BIOTECHNOLOGY

Purification of PEGylated Protein Using Membrane Chromatography

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ABSTRACT: N-terminus-specific PEGylation was used to produce mono-PEGylated lysozyme. However, some di- and tri-PEGylated proteins were also produced due to side chain reaction. The reaction products were characterized by chromatographic and electrophoretic methods. Commercial cation exchange membrane Sartobind S was used for chromatographic purification of PEGylated lysozyme, the basis of separation being the shielding of protein charge by PEG. Using the membrane chromatographic method, lysozyme and mono-, di-, and tri-PEGylated lysozyme could be resolved into separate peaks. Increasing the superficial velocity during chromatographic separation from 24 cm/h to 240 cm/h increased both protein binding capacity and resolution due to enhancement of protein mass transfer coefficient. © 2010 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 99:3326–3333 2010

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INTRODUCTION

PEGylation refers to the modification of (mainly) proteins or peptides by conjugation with polyethylene glycol (PEG). PEGylation prolongs the *in vivo* half-life of protein drugs and reduces enzymatic degradation and immunogenicity, thereby greatly enhancing the clinical efficacy and acceptability of therapeutic proteins. PEGylated proteins comprise an emerging class of biopharmaceuticals, several PEGylated products having been approved by FDA and some having achieved significant clinical and market success, for example, PEG-interferon α2a (Pegsys®),[1] PEG-interferon α2b (PEG-Intron®),[2] and PEG-G-CSF (Neulasta®).[3] Many products such as PEGylated antibody fragments are in different stages of development.

Approaches used for protein PEGylation can be classified into two categories, (a) general and (b) site-specific. The general approach involves random PEGylation, mainly at lysine residues.[4] This results in the formation of a heterogeneous mixture of proteins with varying degrees of PEGylation, necessitating complicated purification processes for fractionating differently PEGylated forms. Where a heterogeneous product is acceptable, batch-to-batch reproducibility of reaction sites and composition become crucial regulatory issues. There have been several attempts to PEGylate proteins through free cysteine or oxidized carbohydrates.[5–7] Due to the scarcity of these groups these are not widely applicable. If proteins do not contain free cysteine but instead contain paired disulfide bonds, PEGylation through these bonds could also be carried out.[8] This approach while being site-specific requires a complex sequence of reactions. N-terminus protein PEGylation which is site-specific to the α-amino group can be carried out with all proteins. This approach utilizes the pK_a difference between the α-amino group (pK_a 7.6–8.0) and the ε-amino group of the lysine residues (pK_a 10.0–10.2).[9] At acidic condition, mPEG-aldehyde can react largely selectively with the α-amino group to produce a Schiff base intermediate.[10] This intermediate can then be reduced to conjugate PEG with the protein through a secondary amide bond. The use of this approach has been reported for production of PEG-G-CSF (Neulasta®)[3] and PEGylated epidermal growth factor (EGF),[11] insulin,[12] interferon β-1b,[13] and consensus interferon.[14]

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Most PEGylated proteins are purified by column chromatography, primarily size exclusion chromatography (SEC) and ion-exchange chromatography (IEC). SEC which is based on solute size difference is limited both by the small amount of sample that can be loaded as well as by the low mobile phase flow rate, and is therefore mainly used for analytical separations. IEC is based on charge differences, uncharged PEG being able to shield the intrinsic charge on the protein, the extent of shielding increasing with the degree of PEGylation. However, column based IEC based on porous particulate chromatography is diffusion limited and this restricts the speed of separation. Typical superficial velocities reported during separation of PEGylated proteins using IEC include 90 cm/h for purification of PEG-G-CSF, 100 cm/h for purification of PEG-GRF (1–29), and 136 cm/h for purification of PEG-IFN.[15–17] The binding capacity of PEGylated proteins on IEC media is normally an order of magnitude lower than that of native proteins.[18,19] This phenomenon has been explained both in terms of charge shielding and increase in hydrodynamic radius (leading to decrease in diffusivity). While this is convenient for separation of native proteins from PEGylated proteins in general, it makes it difficult to fractionate differently PEGylated forms. The current work shows that even with a site-specific reaction strategy, minor quantities of other PEGylated forms are also produced and need to be separated from the desired form. Some researchers have used monolithic chromatographic media such as CIM^{TM} to overcome limitations of particulate media based chromatography.[20] Although high flow rates can be used with the CIM^{TM} media, high back pressure is generated, for example, at 300 cm/h, SO_3-CIM^{TM} gave a backpressure of 3.90 MPa while QA-CIM^{TM} could not be operated at this flow rate due to overpressure. Severe peak broadening was also observed, particularly with SO_3-CIM^{TM}.

Membrane chromatography which uses stacks of microporous membranes as chromatographic media is suitable for rapid separation of proteins and other bio-macromolecules, a feature made possible by the predominance of convective mass transport.[21] We hypothesized that the large flow-through pores present on an adsorptive membrane would make it particularly suitable for processing large bio-macromolecules such as PEGylated proteins at relatively low backpressure. In this article, membrane chromatography is suggested as an alternative to both particulate media based and monolith chromatography for purification of PEGylated proteins. N-terminus PEGylation of lysozyme, a model protein is first systematically optimized. The separation of mono-PEGylated lysozyme from native lysozyme and other PEGylated forms is then attempted using ion-exchange membrane chromatography (IEMC) using commercial Sartobind S membranes. IEMC has been successfully applied for purification of other therapeutic proteins.[22,23] To the best of our knowledge, this is the first study on the use of membrane chromatography for purification of PEGylated proteins. The effects of key process parameters such as flow rate on membrane binding capacity, separation, and backpressure are studied.

EXPERIMENTAL METHODS

Materials

Lysozyme (L6876), glycine (G8898), Trizma base (T1503), sodium chloride (S7653), sodium cyanoborohydride (156159), barium chloride (202738), iodine (326143), hydrochloride acid (258148), 25% glutaraldehyde solution (G6257), and 70% perchloric acid (77227) were purchased from Sigma–Aldrich (St. Louis, MO). Sodium acetate trihydrate (SX0255-1) and glacial acetic acid (AX0073-6) were purchased from EMD (Gibbstown, NJ). Potassium iodide (74210-140) was purchased from Anachemia (Montreal, QC, Canada). mPEG-Propionaldehyde 5000 and 10000 (P1PAL-5 and P1PAL-10) were purchased from Sunbio, Inc. (Anyang, South Korea). Low molecular weight (LMW) protein calibration kit (28-4038-41) was purchased from GE Healthcare Bio-Sciences (Piscataway, NJ). High quality purified water (18.2 MΩ cm) obtained from a Diamond^{TM} NANOpure (Barnstead, Dubuque, IA) water purification unit was used to prepare all the test solutions and buffers. Sartobind S cation exchange membrane (94IEXS42-001, 275 μm thickness) was purchased from Sartorius (Gottingen, Germany).

Lysozyme PEGylation

PEGylation reactions were carried out in the small flasks with continuous magnetic stirring at room temperature. The reaction mixture consisted of lysozyme, mPEG-aldehyde and sodium cyanoborohydride in 100 mM acetate buffer. The concentration of lysozyme was kept fixed at 1 mg/mL. To study the effect of pH on PEGylation, reactions were carried out for 5 h using acetate buffer at different pH values, that is, pH 4.0, 4.5, 5.0, 5.5, and 6.0 while maintaining the mPEG/lysozyme molar ratio at 4:1 and the sodium cyanoborohydride concentration at 10 mM. The effect of mPEG/lysozyme molar ratio (i.e., 2:1, 4:1, and 6:1) on PEGylation was examined at the optimized pH value and the samples were taken at 5 and 20 h. A reaction was also carried out at molar ratio of 4:1 using 20 mM sodium cyanoborohydride to examine the effect of this reagent on PEGylation. To study the effect of reaction time, 4:1 mPEG/lysozyme molar ratio and 10 mM sodium cyanoborohydride concen-