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Separation of PEGylated variants of ribonuclease A and apo-α-lactalbumin via reversed phase chromatography

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ABSTRACT

The covalent attachment of polyethylene glycol (PEG) molecules to pharmaceutical proteins, "PEGylation", often results in a population of conjugate species that includes differing numbers and locations of attached PEG chains. As some portion of this population may be biologically inactive, a challenging separation problem arises. An interesting alternative to the size-based resolution of these conjugates involves the use of reversed phase chromatography (RPC), treating the PEG moieties as hydrophobic purification tags. The use of RPC raises concerns about protein denaturation in the mobile and on the stationary phase. Here, the potential dual role of conjugated PEG chains as both group-specific separation tags and as steric or structural stabilizers in RPC was explored. In this work, RPC with C18-based media was used to resolve PEGylation number variants of ribonuclease A (RNase A) and apo-α-lactalbumin (apo-αLac) in a neutral pH mobile phase. While the attachment of 20 kDa PEG molecules did not modify the structures of RNase A and apo-αLac, as confirmed by structural analysis using circular dichroism, exposure to the mobile phase modifier, acetonitrile, and to the C18 media during separation resulted in perturbations to both the secondary and tertiary structures of all species studied. RNase A experienced small perturbations that were mediated to some extent by PEGylation; these results were consistent with activity assays which showed that PEGylated RNase A species retained native-like activity after RPC separation. Apo-αLac, a more hydrophobic and less stable protein than RNase A, experienced extensive structural perturbations regardless of PEGylation state. The temperature of the mobile phase was found to strongly influence chromatographic separation of PEG-conjugates with conjugate species becoming more strongly retained with increasing temperature. This work shows that it is feasible to employ RPC with neutral pH mobile phases to resolve PEG conjugate number heterogeneity.

1. Introduction

The covalent attachment of polyethylene glycol (PEG) chains to protein drugs, a process called "PEGylation", can mitigate protein susceptibility to destruction by proteolytic enzymes, short circulating half-life, short shelf-life, rapid kidney clearance and propensity to generate neutralizing antibodies [1]. Pharmacokinetic properties can be improved by increasing the in vivo circulation half-life, which implies the prolongation of the therapeutic effects with a concomitant reduction in required dosage and improvement of patient comfort by reducing the number and frequency of required injections [2]. During the last several years, the US Food and Drug Administration has approved several PEGylated therapeutic proteins to treat different diseases in humans, including bovine adenosine deaminase for severe combined immunodeficiency disease, α-interferon for hepatitis C, L-asparaginase for acute lymphoblastic leukemia [3,4], granulocyte colony-stimulating factor [5] to stimulate white blood cell production in chemotherapy patients and a growth hormone antagonist to treat acromegaly [6].

Conjugation may result in the generation of a family of species characterized by a distribution in number and position of attached PEG groups [2]. The composition of the product is dependent on the number and local reactivity of the attendant attachment sites (primary amines and sometimes other nucleophilic groups) on the protein, the reactivity of the activated PEG reagent and the conditions of the modification reaction [7]. As PEGylated variants are

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not equal in their effectiveness [8,9], it is desirable to resolve heterogeneous conjugate mixtures into effective subpopulations using adequate separation strategies [10] and/or to limit the breadth of the conjugate distribution during the conjugation reaction itself [11,12]. Hybrid approaches such as size exclusion reaction chromatography, which combine reaction and separation unit operations, are also under development [13]. Near-term solutions at scale will likely be concentrated on the population resolution route.

Size exclusion chromatography (SEC) and ion exchange (IEX) [14] are the most popular methods for the separation of conjugate species [2,3,15]. SEC is very efficient in removing low molecular weight impurities as well as unreacted protein. SEC has several limitations, including the inability to separate positional isomers of the same molecular weight, poor resolution for PEG-proteins [3], long processing times, high buffer consumption and significant product dilution. For IEX, a difference in the charge properties of the species is required for resolution. The attachment of PEG chains may remove a charged group at the site of covalent modification, sterically protect surface charges or alter the pK_a of neighboring ionizable surface residues via hydrogen bonding interactions. However, in practice, the p*I*s of PEGylation variants do not differ significantly, preventing their separation [15]. PEGylation should affect protein surface hydrophobicity, increasing or decreasing it depending on the native protein's hydrophobicity 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 [16,17].

A possible alternative method for the resolution of PEG-protein conjugate populations at scale is reversed phase chromatography (RPC), making use of the pendant PEG chains as hydrophobic separation tags for the conjugates [16,18]. RPC is a powerful separation technique that has been used in the resolution of proteins with small differences in physical properties, including single amino acid variants. RPC has also been used to separate PEG-conjugates for analytical purposes [8,19]. The present work explores the possibility that conjugated PEG chains can play dual roles as both group-specific separation tags and as structural stabilizers in RPC-based protein purification processes, allowing active PEG-conjugates to be resolved and recovered after processing via RPC.

Our model proteins for this study were ribonuclease A (RNase A) and apo-α-lactalbumin (apo-αLac). Both proteins have therapeutic potential. RNase A has potential as an aspermatogenic and antitumor agent; studies have shown an improvement in the therapeutic effect when RNase A is conjugated to PEG [20]. Some forms of αLac can induce apoptosis in tumor cells, which suggests that the protein can fulfill many important biological functions [21]. RNase A is a small protein (13,686 Da MW) with 124 amino acid residues that lacks tryptophan residues and has an isoelectric point at 9.3 [22]; α-lactalbumin is also a small protein, containing 123 amino acids (14,200 Da MW) and has an isoelectric point between 4 and 5 [21]. These proteins bracket a wide range of solution stability: RNase A is very stable with a neutral pH unfolding free energy ($\Delta G_{unfolding}$) between 8.46 and 14.8 kcal mol^{-1} [23,24] whereas apo-αLac is marginally stable with a neutral pH $\Delta G_{unfolding}$ of 3.4 kcal mol^{-1} [25,26].

In this study, RNase A and apo-αLac were conjugated with 20 kDa PEG chains using an aldehyde-based chemistry that attacks exposed primary amine groups on the protein surface with preference for the N-terminus due to its lower pK_a. This molecular weight was selected as current PEGylated therapeutics favor PEGs with high molecular weight due to the improved in vivo clearance behavior [1]. The resulting PEG-conjugates of both proteins were separated via RPC under neutral pH conditions. The secondary and tertiary structures as well as the enzymatic activity (RNase A) of each species were assessed before and after the RPC processing in order to identify any structural perturbations. The effect of temperature on the RPC separation behavior of PEG-conjugates was also evaluated. The results obtained should be useful in determining the appropriate conditions for the resolution of PEGylation variants via RPC.

2. Materials and methods

2.1. Materials

Bovine pancreatic ribonuclease A (cat. no. R5000) and α-bovine lactalbumin (cat. no. L5385) were purchased from Sigma-Aldrich (St. Louis, MO). Methoxy-poly (ethylene glycol)-butyraldehyde with a nominal molecular mass of 20 kDa was obtained from Nektar Therapeutics (Huntsville, AL) and sodium cyanoborohydride from ICN Biomedicals Inc. (Costa Mesa, CA). Conjugation and purification buffers were prepared from sodium phosphate monobasic and dibasic salts (Fisher Scientific), and potassium chloride from Sigma–Aldrich (St. Louis, MO). HPLC-grade acetonitrile was obtained from Fisher Scientific (Pittsburgh, PA). Silica-based C18 bulk chromatographic media (cat. no. 218TPB1015) with 10–15 μm particle size, 300 Å pore size, 60–110 m^2 g^{-1} surface area and 8 wt% carbon load was obtained from Vydac (Hesperia, CA). All water used throughout was purified by reverse osmosis and polished to >18 mΩ cm using a Barnstead NANOpure Diamond system.

2.2. PEGylation reaction

A solution of RNase A or apo-αLac (6 mg) at 3 mg mL^{-1} 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 molecular mass methoxy poly(ethylene glycol) butyraldehyde [18]. When apo-αLac was reacted, the buffer contained 2 mM EDTA. The reaction mixture was stirred rapidly for 17.5 h at 4 °C [16,27]. After this time, the reaction mixture was stored no longer than 3 days at 4 °C prior to analysis and separation.

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

The reaction mixture (2 mL) was analyzed via size exclusion chromatography with an Äkta Explorer 100 system (GE Healthcare, Uppsala, Sweden) using a Sephacryl S-300 column (1.6 cm inner diameter, 60 cm length, GE Healthcare, 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^{-1} [18,27]. When PEG-α-lactalbumin species were separated, the buffer contained 2 mM EDTA. 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 diafiltered with five volumes of 25 mM sodium phosphate buffer, pH 7.2. RNase A concentration was measured spectrophotometrically using absorbance at 280 nm and a measured extinction coefficient of 8045 AU M^{-1} cm^{-1}. Apo-αLac concentrations were similarly determined using a measured extinction coefficient of 23,556 AU M^{-1} cm^{-1}.

2.4. Mass spectrometry analysis by MALDI-TOF

Each peak collected from SEC experiments was analyzed by mass spectrometry. Prior to analysis the samples were desalted using centrifugal filters (Microcon YM-3, Millipore). MALDI-TOF/MS analysis was performed with a PerSeptive (Framingham, MA) Voyager STR mass spectrometer fitted with a standard 337 nm nitrogen laser. Mass spectra were recorded with the