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# Preparative purification of polyethylene glycol derivatives with polystyrene-divinylbenzene beads as chromatographic packing

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**Abstract**—A clear and powerful chromatographic approach to purify polyethylene glycol derivatives at a preparative scale was reported, which was based on the polystyrene-divinylbenzene beads with ethanol/water as eluants. The validity of this method was verified with the reaction mixture of mPEG-Glu and mPEG propionaldehyde diethylacetal (ALD-PEG) as the model. The target products were one-step achieved with the purity of >99% on the polymer resins column at gram scale. The method developed was free from such disadvantages as utility of toxic solvent and narrow application scope, which was combined with conventional approaches. The method developed provided an appealing and attractive alternative methods for purification of PEG derivatives at a preparative scale.


Over the last three decades, there has been growing interest in the conjugation of therapeutic proteins, peptides and antibodies with polyethylene glycol (referred as PEGylation).[1] Up to now, seven PEGylated protein pharmaceuticals have been approved by FDA for the treatment of several diseases and many other PEGylated proteins and antibodies are currently under development and evaluation.[2–4] Correspondingly, various PEG derivatives (PEGs) were also developed in order to render the pharmaceuticals more appealing properties, including branched PEG,[5] forked PEG,[6] star-like PEG[7], degradable PEG,[8] etc. Like any chemical reaction, the derivatization reaction of PEG often produces a mixture of products. PEG is large molecule and the variation of end group does not bring about much changes in molecular weights and properties, which causes great difficulty in purification of the target polymer.[9] Conventionally, the preparative purification of PEGs mainly employs two chromatographic approaches: ion exchange chromatography on the basis of charge,[10,11] and normal phase chromatography on the basis of polarity.[12,13] However, the ion exchange chromatography is impotent when the target products and PEG impurities have the similar charges or all of them are non-charged.[14] Although the normal phase chromatography on silica gel resins can give significant fractionations of PEGs,[15] it suffers from the marked drawbacks such as utilization of toxic solvent and complicated eluant system. The demerits become especially conspicuous when the product is prepared at an industrial scale. To obtain the target PEG with satisfied purity, optimization of eluants usually requires considerable trial and errors. In some instances, it was still difficult to obtain the highly pure product from the reaction mixture in good yield.[16] At present, the chromatographic approaches are greatly restricted by either narrow application scope or utilization of toxic reagents and optimum of eluants. Therefore, it is of significance to develop a clear chromatography with extensive application scope for purification of PEGs. Polystyrene-divinylbenzene beads (PS/DVB) are polymeric resins and can be operated under low pressure with high resolution due to its rigid, macroporous-structure and high hydrophobic groups on the surface. The columns packed with the polymer could be well applied for the separation of polar and non-polar samples.[17] The polymeric resins were expected to work at the purification of PEGs, and the chromatographic method based on it was investigated.

*Keywords*: Polystyrene-divinylbenzene beads; Purification of polyethylene glycol derivatives; Preparative reversed-phase chromatography.
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All reagent-grade chemicals were purchased from Beijing Chemical Reagents Co. (Beijing, China) except for those specified. HPLC grade acetonitrile was from Fisher Scientific (Leics, UK) and HPLC grade water was prepared by Milli-Q systems (Millipore, USA). Monomethoxyl PEG (mPEG, Mw 5000 Da) was acquired from Fluka Chemicals (Ronkonkoma, NY) and the carboxymethylated mPEG (CM-PEG) was synthesized according to Li et al.[18] PS/DVB resins (particle diameter of 30 μm, average pore size of 147 Å, specific surface area of 745.2 $m^2/g$) were prepared by employing membrane emulsification as uniform porous microsphere.[19]

In this work, we chose the reaction mixture of mPEG-Glu and mPEG propionaldehyde diethylacetal (ALD-PEG), two important reagents in PEGylation chemistry, for the purification studies. The mPEG-Glu is a 'forked' polymer and its terminus has two functional groups, which may render the conjugate more favorable properties when attached to pharmaceutics, such as the improved affinities with dimerised cell surface reporters and increased specificity to target location.[20] The hydrolyzed product of ALD-PEG is well known to specifically react with the N-terminus of proteins at slight acid conditions. The derivatives cannot be purified from reaction mixture using ion exchange chromatography due to their similar charge properties to the macromolecular impurities. For mPEG-Glu, its purification by chromatography on silica gel resins also failed to achieve the product with satisfied purity in our over-and-over trials (data not shown). Therefore, they were meaningful and representative as model for purification studies at a preparative scale.

The synthesis of mPEG-Glu was described in an earlier publication.[21] Briefly, CM-PEG was activated into hydroxysuccinimidylester (CM-PEG-OSu) under *N*-hydroxysuccinimide (NHS) and *N*-dicyclohexylcarbodiimide (DCC), then reacted with L-glutamic acid diethyl ester hydrochloride to give the mPEG-Glu. In the whole reaction, two PEG by-products were produced due to the side reactions of hydrolysis and rearrangement of intermediates as shown in Figure 1a,[22] which was clearly shown on the chromatogram of RP-HPLC (Fig. 2, Graph a).[23] ALD-PEG was synthesized by reaction of mPEG with 3-chloropropionaldehyde diethylacetal under basic conditions (Fig. 1b). However, its conversion ratio was about 50% due to the nature of heterogeneous phase reaction,[24] and the unreacted mPEG was mixed in target product as major impurities (Fig. 3, Graph a).

The purification of target PEG derivative was performed on the column (2.6 × 40 cm i.d.) packed with 200 ml of PS/DVB resins equipped with a peristaltic pump, using ethanol/water as eluants. Initially, 30% ethanol was used to adsorb all PEGs, and the ethanol of increased concentration was applied to elute the species in step-wise manner. The fractions were collected and

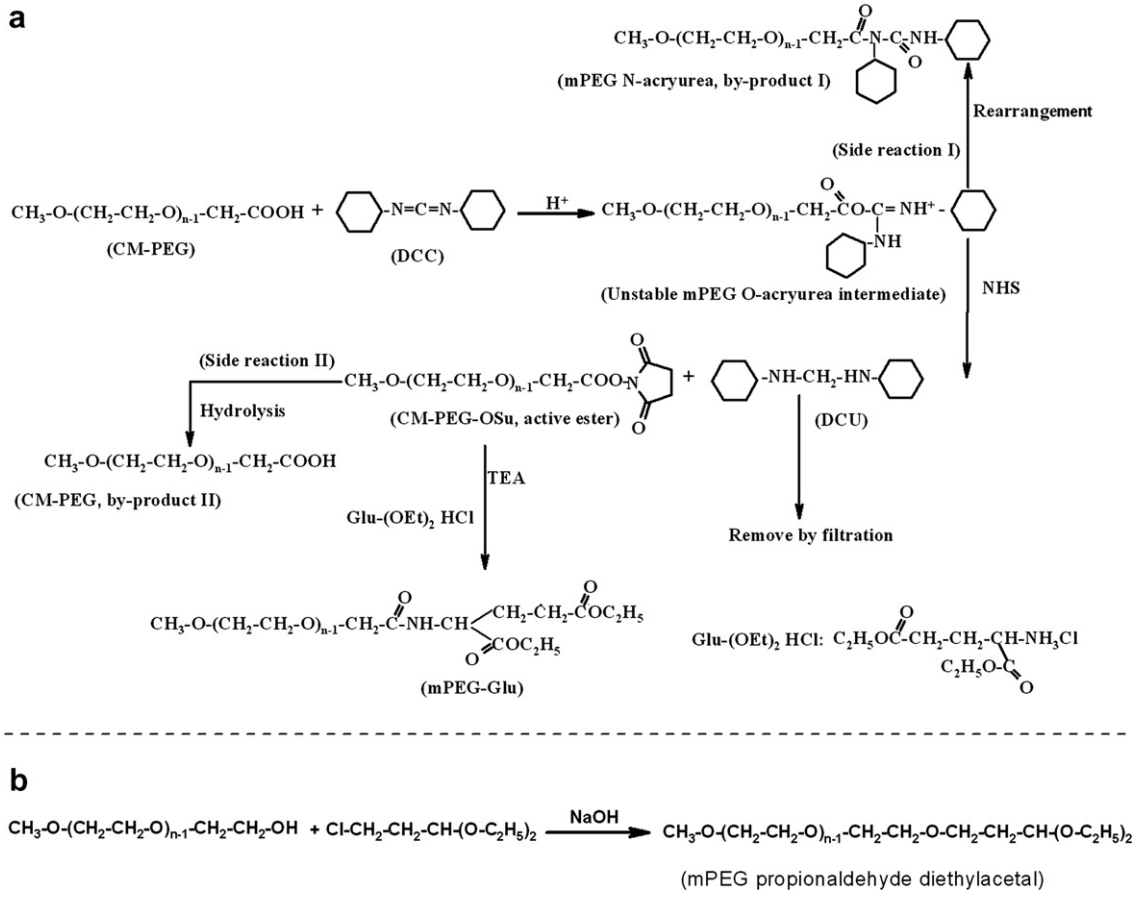


**Figure 1.** Synthesis scheme of mPEG-Glu and ALD-PEG, along with their side reaction. (a) mPEG-Glu; (b) ALD-PEG.

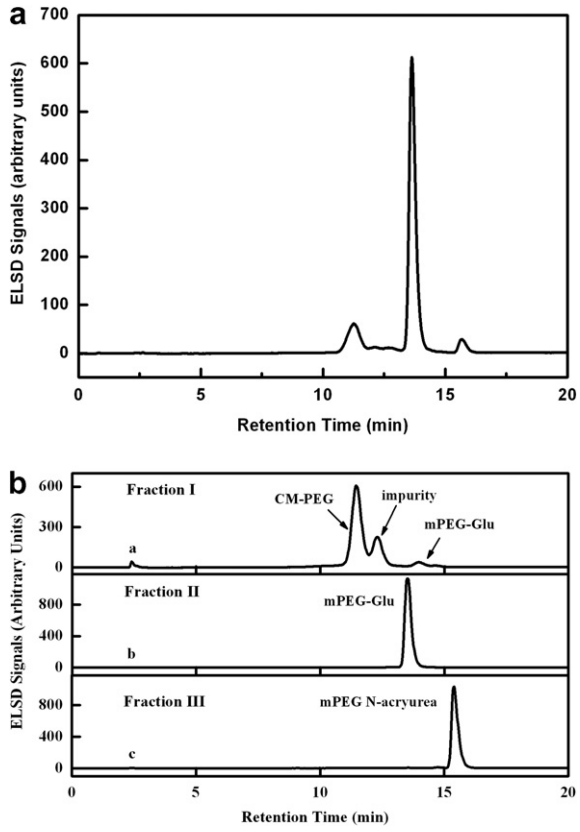


**Figure 2.** RP-HPLC analysis of mPEG-Glu reaction mixture and chromatographic fractions. RP-HPLC was conducted on a Symmetry Shield RP18 column (3.9 × 150 mm i.d., silica-based octadecyl phase, particle diameter 10 μm, Waters, USA). Solvent A was purified water containing 0.1% TFA and solvent B was acetonitrile containing 0.1% TFA. The elution program was set up as 20–35% B in 5 min, 35–50% B in 15 min, 50–100% B over 5 min. Detector: ELSD. Graph a: mPEG-Glu reaction mixture; Graph b: line a, fraction I (CM-PEG, impurity and traced amount of mPEG-Glu); line b, fraction II (pure mPEG-Glu); line c, fraction III (pure PEG *N*-acryurea).

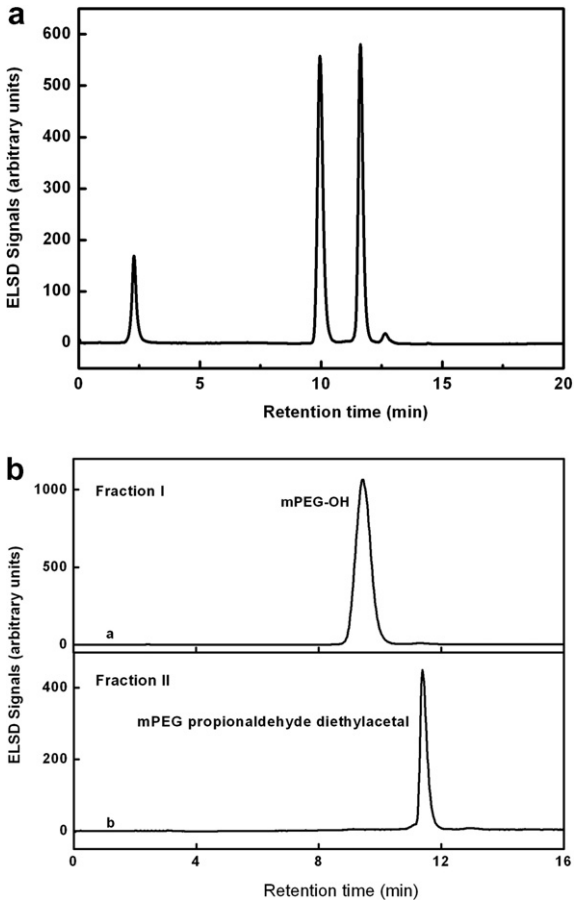


**Figure 3.** RP-HPLC analysis of ALD-PEG reaction mixture and chromatographic fractions. RP-HPLC was conducted on a Symmetry Shield RP18 column (3.9 × 150 mm i.d., silica-based octadecyl phase, particle diameter 10 μm, Waters, USA). Solvent A was purified water containing 0.1% TFA and solvent B was acetonitrile containing 0.1% TFA. The elution program was set up as 20–35% B in 5 min, 35–50% B in 15 min, 50–100% B over 5 min. Detector: ELSD. Graph a: ALD-PEG mixture; Graph b: line a, fraction I (pure unreacted mPEG); line b, fraction II (pure ALD-PEG).

the purity was analyzed by RP-HPLC, which guided us to adjust and optimize the ratio of ethanol in eluants (data not shown). For mPEG-Glu mixture, the best separation profile was achieved as such that 42.5% ethanol for fraction I, 50% ethanol for fraction II, and 90% ethanol for fraction III.[25] In the optimized conditions, the fractions II and III were achieved with high purity, showing the single and symmetric peaks on the chromatograms of RP-HPLC (Fig. 2, Graph b, lines b and c). The fraction II was identified to be target mPEG-Glu and faction III to PEG *N*-acryurea by NMR spectra.[26,27] The fraction I presented three peaks, two of which, respectively, corresponded to CM-PEG and mPEG-Glu and the rest peak was presumed to be impurity from PS/DVB resins. Under the employed chromatographic conditions, the target polymer was absorbed to maximum extent on resins and only trace amount of mPEG-Glu was co-eluted in fraction I (Fig. 2, Graph b, line a). The target mPEG-Glu achieved was free from the by-product mPEG *N*-acryurea or CM-PEG. A 0.56 g of pure mPEG-Glu was obtained from 1 g of mixture by one-step chromatography and the recovery of PEGs reached 69.8%.

To test the general application of this method, the purification of ALD-PEG mixture was also conducted on the chromatographic setup mentioned above. Similarly, ALD-PEG was well separated from unreacted mPEG when 45% ethanol was applied for fraction I and 90% ethanol for fraction II,[28] both of which presented the single and symmetric peak on the chromatograms of RP-HPLC (Fig. 3, Graph b, lines a and b). NMR spectra indicated that fraction I was unreacted mPEG and fraction II was pure ALD-PEG.[29,30] A 0.52 g of target product was obtained from 1 g of reaction mixture, and the recovery of PEGs was calculated to be 90.5%.

Most of the advantages of this purification procedure were clear and simple, where non-toxic ethanol and water were used as eluants and a low-pressure instrument such as peristaltic pump was required. Only 0.02 MPa pressure drop was produced when run at a flow rate of 4 ml/min on the setup investigated.[31] The chromatographic procedure also shared the advantages

of reliability. The purification of PEGs was also performed on different volume of PS/DVB resins (20–1000 ml) and the batches showed high reproducibility. Furthermore, the chromatography had high resolution for PEGs, which was similar to that of RP-HPLC. These appealing properties allow us to conveniently perform the purification of PEGs on a preparative scale, especially at an industrial scale.

We demonstrated PS/DVB resins powerful and efficient as separation packing of PEGs, which may result from the following aspects: (i) PS/DVB resins have rich aromatic groups on the surface and may provide much powerful selectivity to varying PEGs; (ii) The optimum of eluants can be easily obtained by subtly adjusting the ratio of ethanol and water; (iii) the PS/DVB medium has much small particle (30 μm) and larger specific surface area (745.2 $m^2$/g), which renders it high separation performance.

In summary, the PS/DVB resins was first-time used to purify the PEGs under low pressure on a preparative scale and showed promising properties. The target mPEG-Glu and ALD-PEG were favorably achieved with the purity of >99% at gram scale. The studies offered a novel, clear, and powerful alternative approach for the purification of PEGs.

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22. The CM-PEG was reacted with DCC to form unstable mPEG *O*-acryurea intermediate, the major portion of which was reacted with NHS to produce the active ester of polymer (CM-PEG-OSu) and DCU precipitate, and the other small portion was rearranged to give rise to by-product mPEG *N*-acryurea. The active ester of CM-PEG-OSu was unstable, and was readily hydrolyzed by traced water in solvent (methylene chloride) to give CM-PEG, as shown in Figure 1a.
23. RP-HPLC was conducted on Agilent 1100 HPLC System (Agilent Technologies, Palo Alto, USA) equipped with evaporative light scattering detection (ELSD). Nitrogen was used as carrier gas, and the pressure at the nebulizer was set to 2.0 bar. The temperature of the evaporator oven was 40 °C. A Symmetry Shield RP18 column (3.9 × 150 mm i.d., silica-based octadecyl phase, particle diameter 10 μm, Waters, USA) was equilibrated with 80% solvent A (0.1% trifluoroacetic acid in ultra-pure water) and 20% of solvent B (0.1% trifluoroacetic acid in acetonitrile) at the flow rate of 0.5 ml/min. A 5 μl volume of samples (3 mg/ml) was loaded onto the column and the elution program was set up as 20–35% B in 5 min, 35–50% B in 15 min, 50–100% B over 5 min at room temperature.
24. The conversion ratio was calculated based on the area under the curve by integrating chromatogram of RP-HPLC with the elution program mentioned at Ref. 23.
25. The column (2.6 × 40 cm i.d.) packed with PS/DVB resins was fully equilibrated with 30% (v/v) ethanol and then loaded with 1 g of mixture (dissolved in 30% ethanol). The column was washed at a flow rate of 4.0 ml/min using a peristaltic pump about three column volumes. The column was developed with 42.5% ethanol to elute fraction I till no PEG ingredient was detected with iodine assay. This fraction was assigned to fraction I. Subsequently, 50% ethanol was applied for fraction II and 90% ethanol for fraction III in a step-wise manner. The eluted fractions were evaporated to dryness and then redissolved in 80 ml of pure water following the extraction with three portions (20× 3 ml) of $CH_2Cl_2$. The extracts were pooled, dried over $Na_2SO_4$, concentrated, and finally precipitated by cold ether. Recovery (69.8%), fraction 1, 0.087 g; fraction 2, 0.59 g; fraction 3, 0.021 g.
26. $^1$H NMR ($DCCl_3$), $\delta$ (ppm): 1.30 (t, 6H, –$OCH_2CH_3$of ester), 2.3–2.5 (m, 4H of glutamic acid, –$CH_2$–$CH_2$–), 3.38 (s, 3H, $CH_3$–O–$CH_2$–$CH_2$–), 3.5–3.6 (s, multi H, –O–$CH_2$–$CH_2$– of PEG backbone), 4.0–4.1 (m, 4 H, $CH_3$–$CH_2$–O– of ester), 5.3 (m, 1 H, –CH–), 7.3 (d, 1H, –NH–).
27. $^1$H NMR ($DCCl_3$), $\delta$ (ppm): 3.40 (s, 3H, $CH_3$–O–$CH_2$–$CH_2$–), 3.59 (s, multi H, PEG backbone, –O–$CH_2$–$CH_2$–), 4.26 (s, 2H, –O–$CH_2$–CO–N(Phe)–), 6.0 (s, 1H, –CO–NH–Phe), 3.58 (2H, overlapped by the signal of PEG

backbone, –N–C*H*–$CH_2$–$CH_2$–$CH_2$–$CH_2$-$CH_2$–), 1.51 (m, 8H, –N–CH–C*H*$_2$–$CH_2$–$CH_2$–$CH_2$–C*H*$_2$–), 1.29 (m, 12H, –N–CH–$CH_2$–C*H*$_2$–C*H*$_2$–C*H*$_2$–$CH_2$–).

28. ALD-PEG mixture (1.0 g) was dissolved in 30% (v/v) ethanol and purified on the column (26 × 40 cm i.d.) packed with PS/DVB previously equilibrated with 30% ethanol. The column was washed with 30% ethanol until no PEG was detected by iodine assay. Ethanol (45%) was then applied to the column and washed till no PEG ingredient was detected in eluant to get fraction I. The desired polymer was achieved in fraction II by 90% ethanol in a step-wise manner. The eluted fraction was evaporated to dryness and then dissolved in 80 ml of pure water following the extraction with three portions (20× 3 ml) of $CH_2Cl_2$. The extracts were pooled, dried over $Na_2SO_4$, concentrated, and finally precipitated by cold ether. Recovery (90.5%), fraction 1: 0.43 g; fraction 2: 0.475 g.
29. $^1$H NMR ($DCCl_3$), $\delta$ (ppm): 3.60 (s, PEG backbone –O–C*H*$_2$–C*H*$_2$–), 3.38 (s, 3H, C*H*$_3$–O–).
30. $^1$H NMR ($DCCl_3$), $\delta$ (ppm): 3.60 (s, PEG backbone –O–C*H*$_2$–C*H*$_2$–), 3.38 (s, 3H, C*H*$_3$–O–), 4.64 (t, 1H, –C*H*–(O–$C_2H_5$)$_2$), 1.90 (m, 2H, –C*H*$_2$–CH–(O–$C_2H_5$)$_2$), 1.20 (t, 6H, –(O–$CH_2$–C*H*$_3$)$_2$).
31. The column pressure was measured at room temperature in the column (2.6 × 40 cm i.d.) packed with 200 ml of PS/DVB and 30% ethanol as mobile phase, using an AKTA Purifier 10 Protein Purification Systems (GE Healthcare, USA). The flow rate was set up as 4.0 ml/min. The pressure drop was read from the instrument curve.