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Use of ion-exchange chromatography and hydrophobic interaction chromatography in the preparation and recovery of polyethylene glycol-linked proteins

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Abstract

Cation- and anion-exchange chromatography can be used to purify a polyethylene glycol-linked protein dimer (PEG dimer) made with M_r 20 000 PEG bis-vinylsulfone, even when there are no net charge differences between the components that are being separated. The retention time on ion-exchange generally is inversely proportional to the PEG:protein ratio (on a mass basis). One of the biggest challenges in developing the process for making this PEG dimer was the quality of the PEG linker. Reversed-phase HPLC can be used to determine both size heterogeneity and the degree of end-group activation of M_r 20 000 PEG bis-vinylsulfone. In addition, we have found that hydrophobic interaction chromatography can be used make more size homogeneous preparations of M_r 20000 PEG bis-vinylsulfone, which significantly increased the recovery of the PEG dimer.

Keywords: Hydrophobic interaction chromatography; Preparative chromatography; Proteins; Poly(ethylene glycol); Poly(ethylene glycol) bis-vinylsulfone

1. Introduction

Chemical modification with polyethylene glycol (PEG) has been shown to decrease the immunogenicity, improve the physical stability and increase the serum half-life of therapeutic proteins [1,2]. In the latter instance, site-specific attachment of a single M_r 10 000–20 000 PEG can appreciably increase the serum half life of smaller (M_r<30000) peptides and proteins without causing a significant loss of biological activity [3,4].

Because PEGs have a larger hydrodynamic volume than globular proteins of the same mass, PEGylated proteins can be separated from un-PEGylated proteins by size-exclusion chromatography [5–7]. Unfortunately, size-exclusion chromatography has very poor thoughput at production scale and also fails to separate other PEGylation variants that might be formed during the PEGylation reaction. Hydrophobic interaction chromatography and reversed-phase chromatography have been used with some success [3,5–7], but the separation depends on the hydrophobicity of the protein relative to the PEG. Cation-exchange chromatography has been used to purify proteins that are PEGylated at amine groups, where the PEGylated proteins contain one less positive charge per PEG molecule attached [6].

In this paper we report on the use of M_r 20 000 PEG bis-vinylsulfone to cross-link a M_r 18 000 protein through cysteinyl residues to make a make a M_r 20 000 PEG linked dimer (Fig. 1). This PEG dimer not only has a longer serum half-life due to the

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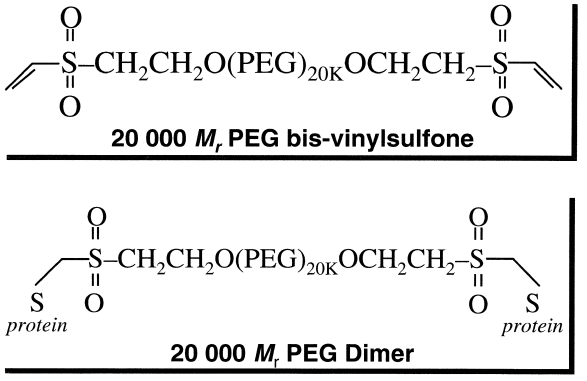

Fig. 1. Structure of M_r 20 000 PEG bis-vinylsulfone (top) and of the PEG dimer. The PEG linker reacts with a cysteinyl residue on the protein, resulting in a protein linked on either end of the PEG molecule via a thioether bond.

M_r 20 000 PEG, but, because it is dimeric, also has a greater avidity for the multimeric ligand to which it binds. In this paper we describe the synthesis of this PEG dimer and demonstrate how ion-exchange chromatography can be used to purify the PEG dimer; even under conditions where there is no net charge difference between the PEG dimer, the PEG monomer (PEG with only a single protein molecule attached) and the unPEGylated protein. We will also describe how reversed-phase HPLC (HP-HPLC) can be used to analyze M_r 20 000 PEG bis-vinylsulfone quality and can be predictive of the PEG dimer yields. Finally, we will demonstrate how hydrophobic interaction chromatography can be used to purify the M_r 20 000 PEG bis-vinylsulfone, leading to greater yields of the PEG dimer.

2. Experimental procedures

2.1. Reagents

M_r 20 000 PEG bis-vinylsulfone was either purchased from Shearwater Polymers (Huntsville, AL, USA) or produced at Amgen. The protein which was PEGylated for this work was an M_r 18 000 recombinant protein produced in *Escherichia coli*. The protein has a single free cysteinyl residue which serves as a PEGylation site. *o*-Thiobenzoic acid (OTBA) was purchased from Sigma (St. Louis, MO, USA).

2.2. PEGylation reaction

The PEGylation reaction is carried out with 1–5 mg/ml protein with ~0.5 mol of M_r 20,000 PEG bis-vinylsulfone per mole of protein in 50 m*M* sodium phosphate buffer, pH 7.5, for ca.~ 15–20 h at room temperature. After PEGylation, the pH of the PEGylation reaction mixture was adjusted to 3.1 with 1*M* hydrochloric acid.

2.3. Purification of the PEG-linked protein dimer

The PEG dimer was purified using SP-Sepharose High Performance (HP) cation-exchange media (Pharmacia, Uppsala, Sweden) packed into a 15 cm bed height×1.6 cm I.D. column. The column was equilibrated with 50 m*M* sodium phosphate buffer, pH 3.1, and loaded with 4–8 mg of protein per ml bed volume. The column was next washed with 3 column volumes of equilibration buffer and eluted with a 20 column volume 0.25–0.50 *M* NaCl gradient in 50 m*M* phosphate, pH 3.1. Flow rate for all steps was 0.15 column volumes per min. The column was run at room temperature and the elution profile was monitored by 280 nm absorbance.

The PEG dimer was also purified by anion-exchange chromatography using Q-Sepharose HP media (Pharmacia) packed into a 6 cm bed height× 1.5 cm I.D. column. The column was equilibrated with 25 m*M* piperidine buffer HCl, pH 11.0, and loaded with 4 mg of protein per ml bed volume in 25 m*M* piperidine HCl buffer, pH 11.0. The column was then washed with 3 column volumes of equilibration buffer and eluted with a 20 column-volume 0 –0.5 *M* sodium chloride gradient in 25 m*M* piperidine buffer, pH 11.0. Flow rate throughout was 0.12 column volumes per min. The column was run at room temperature and the elution profile was monitored by 280 nm absorbance.

2.4. RP-HPLC analysis of M_r 20 000 PEG bis-vinylsulfone

A 1 mg/ml solution of the M_r 20 000 PEG bis-vinylsulfone was first derivatized for 18 h at room temperature with 4 mol of o-thiobenzoic acid (OTBA) per mole of PEG in 20 m*M* sodium phosphate buffer, pH 7.5. The derivatized PEG

linker (50 μg of PEG in 0.05 ml) is then run on an Astec C_4 RP-HPLC column, 150 mm×4.6 mm, 5 μm particle size (Advanced Separations Technologies, Whippany, NJ, USA). The column is eluted using a linear acetonitrile gradient (30–50% acetonitrile in water, 0.1% trifluoroacetic acid) over 36 min at room temperature. Elution of the OTBA modified PEG is monitored by UV absorbance at 254 nm.

2.4.1. Purification of M_r 20 000 PEG bis-vinylsulfone by hydrophobic interaction chromatography (HIC)

ToyoPearl Butyl 650*M* media (Toso Haas, Montgomeryville, PA, USA) was packed into a 12 cm bed height×1.6 cm I.D. column and equilibrated with 4 *M* NaCl. The column was loaded with a 10 mg/ml solution of PEG linker in 4 *M* NaCl at a column load rate of 8–12 mg PEG per ml column bed volume. The column was then washed with two column volumes of 4 *M* NaCl and eluted with a 10 column volume 4–1.5 *M* NaCl gradient at 0.1 column volumes per min at room temperature. One column volume fractions were collected and size-exclusion HPLC was used to determine the molecular mass profile of the PEGs in each fraction.

2.4.2. Size-exclusion HPLC (HPSEC) analysis of M_r 20 000 PEG bis-vinylsulfone

HPSEC analysis was carried out using a Toso Haas G3000 SWXL HPLC column, 300×7.8 mm, 5 μm particle size. The mobile phase was 0.1% sodium dodecylsulfate, 120 m*M* sodium chloride, 10 m*M* sodium phosphate, pH 7.4. A 100 μl PEG sample from the HIC fractions (containing 0.5–5.0 mg/ml PEG) was injected neat and eluted at a flow-rate of 1.0 ml/min. PEG was detected by refractive index using a Wyatt Optilab Model 903 refractive index detector (Wyatt Labs, San Franciso, CA, USA).

2.4.3. Protein concentration determination

Protein concentration was determined by the absorbance at 280 nm using an extinction coefficient of 0.78 for a 1 mg/ml solution. In determining mg/ml concentration of the unPEGylated and PEGylated proteins, only the weight of the protein is taken into account. The same extinction coefficient can therefore be used for the PEGylated and unPEGylated protein because the PEG does not have any intrinsic absorbance at 280 nm.

3. Results

3.1. Purification of the M_r 20 000 PEG-linked protein dimer

The PEG dimer (two protein molecules per one molecule PEG) needs to be separated from PEGylated product that only has one molecule of protein per molecule of PEG (PEG monomer) and from unPEGylated protein. As can be seen in Fig. 2, cation-exchange chromatography can be used to separate all three of these components, even though all three have the same net charge. Since the Protein:PEG ratio correlates with retention time, it was thought that the PEG was weakening the interaction of the protein with the resin. To test this hypothesis, the PEGylation reaction components run on on an anion-exchange column using Q-Sepharose HP media. As can be seen in Fig. 3, the order of elution of the PEGylation reaction components is the same as with cation-exchange chromatography. In this case, the unPEGylated molecule has an additional negative charge due to the ionization of the SH group on cysteine and would be expected to elute later on an anion-exchanger. However, PEG dimer and PEG monomer have the same net charge and elute in the same order as on the cation-exchange resin. These results suggest that the mode of sepa-

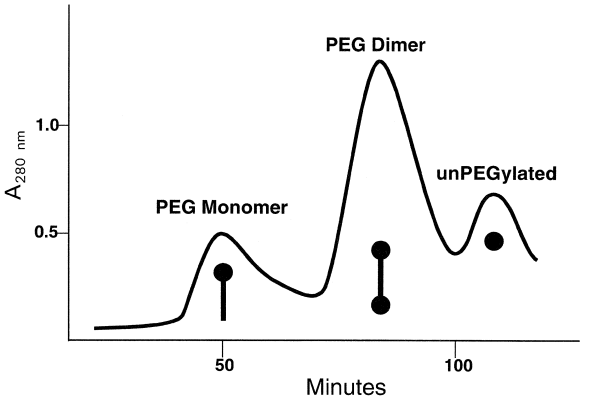

Fig. 2. Separation of the PEG dimer from the PEG monomer and unPEGylated protein by cation-exchange chromatography using SP-Sepharose HP. (See Experimental section for details).

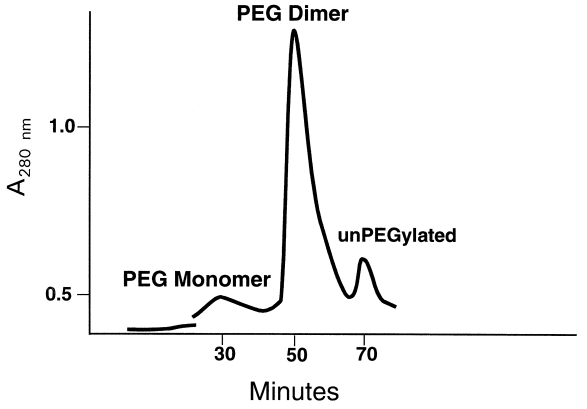

Fig. 3. Separation of the PEG dimer from the PEG monomer and unPEGylated protein by anion-exchange chromatography using Q-Sepharose HP media. (See Experimental section for details).

ration on ion-exchange chromatography may be a ''charge masking'' effect of the PEG which weakens the interaction of the protein with the ion-exchange media.

3.2. RP-HPLC Analysis of M_r 20 000 PEG bis-vinylsulfone

The RP-HPLC analysis of (OTBA) derivatized M_r 20 000 PEG bis-vinylsulfone enables us to determine the quality of the PEG linker prior to using in the the PEGylation reaction. This is very important because the degree of activation as well as the size homogeneity of the PEG linker varies from lot to lot. The RP-HPLC assay can determine the extent of activation of the PEG (bis- versus mono-vinylsulfone) and is also able to partially separate the PEGs on the basis of size (Fig. 4). The PEG mono-vinylsulfone elute ahead of the bis vinylsulfones. Also, the retention time somewhat correlates with the molecular mass of the PEG, with M_r 20 000 bis-vinylsulfones eluting ahead of lower-molecular-mass bis-vinylsulfones and M_r 20 000 mono-vinylsulfones eluting ahead of lower-molecular-mass mono-vinylsulfones (arrow). As shown in Fig. 5, the percent area of the M_r 20 000 PEG bis-vinylsulfone peak is predictive of the amount of M_r 20 000 PEG dimer formed during the PEGylation reaction.

Free OTBA has a longer retention time than any of the PEG linked OTBA molecules (not shown in figure). Although PEGs will bind to reversed-phase

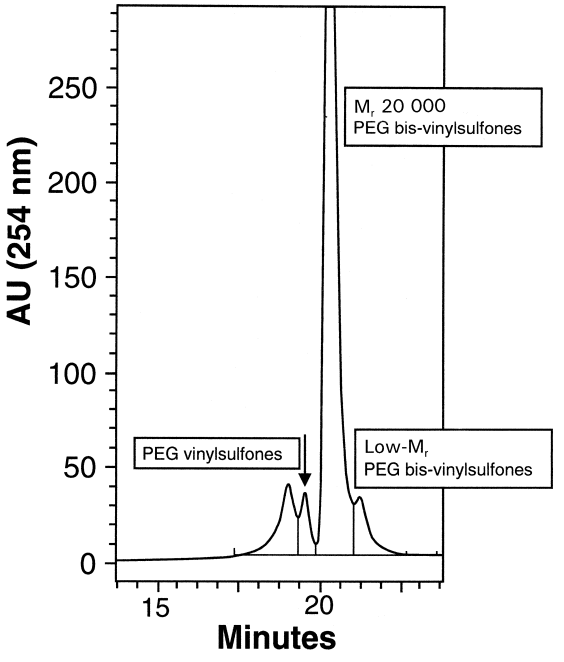

Fig. 4. RP-HPLC chromatogram of *o*-thiobenzoic acid derivatized preparation of M_r 20 000 PEG bis-vinylsulfone. Order of elution is 20,000 M_r PEG mono-vinylsulfone, low-molecular-mass (M_r~ 2000–10 000) PEG mono-vinylsulfone (arrow), M_r 20 000 PEG bis-vinylsulfone and low-molecular-mass PEG bis-vinylsulfone. (See Experimental section for details).

media (unlike ion-exchange media), in this instance it appears that the PEGs serve to weaken the interaction of the OTBA with the solid-phase, with the

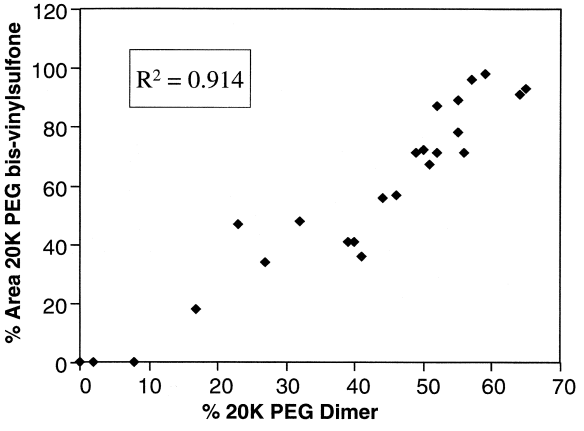

Fig. 5. Correlation of RP-HPLC % area of the Mr 20 000 (20K) PEG bis-vinylsulfone peak vs. % yield of M_r 20 000 PEG dimer made with different batches of PEG linker.

greater PEG to OTBA mass ratio resulting in progressively earlier retention times. This mode of separation is analagous to what occurs with the separation of PEGylated proteins on ion-exchange media (see above).

3.3. Removal of high-molecular-mass PEGs by HIC

Because of problems with oxidative degradation of the PEG backbone [8] during the synthesis of M_r 20 000 PEG bis-vinylsulfone, an alternative route of synthesis for the linker was developed. Although the problems with PEG breakdown were eliminated, this new synthetic route resulted in the presence of as much as 20–30% high-molecular-mass forms of PEG bis-vinylsulfone which were due to an end-to-end polymerization of the M_r 20 000 PEG bis-vinylsulfones, resulting in PEG bis-vinylsulfones of mostly M_r 40 000 and a little M_r 60 000. These high-molecular-mass PEG linkers compete with the M_r 20 000 PEG bis-vinylsulfone for the PEGylation site on the protein. In addition, it is difficult to get adequate removal of the high-molecular-mass PEG dimers from the M_r 20 000 PEG dimer without taking significant losses during the SP-Sepharose HP purification step.

To alleviate this problem, we developed a preparative HIC procedure to eliminate the high molecular PEG linker prior to the PEGylation reaction. As shown in Fig. 6, the high-molecular-mass PEG bis-vinylsulfones can be adequately removed from the M_r 20 000 linker with the collection of as few as 10 fractions. The mode of separation is likely due to larger molecular mass PEGs having more contacts with the butyl ligands on the resin, resulting in a greater avidity for the chromatography media and longer retention times. Vinylsulfones are very stable in water, and the PEG linker can be chromatographically purified, concentrated and diafiltered prior to the PEGylation reaction without any appreciable loss in vinylsulfone reactivity.

Fig. 7 shows the elution profile on the SP-Sepharose HP column using HIC purified M_r 20 000 PEG linker vs. the unpurified PEG linker. If the high-molecular-mass PEG bis-vinylsulfone is not removed prior to the PEGylation reaction, the resulting M_r 40 000 PEG dimer tends to elute on the front side of the M_r 20 000 PEG dimer peak, which requires a much more conservative pooling criteria in order to maintain adequate purity of the M_r 20 000 PEG dimer. However, a much less conservative pool can be taken if the HIC purified PEG linker is used, resulting in a 30–50% increase in overall yield of the M_r 20000 PEG dimer.

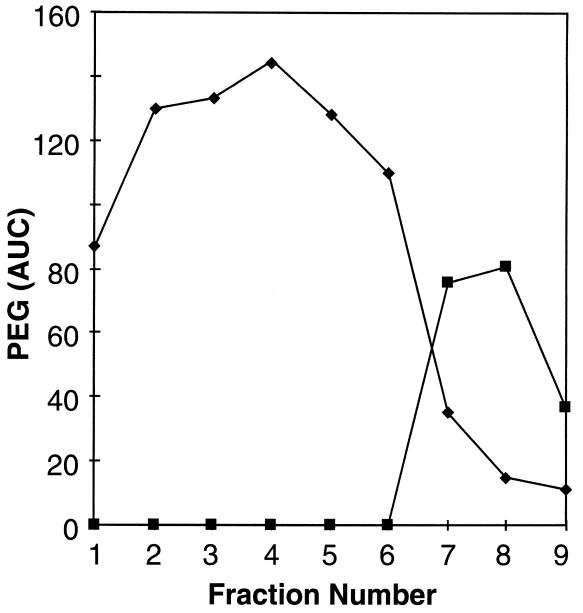

Fig. 6. Separation of M_r 20 000 PEG bis-vinylsulfone (♦) from high-molecular-mass PEG bis-vinylsulfone (■) using hydrophobic interaction chromatography (ToyoPearl Butyl 650M). Amount of PEG is quantified by the area under the curve (AUC) from HPSEC analysis of the column fractions. The volume of each fraction is equivalent to approximately one column volume. (See Experimental section for details).

4. Discussion

The PEGylation at cysteinyl residues does not result in a net charge change to a protein at low pH. However, we are able to get a good separation of the different PEGylated forms of the protein using cation as well as anion-exchange chromatography. The separation of PEGylated proteins by ion-exchange has been described before [6,7]; however, in these instances the proteins were PEGylated at amino groups, resulting in an acidic net charge shift due to neutralization of charge on the amino nitrogen. In the

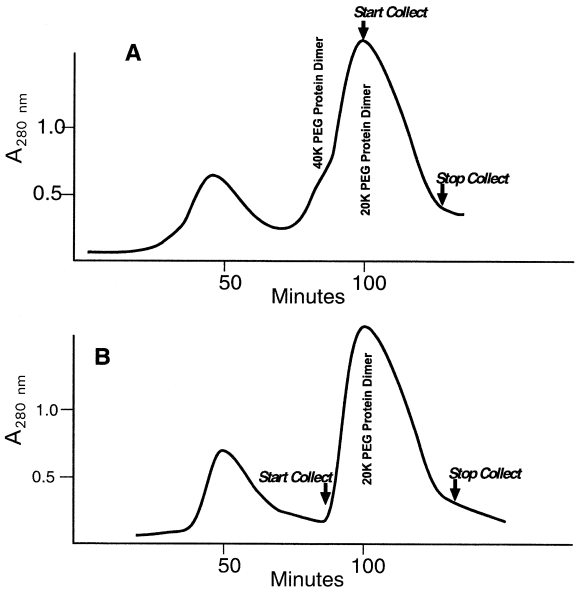

Fig. 7. Cation-exchange chromatographic purification of the M_r 20 000 PEG dimer without (A) or with (B) HIC purification of the M_r 20 000 PEG bis-vinylsulfone prior to the PEGylation reaction. Arrows show where the product pool is collected in each instance.

work described here, the protein:PEG ratio was proportional to the retention time on the SP-Sepharose HP resin (1 protein/1 PEG<2 proteins/1 PEG<protein/0 PEG). This may be due to some sort of general charge shielding effect of the PEG, because the order of elution of these molecules was the same when they were run on an anion-exchanger. It does not appear to be due to a steric effect of the PEG prohibiting penetration of the molecule into the pores of the agarose, since the same results were obtained with a non-porous silica gel cation-exchange resin (unpublished). This separations phenomenon of PEGylated proteins on ion-exchangers is clearly and area for future investigation.

The RP-HPLC analysis of derivatized PEG bis-vinylsulfone is a useful tool for determining the quality of different batches of PEG linker. This method can tell us about both the degree of activation and size homogeneity of PEGs, which will complement other sizing methods such as SEC and light scattering [6,9,10]. A similar method has been described for the analysis of amine specific PEG linkers using *p*-aminobenzoic acid as a derivatizing agent [11].

PEGs have a much larger hydrodynamic volume than globular proteins of the same mass. Therefore, the size of the PEG molecule is one of the main factors in determining the serum half-life of the PEGylated protein. For this reason, the PEG linker should be as size homogeneous as possible from batch to batch in order to ensure a consistent pharmacokinetic profile for the drug. Although preparative SEC can accomplish this, throughput is low due to the limited volume that can be loaded per run. The preparative HIC method described here can be used to make more size-homogeneous PEG linkers, with much greater throughput per run. Use of HIC to purify PEGs can not only help ensure a more consistent PEG linker, but can also increase the yields and reduce the recovery cost for some PEGylated protein therapeutics.

PEG vinylsulfones have a number of advantages over other thiol linkers. Although the reaction kinetics are slower with vinylsulfones compared to iodo-PEGs or PEG maleimides, the specificity is slightly better, as long as the reaction is ran below pH 8 [12]. In addition, the thioether linkage formed with the protein is much more stable than the linkage formed with maleimides [13,14]. One of the biggest advantages, however, is the fact that vinysulfones are very stable in aqueous solution [12]. This has enabled us to use HIC to purify the linker in aqueous buffers without losing any vinylsulfone reactivity. It also simplifies the logistics of handling large quantities of the linker in aqueous solutions. These considerations make PEG vinylsulfones very useful linkers for the large-scale production of PEGylated proteins.

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