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Effect of electrostatic interactions on transmission of PEGylated proteins through charged ultrafiltration membranes

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ABSTRACT

Recent experiments have demonstrated that PEGylated proteins, produced by covalent attachment of a polyethylene glycol chain to a native protein, can be purified using negatively charged ultrafiltration membranes. However, the nature of the electrostatic interactions governing the transmission of these PEG–protein complexes during ultrafiltration has not been examined. Experimental data were obtained for the transmission of PEGylated α-lactalbumin through both unmodified and negatively charged composite regenerated cellulose membranes over a range of solution ionic strength. The effect of the polyethylene glycol on the protein was also studied by measuring the electrophoretic mobility of both PEGylated and acetylated versions of the α-lactalbumin. The attachment of the PEG altered the protein charge, the effective size, and the electrostatic potential surrounding the protein. The PEGylated α-lactalbumin was strongly retained by the negatively charged membrane due to the significant electrostatic interactions in this system. The data were consistent with a simple model of the PEGylated protein as an effective hard sphere with the surface charge shifted to the outer edge of the PEG by accounting for the alteration in the electrostatic potential associated with the reduced electrolyte concentration within the PEG layer. These results provide important insights into the role of electrostatic interactions on the retention of PEGylated proteins during ultrafiltration.

1. Introduction

There is growing clinical interest in the use of protein–polymer conjugates as "second generation" therapeutics, with polyethylene glycol (PEG) being the most commonly used polymer [1–3]. PEGylated proteins can have significantly longer half-lives and lower immunogenicity than the corresponding native protein, particularly for smaller proteins that are rapidly cleared from the bloodstream by the kidney. Clinically approved versions of PEGylated interferon, PEGylated human granulocyte colony-stimulating factor, and PEGylated asparaginase have all shown marked improvements in therapeutic efficacy compared to the recombinant protein alone [4–6].

One of the challenges in producing protein–polymer conjugates is the need to remove residual polymer, native (un-reacted) protein, and any reaction by-products from the final therapeutic formulation. Molek and Zydney [7] recently demonstrated that it is possible to use a two-stage membrane ultrafiltration system for the purification of a model PEGylated protein. The first stage employed a traditional regenerated cellulose membrane, which allowed the small un-reacted protein to be removed in the permeate with the large PEGylated protein (and PEG) retained by the membrane. The second stage employed a negatively charged membrane that provided high retention of the PEGylated protein while passing the neutral PEG. The high retention of the PEGylated protein appeared to be a direct result of the strong electrostatic repulsion between the charged protein and the charged membrane. This strong electrostatic effect was in sharp contrast to the significant reduction in binding seen in ion exchange chromatography of PEGylated proteins in which the polyethylene glycol layer apparently shields or blocks the electrostatic binding interactions [8].

The importance of electrostatic interactions in protein ultrafiltration has been well established over the past decade. Pujar and Zydney [9] showed that a reduction in solution ionic strength caused a 100-fold decrease in albumin transmission through a negatively charged ultrafiltration membrane. Similar effects have been seen with a number of other proteins over a range of solution pH [10] and membrane surface charge density [11]. Several studies [11,12] have shown that the magnitude of these electrostatic interactions can be well described using the theoretical analysis developed by Smith and Deen [13] for the partitioning of a charged sphere in an infinitely long charged cylindrical pore:

$$S_a = (1-\lambda)^2 K_c \exp\left(\frac{-\psi_E}{k_B T}\right) \quad (1)$$

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where S_a is the actual sieving coefficient, defined as the ratio of the protein concentration in the filtrate solution to that in the solution immediately upstream of the membrane. Eq. (1) is only valid at high filtration velocities where solute diffusion across the membrane is negligible relative to the convective flux. The term $(1-\lambda)^2$ describes the steric (hard sphere) exclusion of the sphere from the region within one solute radius of the pore wall (with λ equal to the ratio of the solute radius to the pore radius). K_c is the hindrance factor associated with convection and (ψ_E/k_BT) is the dimensionless electrostatic energy of interaction [13]:

$$\frac{\psi_E}{k_BT} = A_s\sigma_s^2 + A_{sp}\sigma_s\sigma_p + A_p\sigma_p^2 \quad (2)$$

where A_s, A_{sp}, and A_p are functions of the solution ionic strength, solute size, and pore size (equations given in [12]), and σ_s and σ_p are the dimensionless surface charge densities of the solute (protein) and pore. The three terms in Eq. (2) represent the energy of interaction associated with the distortion of the electrical double layer around the solute, direct charge–charge interactions between the solute and the pore, and the distortion of the electrical double layer adjacent to the pore wall, respectively.

Eqs. (1) and (2) were developed for hard sphere solutes in which the charge is uniformly distributed over the external surface of the sphere. There are currently no experimental or theoretical results for the possible effect of the attached polyethylene glycol in a PEGylated protein on the nature of these electrostatic interactions. The objective of this study was to obtain quantitative data for the effect of solution ionic strength and membrane surface charge on the transmission of PEGylated proteins during ultrafiltration and to develop a more fundamental understanding of how the PEG layer alters the intermolecular electrostatic repulsion between the PEGylated protein and the membrane pore. Data were obtained with unmodified and negatively charged membranes using PEGylated α-lactalbumin over a range of ionic strength, with the charge characteristics of the proteins also studied using capillary electrophoresis. A simple theoretical framework was developed to describe the electrostatic interactions of the PEGylated proteins, with the model in good agreement with the experimental data.

2. Materials and methods

2.1. Protein preparation

Experiments were performed with PEGylated α-lactalbumin having one or more 2, 5, 10, 20, or 30 kDa PEG branches. The PEGylated α-lactalbumin was prepared by reaction of the native protein (obtained from Sigma Chemicals, Catalog Number L5385, MW = 14.2 kDa) with an N-hydroxysuccinimide (NHS) ester-activated PEG obtained from either Nektar Therapeutics (Huntsville, AL) or NOF Corporation (Tokyo, Japan). The α-lactalbumin was dissolved in a 10 mM Bis–Tris buffer at pH 7. The activated PEG was then added and the solution was slowly stirred at room temperature (21–24 °C) for a minimum of 8 h to allow the reaction to go essentially to completion. The resulting product solution, which contained the PEGylated α-lactalbumin, the un-reacted protein, the hydrolyzed PEG reagent, and N-hydroxysuccinimide (produced from hydrolysis of the activated PEG), was then diluted approximately 4-fold with Bis–Tris buffer. The solution was prefiltered through a 0.2 μm pore size Acrodisc syringe filter (Pall Corporation, Ann Arbor, MI) to remove any particulate matter and larger aggregates prior to use. The solution ionic strength was adjusted to the desired value by addition of 1 M KCl or NaCl containing the buffer species of interest or the solution was diafiltered through a 10 kDa Ultracel™ membrane using an appropriate diafiltration buffer. PEGylated proteins were stored at 4 °C when not in use. Additional details on the preparation of the PEGylated protein are provided elsewhere [14].

To obtain additional insights into the nature of the electrostatic interactions, experiments were also performed with acetylated α-lactalbumin in which the free amine group on one or more lysine amino acids was reacted with acetic anhydride (instead of an activated PEG). Acetylated α-lactalbumin was synthesized using the general procedure described by Gao and Whitesides [15]. α-lactalbumin was added to deionized water to a concentration of 2 g/L. The solution was chilled in an ice bath to 5 °C and the pH adjusted to 12 by addition of 0.1 M NaOH. Four molar equivalents of acetic anhydride (as a 9.75 g/L solution in dioxane) were then added to the protein solution. The reaction mixture was stirred continuously for 15 min while slowly adding 0.1 M NaOH to maintain pH ≈ 12. The reaction was then quenched by adding 0.5 M HCl to rapidly reduce the pH to approximately 6. A constant volume diafiltration was performed through a 10 kDa Ultracel™ membrane (Millipore Corp., Bedford, MA) using 10 mM Bis–Tris buffer for a minimum of five diavolumes to remove acetic acid, un-reacted acetic anhydride, and other small impurities. The resulting solution was then filtered through a 0.2 μm Pall Supor Acrodisc syringe filter prior to use.

2.2. Protein ultrafiltration

Ultrafiltration experiments were performed in a 25 mm diameter stirred ultrafiltration cell (Amicon Model 8010, Millipore Corp., Bedford, MA) using Ultracel™ composite regenerated cellulose membranes (Millipore Corp.) with a 100 kDa nominal molecular weight cut-off. Negatively charged versions of the Ultracel™ membranes were produced by chemical modification of the base cellulose by attachment of sulfonic acid groups using the base activated chemistry described by van Reis [16]. Membranes were first soaked in 0.1 M NaOH for 12 h and then immersed in a 2 M solution of 3-bromopropanesulfonic acid sodium salt (Catalogue #B2912, Sigma Chemical) in 0.1 N NaOH for an additional 12 h. The membrane was then thoroughly washed with 0.1 M NaOH followed by DI water, 0.2 M acetic acid, DI water again, and finally the buffer solution that was to be used in the ultrafiltration experiment.

Membranes were placed in the bottom of the stirred cell and flushed with at least 40 L/m^2 of Bis–Tris buffer prior to exposure to protein. The membrane hydraulic permeability (L_p) was evaluated by measuring the filtrate flux as a function of the transmembrane pressure using at least five different pressures between 3.4 and 76 kPa (0.5 and 11 psi). The membrane was then soaked overnight in a solution containing the PEGylated protein. The device was then air-pressurized, with the filtrate flux controlled by a pressure regulator (Scott Specialty gases, Plumsteadville, PA). The pressure was measured by an Ashcroft Model 0518 (0–30 psi) or Model 8920 (0–15 psi) pressure gauge. A minimum of 4 mL of filtrate was passed through the membrane to ensure stable operation; this also served to flush the dead space beneath the membrane in the stirred cell. For each experimental condition, a small filtrate sample was collected followed directly by a small sample of the bulk solution from the stirred cell. The stirred cell was then refilled with the PEGylated protein solution and a repeat measurement obtained. The stirred cell was then emptied and refilled with 10 mM Bis–Tris with 500 mM KCl at pH 7.0 to re-evaluate the hydraulic permeability. The process was repeated using solutions of different ionic strength.

Size exclusion chromatography was used to evaluate the concentrations of the PEGylated α-lactalbumin, the un-reacted protein, and the PEG in the filtrate and bulk samples. Data were obtained using a Superdex 200 G/L (GE Healthcare, Uppsala, Sweden) column with a running buffer of 150 mM NaCl with 50 mM phosphate buffer at pH 7.0 using a flow rate of 0.3 mL/min. Sample detection was performed using an Agilent 1100 series refractive index detec-