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Heterobifunctional PEGs: Efficient synthetic strategies and useful conjugation methodologies

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ABSTRACT

The desire to develop nanoparticle and liposomal formulations as drug carriers capitalizing on active transport mechanisms requires constant development of novel heterobifunctional polyethyleneglycol (PEG) constructs. Such constructs should be capable of sequentially reacting with extracellular binding ligands and structural components of nanoparticles and/or liposomes. This paper describes two syntheses of heterobifunctional PEGs useful for tethering small molecule ligands to synthetic lysine-bearing polymers.

Over the past 10 years, therapeutic siRNA has attracted much interest as a new class of pharmaceutical agents.[1–3] However, systemic delivery of siRNA to biologically relevant tissues and cell types is difficult due to many obstacles including rapid degradation and clearance.[4] In order to counter these problems, groups have explored strategies which include chemical modification of siRNA,[5,6] encapsulation within liposomes[7,8] and complexation of siRNA within nanoparticles comprising cationic polymers.[9,10] In addition, variations and combinations of these main strategies are under investigation.

Among the challenges associated with self-assembling cationic nanoparticles are particle size, particle stability, cell binding, cellular uptake, circulating half-life and endosomal escape.[11] Several of these issues can be addressed through PEGylation.[12,13] Specifically, coating nanoparticle surfaces with polyethylene glycol can shield the cationic nature of the particles from the electrolytes and anionic environment of the blood stream. This effect also reduces indiscriminant cell binding and potentially improves circulating half-life.

While PEGylation has the potential to improve pharmacokinetic profiles, it does little to direct nanoparticle formulations to specific cell types. This can be achieved by targeting cell-specific extracellular receptors. Through incorporation of appropriate ligands, cell-targeting[14,15] and active transport processes[16,17] can be exploited. Important advantages regarding ligand tethering through PEG include multivalent presentation of ligands[18] and dramatic extension of the ligand interaction range.[19] To this end, heterobifunctional PEGs can serve as tethers capable of both imparting favorable pharmacokinetic properties and presenting cell-targeting ligands. Herein, we describe the syntheses of two useful heterobifunctional PEGs, their reaction with modified RGD and their conjugation to a synthetic lysine-bearing polymer.

PEGs, being relatively inexpensive polymers and available in a range of sizes, present two hydroxyl groups available for reaction. Unfortunately, due to the distances between these hydroxyl groups, differentiation based upon reactivity is not a practical approach for the preparation of heterobifunctional structures. However, through stoichiometric introduction of reagents coupled with incorporation of ionizable functional groups, heterobifunctional PEGs can be easily prepared and purified through ion-exchange chromatography.[20–22]

Development of a scheme for elaboration of compound **1** (prepared in two-steps with one ion-exchange chromatography, 35% overall yield)[21] into a heterobifunctional PEG bearing two electrophilic functional groups presented some challenges in that the chemistry required for generation of the first functional group had to be compatible with the chemistry required for the second. Evaluation of the literature revealed a vinyl sulfone preparation that could be utilized with no impact upon the carboxymethyl functionality.[23,24] The fully elaborated sequence is illustrated in Scheme 1. As shown, the carboxylic acid of compound **1** was initially converted to a methyl ester. The hydroxyl group residing

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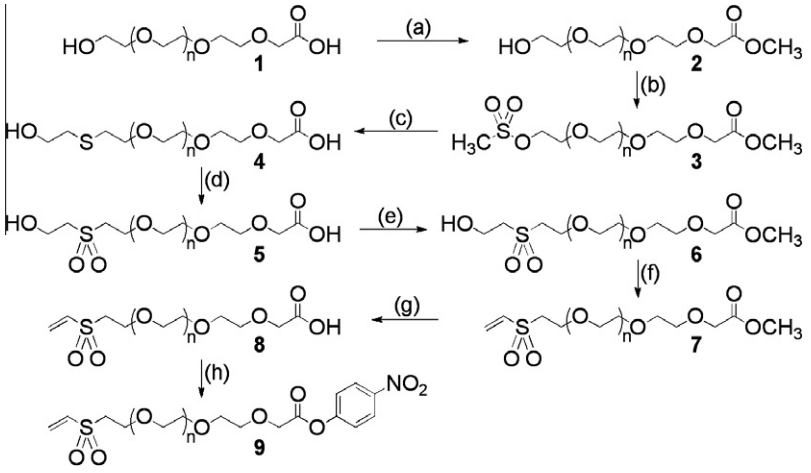


Scheme 1. Reagents and conditions: (a) HCl, MeOH (98% yield); (b) Ms-Cl, DIEA, CH_2Cl_2 (98% yield); (c) mercaptoethanol, NaOH, H_2O (95% yield); (d) H_2O_2, tungstic acid, H_2O (96% yield); (e) HCl, MeOH (98% yield); (f) Ms-Cl, DIEA, CH_2Cl_2 (98% yield); (g) NaOH, H_2O (98% yield); (h) nitrophenyl 4-trifluoroacetate, pyridine (98% yield).

on the resulting product was then converted to a mesylate using methanesulfonyl chloride. On treatment with mercaptoethanol and sodium hydroxide, both ends of compound **3** were affected. Specifically, the mesylate was displaced forming the desired hydroxyethyl sulfide. At the same time, the methyl ester was hydrolyzed regenerating the carboxylic acid.

While compound **4** now contained a carboxylic acid, this was of no consequence for the next step because oxidation of the thioether to a sulfone had no impact on the carboxylate terminus. However, in order to prevent esterification and/or polymerization in the following step, protection of the carboxylic acid was required. This was accomplished, as before, utilizing methanolic hydrochloric acid. With compound **6** in hand, formation of the required vinyl sulfone group was achieved in a tandem mesylation–elimination process on treatment with methanesulfonyl chloride and diisopropylethylamine. In preparation for final activation of the carboxylate group, the ester of compound **7** was hydrolyzed with aqueous sodium hydroxide generating the desired vinyl sulfone carboxymethyl PEG, **8**.

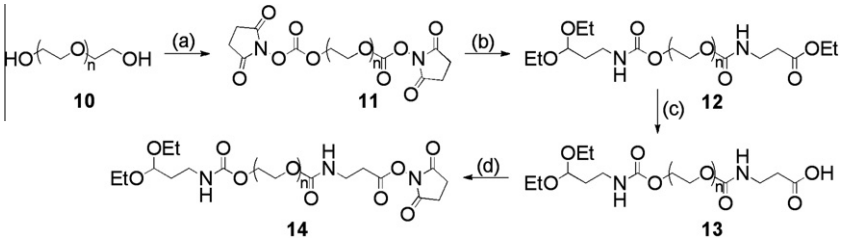
Initial attempts for conversion of the carboxylic acid of compound **8** to an active ester were directed at formation of a succinimidyl ester. Unfortunately, significant precedence exists suggesting that a NHS ester residing on a carboxymethyl PEG is hyperreactive rendering isolation and storage highly problematic.[24,25] Due to these problems, attention was directed towards formation of the less reactive 4-nitrophenyl ester. In support of this decision, 4-nitrophenyl esters of carboxymethylated PEGs are known to exhibit reactivities that are similar to succinimidyl esters of related propionate derivatives.[26]

The first attempt at formation of a 4-nitrophenyl ester incorporated DCC as a coupling agent. While the desired product was formed, these conditions resulted in a 15% impurity identified as the DCC-derived *N*-acyl urea.[27,28] This problem was circumvented by avoiding DCC and utilizing nitrophenyl 4-trifluoroacetate in pyridine.[29] Under these conditions, the desired heterobifunctional PEG was isolated in a 98% yield from the starting carboxylic acid, **8** (Scheme 1). Furthermore, this product, **9**, had 100% functional purity at each end of the PEG.

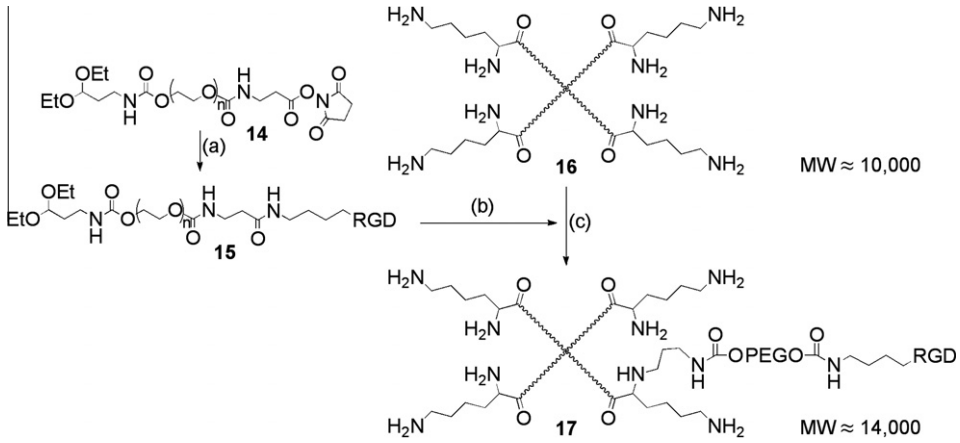
Upon examination of the sequence represented in Scheme 1, one recognizes that a methyl ester is introduced twice and hydrolyzed twice. Furthermore, considering the preparation of compound **1**, there is an additional hydrolysis step.[21] While, arguably, one of the hydrolysis steps is an artifact of the conditions utilized for incorporation of the thioether, there are four protection/deprotection steps that are incorporated by necessity. Thus, while this 10 step process is high yielding, the sequence is somewhat lengthy and inefficient. Therefore, an alternative strategy for the preparation of a similar heterobifunctional PEG was developed. This strategy, illustrated in Scheme 2, relies upon PEG-bearing active carbonates.

As shown in Scheme 2, PEG was treated with disuccinimidyl carbonate[22] generating the bis-succinimidyl carbonate, **11**.[30] Sequential treatment of this compound with β-alanine ethyl ester followed by 3,3-diethoxypropylamine led to isolation of a statistical mixture of PEG-bis-acetal, PEG-bis-ethyl ester and the desired heterobifunctional PEG, **12**. Upon hydrolysis, a similar mixture, now containing carboxylic acids, was isolated. From this mixture, compound **13** was isolated via ion-exchange chromatography. With compound **13** in hand, the final active ester was prepared on treatment with disuccinimidyl carbonate and pyridine.

Synthesis of both compounds **9** and **14**, similar to previously reported preparations of heterobifunctional PEGs,[20–22] rely on one ion-exchange chromatography step. Furthermore, both are highly scalable routes utilizing relatively inexpensive reagents. However, where there are similarities, the advantages of compound **14** are



Scheme 2. Reagents and conditions: (a) Disuccinimidyl carbonate, pyridine, CH_2Cl_2 (99% yield); (b) (i) ethyl 2-aminopropionate hydrochloride, DIEA, CH_2Cl_2; (ii) 3,3-diethoxypropylamine, DIEA, CH_2Cl_2 (97% yield); (c) (i) NaOH, H_2O (94% yield); (ii) DEAE-Sephadex purification (6 mM to 22 mM ammonium bicarbonate/H_2O in 2 mM steps, 39% yield); (d) disuccinimidyl carbonate, pyridine, CH_3CN, CH_2Cl_2 (90% yield).



Scheme 3. Reagents and conditions: (a) $H_2N(CH_2)_4RGD$, H_2O; (b) TFA, H_2O; (c) $NaCNBH_3$, H_2O, pH 5. 35% overall yield.

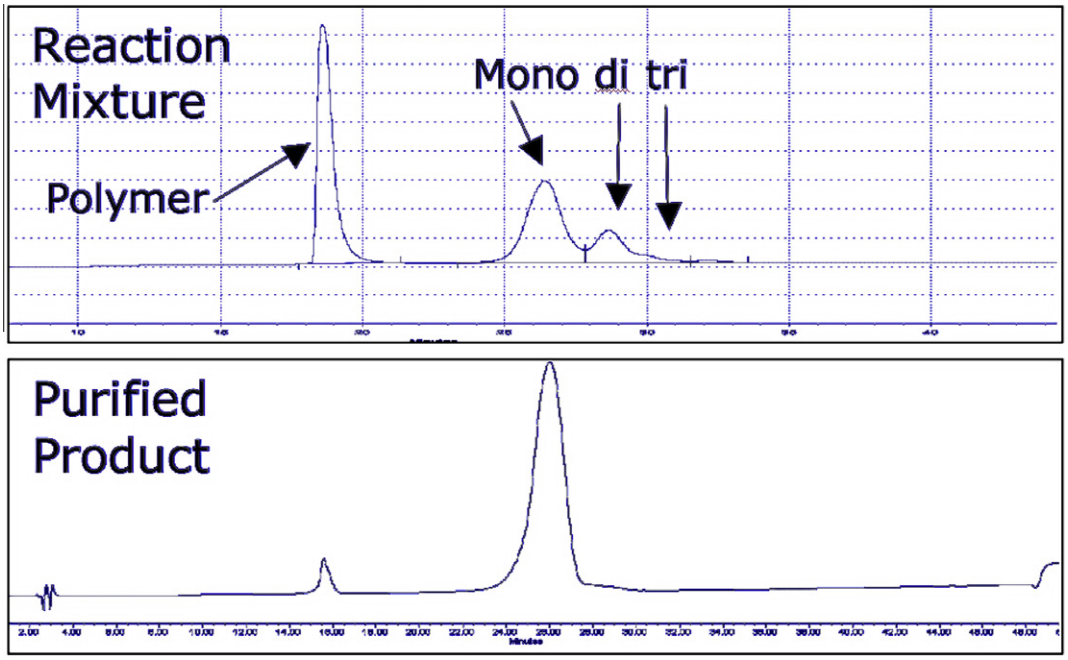


Figure 1. Preparation of compound **17**. Top frame—reverse phase HPLC of the reaction mixture obtained from the PEGylation of a lysine-bearing polymer (Scheme 3). Bottom frame—reverse phase HPLC of compound **17** purified from the reaction mixture by ion-exchange chromatography.

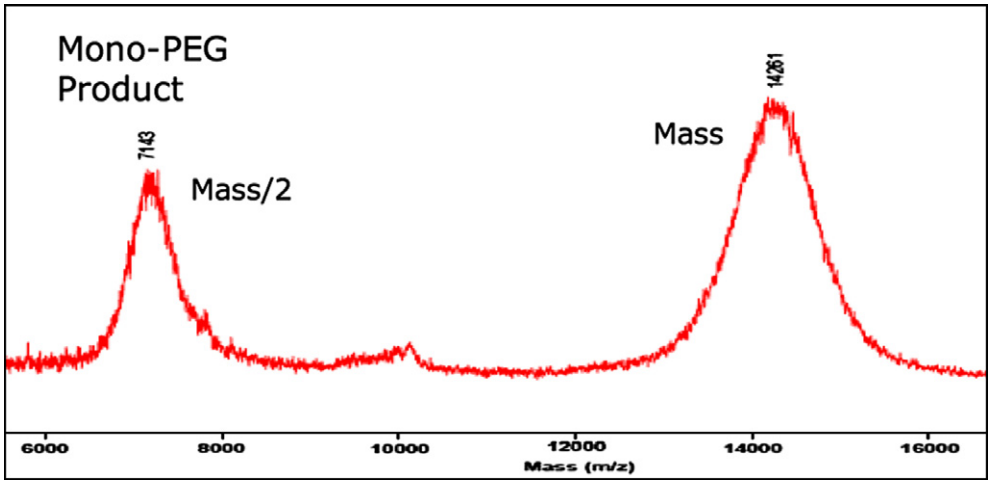


Figure 2. MALDI-TOF of purified compound **17** (M_W ≈14,000) from Scheme 3.

significant. Specifically, the shorter sequence translates to reduced time for synthesis. Furthermore, the yield, calculated over four steps and one chromatography is 32% while a comparable calculation for compound **10** reveals a 27% overall yield from PEG.[21]

Once prepared, the heterobifunctional PEGs of this study were sequentially conjugated with a ligand and then a synthetic lysine-bearing polymer. As **9** and **14** each contain one active ester and one alkylating functionality, both lead to similar constructs. In this context the differences between a protected aldehyde versus a vinyl sulfone, are minor. As shown in Scheme 3, compound **14** is reacted with a lysine-containing RGD derivative bearing only one exposed amino group. The resulting liganded PEG, compound **15**, is then treated with trifluoroacetic acid to liberate the necessary aldehyde group. The resulting solution is then added directly into a solution of lysine-bearing polymer, **16**,[31] buffered at pH 5 with sodium phosphate. At this pH, only the less-basic α-amino groups are available for reaction.[32] Subsequent introduction of sodium cyanoborohydride completes the conjugation via reductive amination. While conjugates similar to compound **17** were accessible through vinyl sulfone chemistry, it is important to note that conjugation proceeded only in DMSO with DIEA as the base. Furthermore, this conjugation occurred randomly at any amine and in low yield (<10%). Thus overall, **14** offers more selectivity and better yields compared to **9**.

As with any reaction of a single reagent with a polyfunctional substrate, statistical mixtures of products are formed. The lysine-bearing polymer utilized in the present study, being tetrameric,[31] is no exception. As illustrated in Figure 1, in addition to unreacted lysine-bearing polymer, a mixture of mono-, di- and tri-PEGylated polymers is formed. This mixture is easily separated by ion-exchange chromatography[33] which allows for both isolation of the desired product (30% yield) and recycling of unreacted polymer. For all peaks, structural confirmation was supported by MALDI-TOF (Fig. 2).

In summary, syntheses for two heterobifunctional PEG reagents are described. Use of these reagents as polymeric tethers between an RGD-based ligand and a polymeric substrate are illustrated. Finally, through use of the chemistry described herein, exploitation of cellular targeting and active transport mechanisms may be realized for the delivery of liposomal or polymer-based drug delivery systems.

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