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Solubility and binding properties of PEGylated lysozyme derivatives with increasing molecular weight on hydrophobic-interaction chromatographic resins

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ABSTRACT

Dynamic binding capacities and resolution of PEGylated lysozyme derivatives with varying molecular weights of poly (ethylene) glycol (PEG) with 5 kDa, 10 kDa and 30 kDa for HIC resins and columns are presented. To find the optimal range for the operating conditions, solubility studies were performed by high-throughput analyses in a 96-well plate format, and optimal salt concentrations and pH values were determined. The solubility of PEG-proteins was strongly influenced by the length of the PEG moiety. Large differences in the solubilities of PEGylated lysozymes in two different salts, ammonium sulfate and sodium chloride were found. Solubility of PEGylated lysozyme derivatives in ammonium sulfate decreases with increased length of attached PEG chains. In sodium chloride all PEGylated lysozyme derivatives are fully soluble in a concentration range between 0.1 mg protein/ml and 10 mg protein/ml. The binding capacities for PEGylated lysozyme to HIC resins are dependent on the salt type and molecular weight of the PEG polymer. In both salt solutions, ammonium sulfate and sodium chloride, the highest binding capacity of the resin was found for 5 kDa PEGylated lysozyme. For both native lysozyme and 30 kDa mono-PEGylated lysozyme the binding capacities were lower. In separation experiments on a TSKgel Butyl-NPR hydrophobic-interaction column with ammonium sulfate as mobile phase, the elution order was: native lysozyme, 5 kDa mono-PEGylated lysozyme and oligo-PEGylated lysozyme. This elution order was found to be reversed when sodium chloride was used. Furthermore, the resolution of the three mono-PEGylated forms was not possible with this column and ammonium sulfate as mobile phase. In 4 M sodium chloride a resolution of all PEGylated lysozyme forms was achieved. A tentative explanation for these phenomena can be the increased solvation of the PEG polymers in sodium chloride which changes the usual attractive hydrophobic forces in ammonium sulfate to more repulsive hydration forces in this hydrotrophic salt.

1. Introduction

PEGylation is a procedure of increasing importance for enhancing the therapeutic potential of biologically active proteins and polypeptides. Properly PEGylated proteins maintain their biological function, such as enzymatic activity or receptor binding and antigen recognition. On the other hand, poly (ethylene) glycol (PEG) conjugation masks the surface and increases the molecular size of the original protein. It results in reduction of many negative biological features such as its immunogenicity and sensitivity to proteolytic degradation. In most cases PEGylation also reduces proteins renal ultrafiltration, resulting in much longer half-life of the modified therapeutics [1–5]. When PEG is covalently linked to a protein it also modifies many of its physicochemical properties, such as biodistribution, thermal stability and solubility. PEGylation also increases water solubility of biologically active macromolecules, resulting in significant improvement of their administration [6].

The change of physicochemical properties of PEGylated proteins also influences their behavior during the whole purification process [7–9]. More specifically, proteins whose molecules have been modified by covalently attaching PEG show altered size, charge, hydrophobicity, metal chelation, and generally interaction with other molecules. It resulted in the change in their behavior in practically all modes of chromatographic separation such as size-exclusion chromatography (SEC), ion-exchange chromatography (IEX), hydrophobic-interaction chromatography (HIC), immobilized metal affinity chromatography (IMAC), and affinity chromatography (AC) [7,8]. Other important separation methods frequently used for down-stream processing, e.g. filtration, are also affected [9]. Changes of protein behavior during all kinds of electrophoretical separations (SDS-PAGE, isoelectric focusing, 2D electrophoresis and different modes of capillary electrophoresis) can also be expected [10].

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Purification of a PEGylated protein involves removal of all molecular species that are not part of the product. 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 (PEGamer resolution). The chromatographic methods used for the purification of PEGylated proteins are mainly ion exchange (e.g. cation exchange), size-exclusion chromatography [10], and reversed-phase chromatography, the last one used for PEGylated peptides [11]. Ultrafiltration and diafiltration were less frequently applied [10]. PEGylation affects the protein hydrophobicity, and consequently, HIC can be considered to be used as an additional method for separation of PEG modified proteins. Surprisingly, HIC is rather rarely applied for the separation of PEGylated proteins. Clark et al. [12] used HIC for the separation of PEGylated growth hormone, Vincentelli et al. [13] have separated PEGylated bovine β-lactoglobulin from PEG and native protein and Cisneros-Ruiza et al. [14] have investigated separation of mono- and di-PEGylated forms from unmodified form of ribonuclease A.

2. Theory

2.1. Interaction of PEG with water, salt and proteins

Poly (ethylene) glycols (PEG) are water-soluble synthetic polymers with a general formula HO–$(CH_2CH_2O)_n$–H or CH_3O–$(CH_2CH_2O)_n$-reactive group (e.g. aldehyde, NHS) have been utilized in various applications in the biotechnology industry. PEG and methoxy-PEG (MPEG) are widely used as precipitants and crystallization agents for proteins and as chemical agent for PEGylation of proteins [15].

Polyethylene glycol itself is an amphiphatic molecule. Such a polymer is neutral, hydrophilic and soluble in various aqueous solutions, as well as hydrophobic and soluble in the organic solvents. PEG also tends to behave like a weak detergent [16,17].

In aqueous solutions PEG can be classified as electron donor or Lewis base because of the two lone pair electrons on the oxygen atom. Hydrogen bonds between oxygen electrons and water hydrogen result in a negative enthalpy of interaction [18]. Because of the strong interaction with water, PEG polymers are strongly hydrated in aqueous solutions. The Lewis base character of the ether bond is important for the PEG structure and function in electrolyte solutions. Takeuchi et al. [19] demonstrated that multivalent cations bound on the oxygen atoms from PEG chains by ion–dipole interactions lend them to anion-exchange sites. Structural changes of the PEG polymer in higher concentrated electrolyte solutions are reported in the literature [20]. Neutron scattering studies and measurements of the intrinsic viscosities have shown that some anions like nitrate or chloride do not change the coiled PEG structure, whereas phosphate, carbonate and sulfate ions increases the PEG tendency to aggregate [21]. These structural changes of PEG polymers in salt solutions are expected to influence the adsorption properties of PEG-proteins on HIC resins.

There is currently no theory which describes the solubility of PEGylated proteins and adsorption to HIC resins, but the PEG is frequently used in protein purification.

Precipitation of proteins by adding PEG is broadly described in the literature by experimental values and by statistical models [22–24]. Usually the addition of PEG decreases the solubility of proteins in water. In general, the increase of the molecular weight of the PEG has a greater effect on the solubility than the addition of electrolytes which either increase or decrease solubility according to their position in the lyotrophic series [25].

Precipitation of proteins in the presence of PEG has been described by a statistical–mechanical perturbation theory with a volume-exclusion potential from Asakura and Oosawa [26]. According to this theory, protein molecules are excluded from the regions of the solvent occupied by PEG molecules. As a result, protein is concentrated and precipitates out when its solubility limit is exceeded. In this theory, a linear trend was found of the logarithm protein solubility against added PEG [23].

Aqueous PEG electrolyte mixtures were also extensively used as components for liquid–liquid partitioning (LLP) systems for protein purification [27]. Here, the logarithm of the distribution coefficients for the partitioning of biomolecules between two phases is proportional to the molecular weight of the molecule being partitioned [28].

Despite some analogies found for the solubilities of PEGylated proteins the above presented theories describing interactions between PEG and proteins in aqueous solutions are not applicable, because PEG is not covalently linked to the protein.

Because there is still no molecular theory that describes the interaction of PEGylated protein with water and electrolytes, the empirical solubility models might be applicable. The solubility of macromolecules in electrolyte solutions can be often successfully described by the empirical Cohn's [29] or Setschenow's equation [30].

$$\text{Log } S = \beta' - K' \cdot \left(\frac{\Gamma}{2}\right) \tag{1}$$

where S is the solute concentration at the experimental salt concentration, g/L; β' is the extrapolated solubility intercept which represents the logarithm of the hypothetical concentration of the solute at zero ionic strength; $\Gamma/2$ is the ionic strength, mol/L ($\Gamma/2 = (1/2)\sum z_i^2 c^2$); Z_i is the valences of cationic and anionic species; c is the molar concentration of cationic and anionic species, mol/L, for ammonium sulfate $\Gamma/2 = 3c$ and K' is the constant obtained from slope of solubility curve.

The slope of Eq. (1) can be considered as the difference between "salting out" and "salting in" effect [31]:

$$K' = K_{\text{Si}} - K_{\text{So}} \tag{2}$$

where K_{Si} is the "salting in" constant and K_{So} is the "salting out" constant.

2.2. PEGylated model proteins

The influence of PEGylation on solubility and binding properties of modified proteins in HIC was investigated with hen egg white lysozyme as model substance. This protein was used by Yamamoto et al. [3,8] for binding studies of PEGylated forms onto ion-exchanger resins. Lysozyme is also often used as a standard protein for characterization and binding capacity studies in HIC. Consequently, binding and elution conditions for this protein in HIC mode are thoroughly investigated [32].

Hen egg white lysozyme is well characterized and is available in large quantities. It contains a total of six lysine residues. Only three of the residues: 33, 97 and 116 are surface accessible for large molecules and can be PEGgylated [43]. Lee and Park have found following reactivity increments for Lys 33 > Lys 97 > Lys 116 were found if amino reactive coupling methods for protein PEGylation were applied [33]. Because of the three reactive lysine residues, in random PEGylation three mono-PEGylated, three di-PEGylated and one tri-PEGylated forms of this protein are expected. The number of PEGylation products is relatively small, and individual species can be easily separated and isolated by cation-exchange chromatography [3,8].

There is no systematic study available for describing the influence of the size of PEG residues on the solubility and binding