all resins. The *PEGylation* effect changes the hydrophobicity of the protein. However, it is not sufficient to obtain a high resolution separation of the *PEGylated* species. Although there exist some differences between retention volumes of *PEGylated* proteins when employing Octyl and Phenyl sepharose (Fig. 1B and C), *diPEGylated* RNase A is completely included into the *monoPEGylated* RNase A. Namely, a stronger hydrophobic resin shows less resolution in the separation of *PEGylated* proteins. Moreover, it is observed that *PEGylated* proteins are not completely soluble at the concentrations of ammonium sulphate used in Fig. 2, while the native protein is completely soluble. Muller et al. [10] conducted a detailed study on the solubility of *PEGylated* conjugates of lysozyme, finding that the solubility in ammonium sulphate decreases with increased length of PEG chains linked to the protein. In our case the solubility decreases as the degree of *PEGylation* increases. When Butyl sepharose is used (Fig. 2A) a slight separation between the *PEGylated* proteins is observed, in fact this is the only profile where it is clear that the *monoPEGylated* protein is not totally pure. For this reason it was decided to refine the parameters of purification using Butyl sepharose.

The effect of AS concentration and gradient length on product separation is depicted in Fig. 3. It is observed that decreasing the concentration of salt did not result in native protein retention on the resin; however, the separation of *PEGylated* proteins is better. When

increasing the gradient length the difference was more evident. It is clear that there exists a difference between the hydrophobicity of proteins as a result of the *PEGylation* process, in this case the less hydrophobic protein is the native protein whereas the most hydrophobic is *diPEGylated* RNase A. The best conditions for the separation of *PEGylated* proteins include the use of 1 M AS and gradient elution of 35 CVs (Fig. 3D), under these conditions the native protein it is not retained by the adsorbent and all proteins are completely soluble in 1 M AS. It is not possible to work at a lower concentration than 1 M ammonium sulphate because all proteins are eluted in the dead time.

As mentioned before, the purity and yield were calculated using the plate model for the chromatograms shown in Fig. 3. Calculations were performed focusing on the purity of *monoPEGylated* RNase A. Table 1 shows that whilst the purity remained practically unchanged, the yield increases with decreasing salt concentration (AS). Fig. 4 clearly shows the fractionation of *PEGylated* proteins, using 1 M AS and 35 CVs. The use of these conditions resulted in a yield of up to 84.80% with a purity of 97%. Purity of *monoPEGylated* RNase A is comparable to that obtained when the separation is performed using SEC, but the yield increases from 65% with SEC to \sim 85% with HIC. On the other hand, Butyl sepharose is unable to retain the unmodified RNase A using 4 M NaCl (data not shown). The *PEGylated* proteins appear immediately at the start of the elution gradient at the same retention volume (\sim 10 mL).

Fig. 5 shows the effect of sodium chloride concentration on product separation using Phenyl sepharose as adsorbent. The unmodified protein is not retained by the resin; nevertheless, the *PEGylated* proteins are strongly adsorbed with a slight change in their retention volumes (\sim 3 mL Fig. 5A and \sim 5.5 mL Fig. 5B). The order of elution of the proteins remains equal to that obtained with Butyl sepharose – ammonium sulphate. Since the resolution of the peaks is lower, only two concentrations of NaCl were used with the same length of gradient elution (25 CVs). Unlike their behaviour with ammonium sulphate, in this case all proteins were completely soluble at the two concentrations of NaCl used, but that was not enough to achieve a definite separation between the *PEGylated* conjugates. Table 2 shows the yield and purity obtained from the chromatograms of Fig. 5. With purity similar to that obtained with Butyl sepharose – AS, the yield of *monoPEGylated* RNase A is between 46.9 and 57.5%.

Fig. 6 shows the results obtained for the separation of the products of the *PEGylation* reaction (without using SEC) after selection of the best conditions for the separation of bioconjugates. The mixture

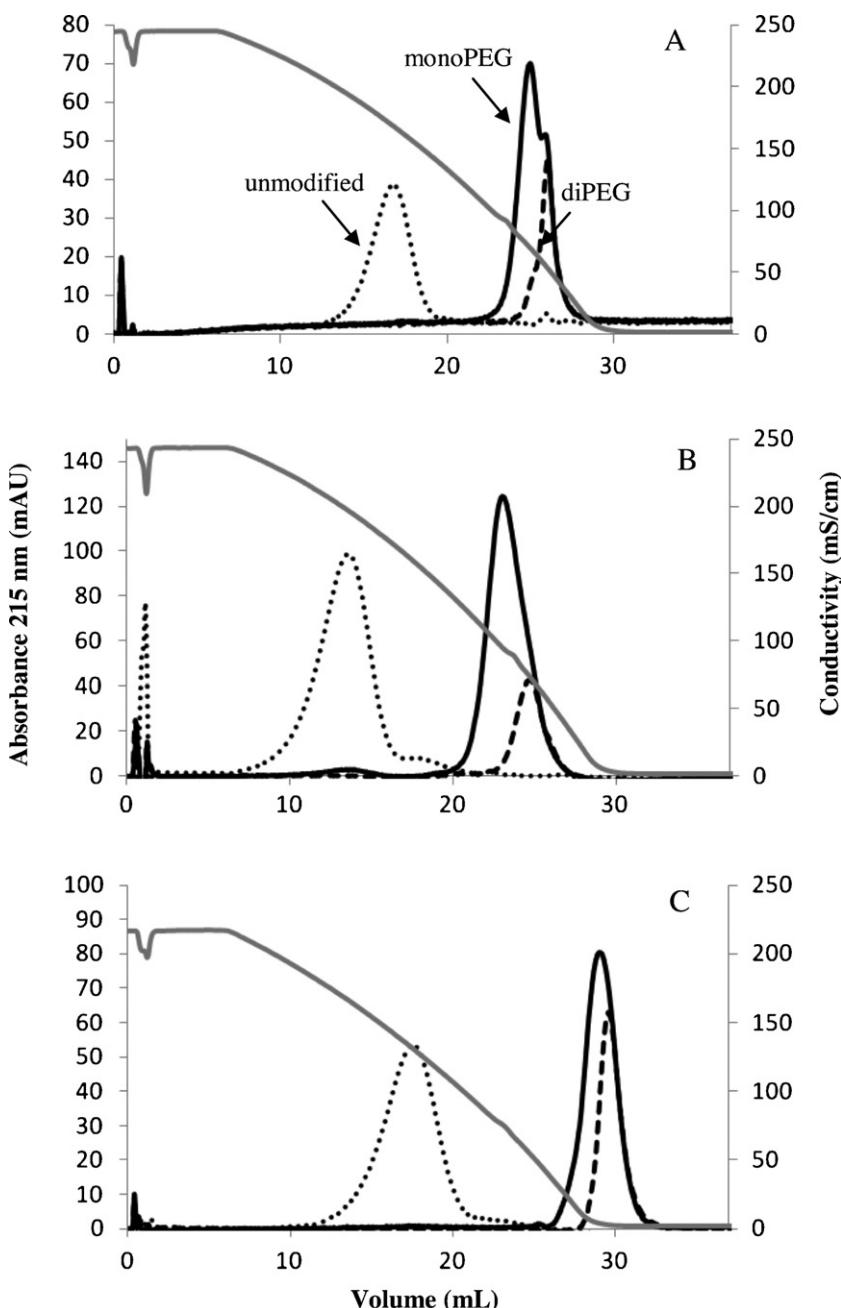


Fig. 2. Effect of type of resin in the purification of *monoPEGylated* RNase A on product separation. Buffer A: Tris-HCl 20 mM pH 7.0 Buffer B: Tris-HCl 20 mM pH 7.0 containing 2 or 2.5 M $(\text{NH}_4)_2\text{SO}_4$. Loop: 100 μL , flow rate: 0.8 mL/min. CV: 25. Gradient lineal from B to A. (a) Butyl sepharose, 2.5 M $(\text{NH}_4)_2\text{SO}_4$; (b) octyl sepharose, 2.5 M $(\text{NH}_4)_2\text{SO}_4$; (c) phenyl sepharose, 2 M $(\text{NH}_4)_2\text{SO}_4$. Each PEGylated protein and unmodified RNase A was analyzed separately. The chromatograms were superimposed.

Table 1

Recovery and purity of *monoPEGylated* RNase A using Butyl sepharose and different concentrations of ammonium sulphate. Data were calculated from the chromatograms of Fig. 3.

	AS (M)	CVs	Volume (mL)	Yield (%) ^a	Purity (%) ^a
Butyl sepharose	2.00	45	33.37	54.66	99.26
	1.75	35	26.80	62.30	98.50
	1.50	35	25.12	66.88	97.80
	1.00	35	17.10	84.80	97.00

AS, ammonium sulphate; CVs, column volumes.

^a Calculated using the plate model.

Table 2

Recovery and purity of *monoPEGylated* RNase A using phenyl sepharose and sodium chloride. Data were calculated from the chromatograms of Fig. 5.

	NaCl (M)	CVs	Volume (mL)	Yield (%) ^a	Purity (%) ^a
Phenyl sepharose	2.5	25	17.43	46.9	98.52
	2.0	25	15.06	57.5	97.62

CVs, column volumes.

^a Calculated using the plate model.

was injected directly into the column packed with Butyl sepharose. This validated the results obtained individually for each product of the reaction.

Previously we reported that the separation of the reaction of *PEGylation* of RNase A using a Tris-capped CH Sepharose 4B media

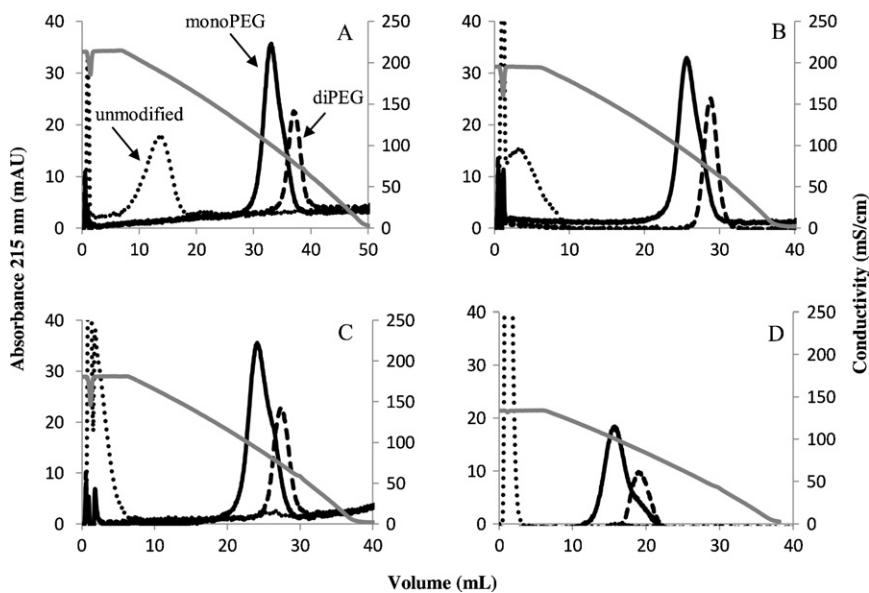


Fig. 3. Effect of ammonium sulphate concentration and gradient length on product separation. Resin: Butyl sepharose. Buffer A: Tris-HCl 20 mM pH 7.0 Buffer B: Tris-HCl 20 mM pH 7.0 containing 2, 1.75, 1.5 or 1 M $(\text{NH}_4)_2\text{SO}_4$. Loop: 100 μL , Flow rate: 0.8 mL/min. Gradient linear from B to A. [monoPEG RNase A]: 1.5 mg/mL, [diPEG RNase A]: 0.5 mg/mL, [RNase A]: 0.5 mg/mL. (A) 2 M, 45 CV; (B) 1.75 M, 35 CV; (C) 1.5 M, 35 CV; (D) 1 M, 35 CV. Each PEGylated protein and unmodified RNase A was analyzed separately. The chromatograms were superimposed.

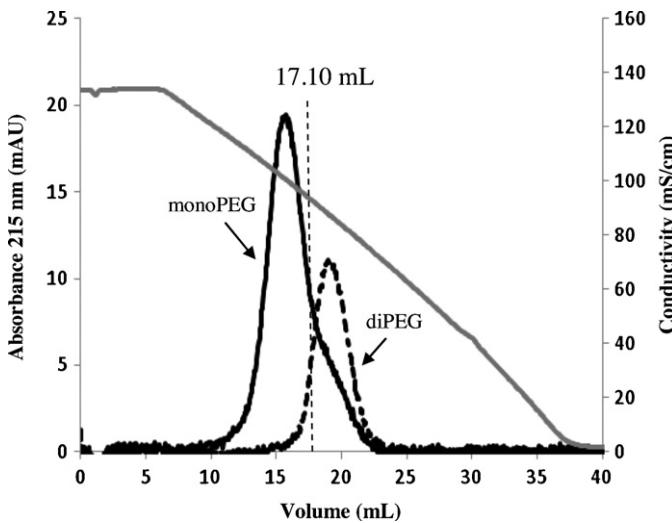


Fig. 4. Fractionation of monoPEGylated RNase A highly pure. Resin: butyl sepharose. Buffer A: Tris-HCl 20 mM pH 7.0 Buffer B: Tris-HCl 20 mM pH 7.0 containing 1 M $(\text{NH}_4)_2\text{SO}_4$, 35 CV. Yield: 84.80%, purity: 97%. Each PEGylated protein and unmodified RNase A was analyzed separately. The chromatograms were superimposed.

with salt gradient elution, resulted in the separation of only unmodified protein and the PEGylated conjugates eluted at virtually the same retention time [14]. Using hydrophobic resins such as Butyl and Phenyl sepharose, it is possible to separate PEGylated proteins. It is noteworthy that the elution order is the same for the system reported by Cisneros-Ruiz et al. [14] and mentioned here, regardless of the resin and/or salt used.

Muller et al. [10] reported the separation of reaction products of PEGylation of lysozyme with 5 kDa PEG, using a TSKgel Butyl-NPR column with 1.2 M ammonium sulphate; under these conditions, they achieved complete separation of the PEGylated conjugates from the unmodified lysozyme. Furthermore, contrary to our observations, the elution order changes depending on the type of salt. They inferred that the shift in the elution order can be explained

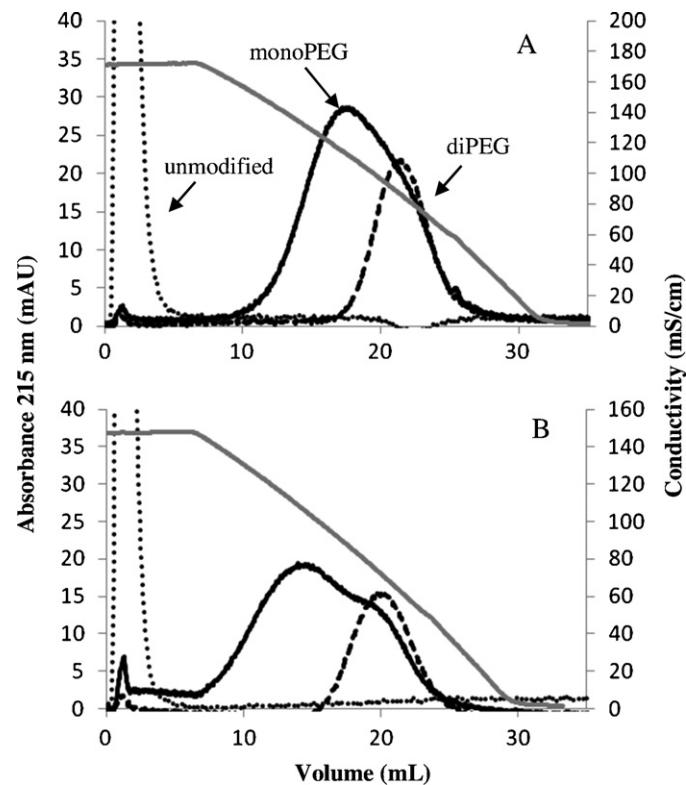


Fig. 5. Effect of sodium chloride concentration on product separation. Resin: phenyl sepharose. Buffer A: Tris-HCl 20 mM pH 7.0 Buffer B: Tris-HCl 20 mM pH 7.0 containing 2 or 2.5 M NaCl. Loop: 100 μL , flow: 0.8 mL/min. Gradient linear from B to A. [monoPEG RNase A]: 1.5 mg/mL, [diPEG RNase A]: 0.5 mg/mL, [RNase A]: 0.5 mg/mL. (A) 2 M, 25 CV; (B) 2.5 M, 25 CV. Each PEGylated protein and unmodified RNase A was analyzed separately. The chromatograms were superimposed.

by the different solubilities of the PEGylated lysozymes in the two different salt solutions (AS and NaCl). One possible explanation for this difference may be due to the length of the PEG and the intrinsic properties of the proteins.

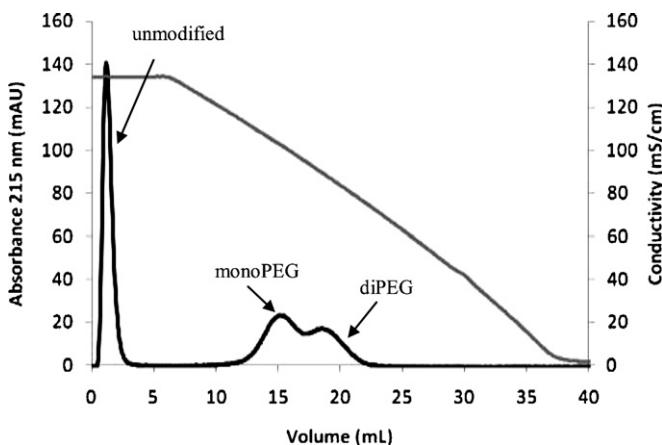


Fig. 6. Separation of products of RNase A PEGylation reaction using HIC. Resin: butyl sepharose. Buffer A: Tris-HCl 20 mM pH 7.0 Buffer B: Tris-HCl 20 mM pH 7.0 containing 1 M $(\text{NH}_4)_2\text{SO}_4$, 35 CV.

The PEGylated protein separation using HIC has been little investigated, especially as a first step in the purification process. It has been suggested that SEC followed by IEX and then HIC may form the basis for a general approach for the purification of PEG-protein conjugates [4,20]. The results shown here suggest that HIC can be used as the first stage in the PEGylated protein purification; however, in order to propose the general application it is necessary to optimize the process and analyze the separation of other monoPEGylated proteins. The results obtained here can serve as a basis for modelling elution curves of PEGylated conjugates and to facilitate the optimization of the purification process [18].

4. Conclusions

It is clear that HIC can be used to separate monoPEGylated RNase A, diPEGylated RNase A and native RNase A. Native RNase A could be separated completely from PEGylated proteins under all conditions assayed; demonstrating that the PEGylation affected the hydrophobicity of the protein, increasing it with increasing degree of PEGylation. The difference between the retention volumes of PEGylated proteins is higher when using a weak strength

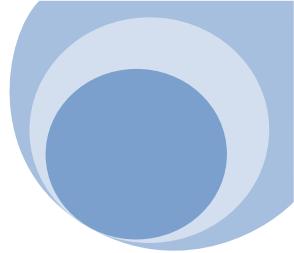
hydrophobic resin such as Butyl sepharose and ammonium sulphate mobile phase. While all proteins were soluble in sodium chloride; the resolution, yield, and purity were very low. The best conditions for the purification of monoPEGylated RNase A include the use of Butyl sepharose, 35 CV and 1 M ammonium sulphate; under these conditions, it is possible to obtain monoPEGylated RNase A with a yield as high as 85% and 97% purity.

Acknowledgements

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Anexo 2.

Presentaciones en congresos





hHydrophobic Interaction Chromatography for Purification of monoPEGylated Ribonuclease A

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Pegylated proteins are an important class of modern therapeutic drugs. When PEGylated proteins are synthesized a mix of products is generated that include the unmodified protein, PEG excess, proteins with different grades of PEGylation and in some cases, isomers. Due to the complexity of the reaction, the protein of interest must be highly purified. Chromatographic techniques are mainly used to resolve such mixtures, particularly size exclusion chromatography (SEC) and ion exchange chromatography (IEC). Very little work has been published regarding the use of hydrophobic interaction chromatography (HIC) in the resolution of PEGylated proteins mixture. In this work, we describe the purification of monoPEGylated RNase A using HIC. Butyl, octyl, and phenyl sepharose were used. The PEGylation reaction generates three main products: unmodified RNase A, monoPEGylated RNase A and diPEGylated RNase A. In this case, all resins are able to completely separate the unmodified protein. Analyzing the effects of the resin type, alt type, salt concentration, and salt gradient, it is possible to separate the PEGylated species. Considering the diPEGylated RNase A as the main impurity, yield and purity were calculated using the plate model. At its best conditions, butyl sepharose is able to separate the monoPEGylated RNase A with 97% of purity and a yield up to 80%. These results show that HIC can be implemented in one purification step to separate the PEGylation reaction products, without using another chromatographic process such as SEC.

Hydrophobic Interaction Chromatography for Purification of monoPEGylated Ribonuclease A

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One of the most frequently used methods for protein modification is the covalent attachment of poly(ethylene glycol), which is also called PEGylation [1]. PEGylated proteins are an important class of modern therapeutic drugs. When PEGylated proteins are synthesized a mix of products is generated that include the unmodified protein, PEG excess, proteins with different grades of PEGylation and in some cases, isomers [2]. Due to the complexity of the reaction, the protein of interest (generally the monoPEGylated protein) must be highly purified. Chromatographic techniques are mainly used to resolve such mixtures, particularly size exclusion chromatography (SEC) and ion exchange chromatography (IEX). Very little work has been published regarding the use of hydrophobic interaction chromatography (HIC) in the resolution of PEGylated proteins mixtures [3]. In this work, we describe the purification of monoPEGylated RNase A using HIC. Butyl, octyl, and phenyl sepharose were used. The PEGylation reaction generates three main products: unmodified RNase A, monoPEGylated RNase A and diPEGylated RNase A. Separation of the unmodified protein from PEGylated species is relatively easy. In this case, all resins are able to completely separate the unmodified protein. Analyzing the effects of the resin type, salt type (ammonium sulphate and NaCl), salt concentration, and salt gradient, it is possible to separate the PEGylated species. Considering the diPEGylated RNase A as the main impurity, yield and purity were calculated using the plate model. At its best conditions, butyl sepharose is able to separate the monoPEGylated RNase A with 97% purity and a yield up to 80% (Figure 1). These results show that HIC can be implemented in one purification step to separate the PEGylation reaction products, without using another chromatographic process such as SEC.

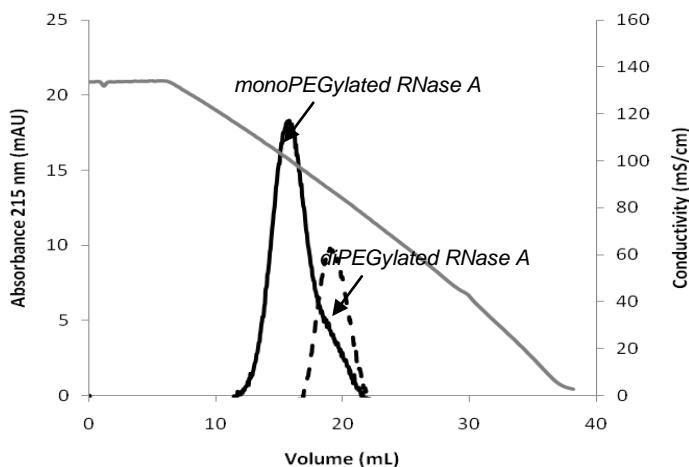


Figure 1. Purification of monoPEGylated RNase A using butyl sepharose. Conditions: ammonium sulphate 1M, 35 CV gradient.

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XIV Congreso Nacional de Biotecnología y Bioingeniería



PURIFICACION DE RNasa A MONOPEGILADA UTILIZANDO CROMATOGRAFIA DE INTERACCION HIDROFOBICA (HIC)

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Palabras clave: PEGilación, purificación, HIC.

Introducción. Las proteínas PEGiladas son una importante clase de drogas terapéuticas modernas. Al producir proteínas PEGiladas se genera una mezcla de reacción que incluye la proteína nativa, el exceso de polietilenglicol (PEG), proteínas con diferentes grados de PEGilación y en algunos casos, isómeros[1]. Debido a la complejidad de la reacción, la proteína de interés (generalmente la proteína monoPEGilada) debe de ser altamente purificada. Las técnicas cromatográficas son las más utilizadas para resolver dichas mezclas, especialmente las cromatografías de exclusión molecular (SEC) e intercambio iónico (IEX). En comparación, muy poco trabajo se ha publicado sobre el uso de Cromatografía de Interacción Hidrofóbica (HIC) en la resolución de mezclas de proteínas PEGiladas [2]. El objetivo de este trabajo es establecer las condiciones óptimas de separación de RNasa A monoPEGilada utilizando Cromatografía de Interacción Hidrofóbica.

Metodología. RNasa A fue PEGilada utilizando mPEG (20kDa) [2]. Los productos de la reacción fueron separados por SEC, concentrados por UF (10 kDa) y lyophilizados [2]. Para la separación en HIC se utilizó una columna de 1 mL (100mm x 5mmID) y las resinas: *phenyl*, *octyl* y *butyl sepharose*. Los experimentos se realizaron a un flujo de 0.8 mL/min. Se analizaron los efectos del tipo de resina, tipo y concentración de sal (NaCl y sulfato de amonio (SA)) y CV. El rendimiento y la pureza se calcularon utilizando el modelo de platos.

Resultados. La reacción de PEGilación fue analizada y separada por SEC, de dicha reacción se obtuvieron: RNasa A monoPEGilada, RNasa A diPEGilada y la proteína no modificada. Los productos de la reacción fueron analizadas por separado en columnas empacadas con resinas hidrofóbicas. Utilizando un gradiente de 25 CV y SA, la *butyl sepharose* es la única resina en la que se aprecia una mayor diferencia en los tiempos de retención de las proteínas PEGiladas, mientras que las 3 resinas son capaces de separar la proteína nativa de las especies PEGiladas dentro del gradiente de elución. Cuando se utiliza NaCl es posible separar las proteínas utilizando *phenyl sepharose*, sin embargo la resolución es menor. Por lo anterior se seleccionó a la resina *butyl sepharose* para optimizar la separación de RNasa A

monoPEGilada. En la tabla 1 se muestran los resultados obtenidos variando la concentración de la sal y los CV.

Tabla 1. Rendimiento y pureza de la RNasa A monoPEGilada obtenidos cuando se utiliza la resina *butyl sepharose*.

	SA	CV	Rendimiento (%)	Pureza (%)
Butyl sepharose	2	45	54.66	99.26
	1.75	35	62.30	98.50
	1.5	35	66.88	97.80
	1	35	84.80	97.00

Al disminuir la concentración de sal el rendimiento aumenta y el % de pureza sigue siendo alto. En la Fig. 1 se muestra parte del perfil cromatográfico en donde se aprecia la separación de las proteínas PEGiladas, bajo estas condiciones, la proteína nativa ya no es retenida por el gradiente y sale en el tiempo muerto.

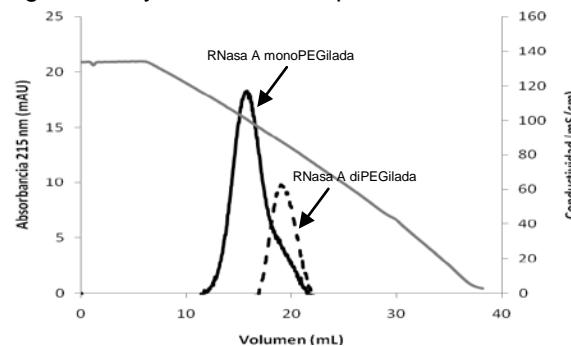


Fig. 1. Purificación de RNasa A monoPEGilada utilizando *butyl sepharose*. Condiciones: 1M SA, 35 CV.

Conclusiones. HIC puede ser utilizada para purificar RNasa monoPEGilada con una pureza del 97% y un rendimiento mayor al 80%, cuando se utiliza *butyl sepharose* y SA 1M. HIC es capaz de resolver la mezcla de la reacción de PEGilación en un solo paso.

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