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Solid-phase polyethylene glycol conjugation using hydrophobic interaction chromatography

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ABSTRACT

PEGylation is a widely applied approach to improve the pharmacokinetic and pharmacodynamic properties of protein therapeutics. The current solution-phase PEGylation protocols often suffer from poor yield of homogeneously PEGylated bioactive products and hence fall short of being commercially attractive. To improve upon these techniques, here we developed a novel, solid-phase PEGylation methodology using a hydrophobic interaction chromatography (HIC) resin. Two variations of the HIC-based PEGylation are described that are tailored towards conjugation of proteins with hydrophobicity index above (lysozyme) and below (fibroblast growth factor 1, FGF-1) that of the mPEG-butyraldehyde (mPEG) chain used. In the case of lysozyme, the protein was first immobilized on the HIC, and the HIC-bound protein was then conjugated by passing over the column. In the case of FGF-1, the mPEG solution was first immobilized on the HIC, and the FGF-1 solution was then passed through the column. Circular dichroism (CD) spectroscopy demonstrated HIC-based PEGylation almost retained the secondary structures of proteins. Bioactivity assay showed that the recovery of activity of HIC-based PEGylated rhFGF-1 (i.e. 92%) was higher than that of liquid-phase PEGylated rhFGF-1 (i.e. 61%), while HIC-based PEGylated lysozyme showed the same activity recovery (i.e. 7%) as the liquid-phase PEGylated form. For specific proteins, the HIC-based solid-phase PEGylation maybe offer a more promising alternative than the current PEGylation methods and is expected to have a major impact in the area of protein-based therapeutics.

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1. Introduction

Recombinant protein therapeutics have emerged as effective treatments for a variety of conditions, ranging from cancer to metabolic disorders and wound diseases, but they are commonly limited by their poor pharmacokinetics and immunogenicity [1–3]. Arguably, the most widely used method for improving the pharmacokinetics of protein therapies is through polyethylene glycol conjugation (PEGylation) [4,5]. The covalent attachment of PEG chains on protein surfaces effectively increases the half-life of the protein in vivo by decreasing renal clearance and masking potentially immunogenic epitopes and protease cleavage sites [6,7]. There are several food and drug administration (FDA)-approved PEGylated drugs in the market (such as PEGylated asparaginase (Onspar), PEGylated adenosine deamidase (Adagen) and PEGylated interferon (PEG-Intron and PEG-Asys)) [8] and many currently in clinical trials [9]. PEGylation is mostly performed in solution, which causes various complications. Solution-phase chemistry is commonly associated with difficulties in controlling the reaction selectivity and the extent of covalent conjugation, isomerization, low efficiency in the separation of various end-products and unreacted starting materials and often low yields of the desired reaction product [10–14].

In lieu of the shortcomings of the solution-phase PEGylation, solid-phase PEGylation method has been proposed to minimize the variations between batches and increase the specificity and yield of the desired reaction product [15–19]. Solid-phase PEGylation is often carried out by immobilizing the protein on a solid matrix packed in a column, followed by passing the activated PEG reagent through a column [15–19]. The PEGylated protein is then separated from the unreacted protein and undesirable PEGylated isomers by gradient elution. Utilizing the ion exchange interactions between proteins and an ion exchange column (IEX), Monkarsh et al. [20] prepared and separated various constitutional isomers of PEGylated interferon-α-2a (PEG-INF). As one of the earliest solid-phase purification techniques, ion exchange chromatography has also been applied in the PEGylation of bovine hemoglobin, human serum albumin (HSA) and staphylokinase (SAK) [16,21]. However, studies have shown that IEX-based PEGylated proteins modified on

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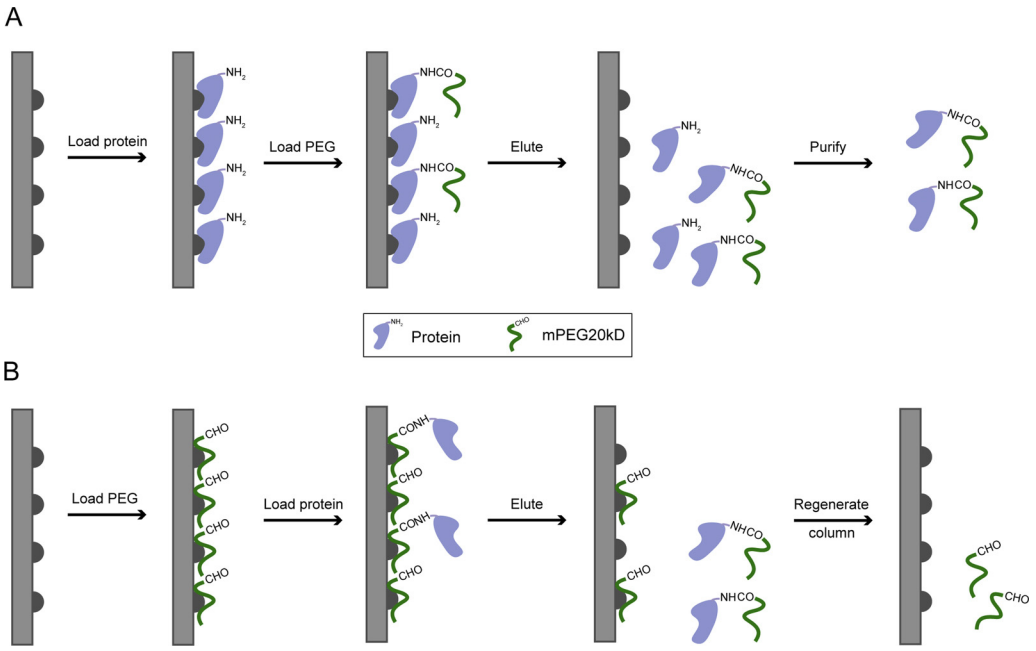

Fig. 1. Schematic illustration of the two strategies of solid-phase PEGylation using an HIC. (A) and (B) facilitate the conjugation of proteins with a higher or lower hydrophobicity than mPEG, respectively.

an ion exchange matrix often lose some bioactivity [16,21], and the efficiency for mono-PEGylation varies between proteins and hence this method is not broadly applicable. Moreover, we found that the electrostatic interactions between proteins and the matrices of the IEX block PEGylation sites. Additionally, the basic pH (i.e., pH >7.0) at which proteins are immobilized on ion exchange matrices is not suitable for N-terminal PEGylation [15]. Our previous research developed another novel solid-phase PEGylation strategy using heparin sepharose affinity chromatography (AC) for recombinant human keratinocyte growth factor 1 (rhKGF-1) and recombinant human fibroblast growth factor 2 (rhFGF-2) [18,19]. However, this solid-phase strategy is more specific because it is only suitable for a limited number of proteins that possess an intrinsic affinity for a heparin sepharose matrix.

To improve upon the currently established solid-phase PEGylation strategies, here we devised a novel approach using a hydrophobic interaction (HIC) resin as a solid phase. This strategy takes advantage of the non-specific pH-insensitive nature of hydrophobic interactions thus increasing the likelihood of an exposed N-terminus available for PEGylation [17]. In this study, we present two variations of HIC-based PEGylation tailored towards proteins with higher (lysozyme) and lower (rhFGF-1) hydrophobicity indices than the mPEG used for conjugation (Fig. 1A and B). In the case of lysozyme, the protein is first immobilized on a commercially available hydrophobic matrix, HiTrap Phenyl FF, followed by the passage of an mPEG solution in the presence of a reducing agent. For rhFGF-1, activated mPEG is first immobilized on the HiTrap Phenyl FF, followed by the passage of a protein solution through the column in the presence of a reducing agent. With the help of hydrophobic interactions between proteins and mPEG on the HIC platform, we successfully modified lysozyme and rhFGF-1. Circular dichroism (CD) spectroscopy demonstrated HIC-based PEGylation almost retained the secondary structures of proteins. Bioactivity assay showed that the recovery of activity of HIC-based PEGylated rhFGF-1 was higher than that of liquid-phase PEGylated rhFGF-1, while HIC-based PEGylated lysozyme showed the same activity recovery as the liquid-phase PEGylated form. The HIC-based PEGylation offers a robust strategy for PEGylating proteins with different degrees of hydrophobicity.

2. Experimental procedures

2.1. Materials

PEG20 kDa-butyraldehyde (mPEG20K, its polydispersity is about 1.1), sodium cyanoborohydride ($NaBH_3CN$) and lysozyme were purchased from Sigma-Aldrich (St. Louis, MO, USA). Purified rhFGF-1 was produced by the Key Laboratory of Biotechnology and Pharmaceutical Engineering of Zhejiang Province, Wenzhou Medical University, China. HiTrap Phenyl fast flow (FF) columns and carboxymethyl (CM) Sepharose FF columns were purchased from GE Healthcare (Piscataway, NJ, USA). The protein assay reagent used for quantitative protein analysis was purchased from Bio-Rad (Hercules, CA, USA). Other reagents, unless otherwise indicated, were of the highest quality commercially available. All chromatographic separations and solid-phase PEGylation reactions were performed on an ÄKTA explorer purchased from GE Healthcare (Piscataway, NJ, USA) and equipped with an ÄKTA explorer workstation (Amersham Biosciences).

2.2. Solid-phase PEGylation of lysozyme using an HIC

The solid-phase PEGylation of lysozyme, which has higher hydrophobicity index than mPEG20K, entailed the following three steps: (1) Two milliliters (mL) of 1.5 mg/mL lysozyme, prepared in a binding buffer (3 M NaCl in 20 mM Phosphate Buffer (PB), pH 6.0), was passed at 0.5 mL/min through an HIC (HiTrap Phenyl FF) column (2 mL bed volume), which was pre-equilibrated with the binding buffer at 0.5 mL/min. (2) Binding buffer containing 3.75 mg/mL mPEG20K and 20 mM $NaBH_3CN$ was passed through the column at 0.5 mL/min for 20 min, followed by elution of the excess mPEG20K with binding buffer. (3) The reaction complex was completely eluted with elution buffer (20 mM PB, pH 6.0) at a rate of 1 mL/min. The elution fractions were collected and analyzed by 12% SDS-PAGE.

In compared with solid-phase PEGylation of lysozyme, liquid-phase PEGylation of lysozyme was performed. The reaction mixture including 1.5 mg/mL lysozyme, 3.75 mg/mL mPEG20K and 20 mM NaBH3CN in 20 mM Phosphate Buffer (pH 6.0) was incubated at

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