Articles

A Practical Synthesis of *N*ϵ-Monomethoxypoly(ethylene glycol)carbonyl-L-lysine Hydrochloride: A Precursor to Chiral and Mixed Branched Pegylated Reagents

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ABSTRACT: Starting from monomethoxypoly(ethylene glycol) succinimido carbonate (**2**), an efficient two step synthesis of *N*ϵ-monomethoxypoly(ethylene glycol)carbonyl-L-lysine hydrochloride (**8**) is described. The hydrochloride salt **8** was used to prepare *N*α,*N*ϵ-bis(monomethoxypoly(ethylene glycol)carbonyl)-L-lysine (**9**). This is the first reported example of the synthesis of a chiral lysine PEG precursor to an active pegylated reagent.

Introduction

Pegylation of proteins is an extensively used strategy designed to improve the biochemical and pharmacological properties of therapeutic proteins.[1,2] The most common conjugated product is formed from the reaction of an activated ester of racemic *N*α,*N*ϵ-bis-(monomethoxypoly(ethylene glycol)carbonyl) -lysine (**1**) and a protein. Because of the route, the molecular weights of each polymer fragment are equivalent and are typically 20Kda.[3] Succinimido (Su) carbonate esters **1a** are frequently used as the activated ester in the bioconjugation reaction (Scheme 1).[4] Branching of the poly(ethylene glycol) (PEG) portion caused by the two PEG polymer substituents is thought to increase protection of the polymer by an "umbrella-like effect" and allows for three-dimensional coverage of the protein surface.[5] Recent studies based on the viscosity radii have shown that the increased circulation half-life for branched-PEG proteins can also be explained by more efficient masking of the protein surface when compared with linear-PEG proteins.[6] Despite the commercial importance of such PEG reagents, a practical route to chiral and mixed branched precursors has not yet been reported.[7]

The first synthesis of racemic $(mPEG)_2$-lysine **1** was published by Monfardini in their seminal paper on the bioconjugation and protection of proteins by pegylation. In this work, the synthesis of $(mPEG)_2$-lysine **1** parallels the chemistry that is used in the bioconjugation reaction and involves the reaction of lysine in basic, buffered media for twenty-four hours with mPEG-SC **2** to afford mixtures of mPEG-OH **4**, mPEG-lysine **3**, and $(mPEG)_2$-lysine **1** (Scheme 2).[4] The desired bis acylated product **1** was separated by preparative ion exchange or gel permeation chromatography.[4,8]

When the ethyl ester of lysine is used, organic solvents can be employed in the coupling reaction and the formation of mPEG-OH **4** by hydrolysis of the starting material **2** can be avoided (Scheme 3).[9] Alkaline hydrolysis of $(mPEG)_2$-lysine ethyl ester **5** will produce a racemic product[10] and this chemistry does not allow for the formation of mixed branched pegylated reagents. A route in which chromatographic separations are avoided and in which chiral reagents are formed is highly desirable as the effect of chirality and different molecular weight branches of the pegylated reagents on the pharmacokinetic and pharmacodynamic properties of the conjugated protein has not yet been investigated.

Felix has reported on complimentary routes to symmetrically and asymmetrically branched pegylated reagents.[11,12] Tris-branched pegylated reagents, which contained either free amino[11] or carboxylic acid[12] groups were available by this chemistry. These reagents were also found to be suitable for coupling to model peptides. In these routes, an orthogonal protection strategy was employed to prepare the reagents and differentiation of the α- and ϵ nitrogens of lysine was accomplished by the use of Fmoc-Lys(Boc)-OSu as the starting material.[11,12]

A practical method for the chemoselective differentiation of the α and ϵ nitrogens of lysine has recently been reported. This chemistry relies on the formation of a stable complex of lysine by reaction with 9-borabicyclononane (9-BBN). These 9-BBN complexes of lysine and other amino acids are soluble in organic solvents and can be readily deprotected by an exchange with ethylenediamine or by hydrochloric acid in methanol. No epimerization was observed for either of these deprotection conditions (Scheme 4).[13] This protecting group has found recent utility for the simultaneous protection of the α-amino and α-carboxyl groups of hydroxy lysine,[14] N-methyl ornithine[15] and 3-iodo-tyrosine.[16]

As our goal was to develop a practical multigram route to chiral mPEG lysine **3**, the 9-BBN protecting group was ideally suited for our needs as both synthetic transformations, namely carbamate formation and removal of the 9-BBN protecting group can occur in organic solvents. If water is not the reaction solvent, hydrolysis of mPEG-SC **2** to mPEG-OH **4** can be avoided and chromatographic separations can be eliminated. Simplifications of the reaction workup process will also be forthcoming with such improvements as only straightforward solvent transfers are required to isolate intermediates and final products. We wish to report on the synthesis of *N*ϵ-monomethoxypoly(ethylene glycol)carbonyl-L-lysine hydrochloride (**8**) and its use in the

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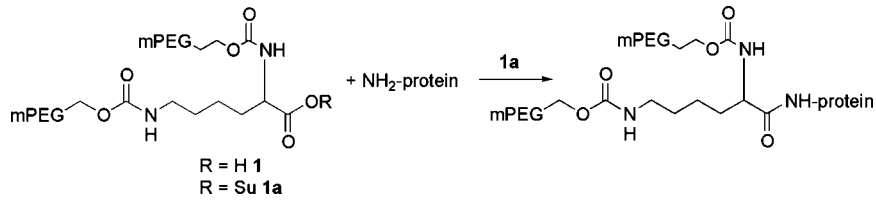
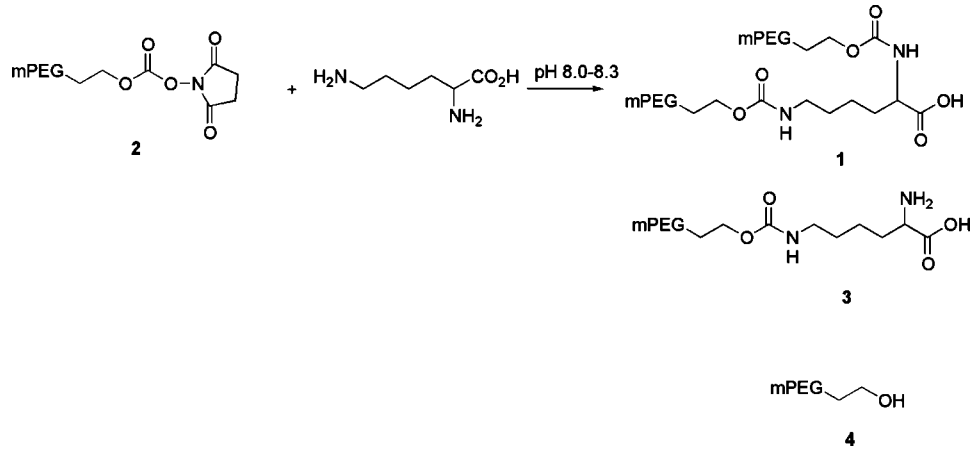
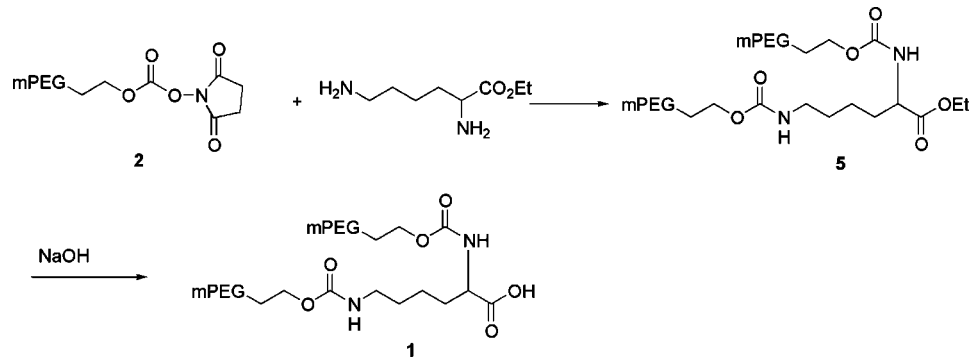
Scheme 1. Reaction of $(mPEG)_2$ Lysine-Su 1a with a Protein



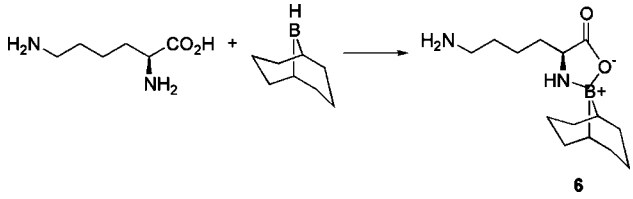
Scheme 2. First Generation Synthesis of $(mPEG)_2$-lysine 1



Scheme 3. Second Generation Synthesis of $(mPEG)_2$-lysine 1



Scheme 4. Preparation of 9-BBN Lysine 6



synthesis of *N*α, *N*ϵ-bis(monomethoxypoly(ethylene glycol)carbonyl)-L-lysine (**9**) (Scheme 5).

Results

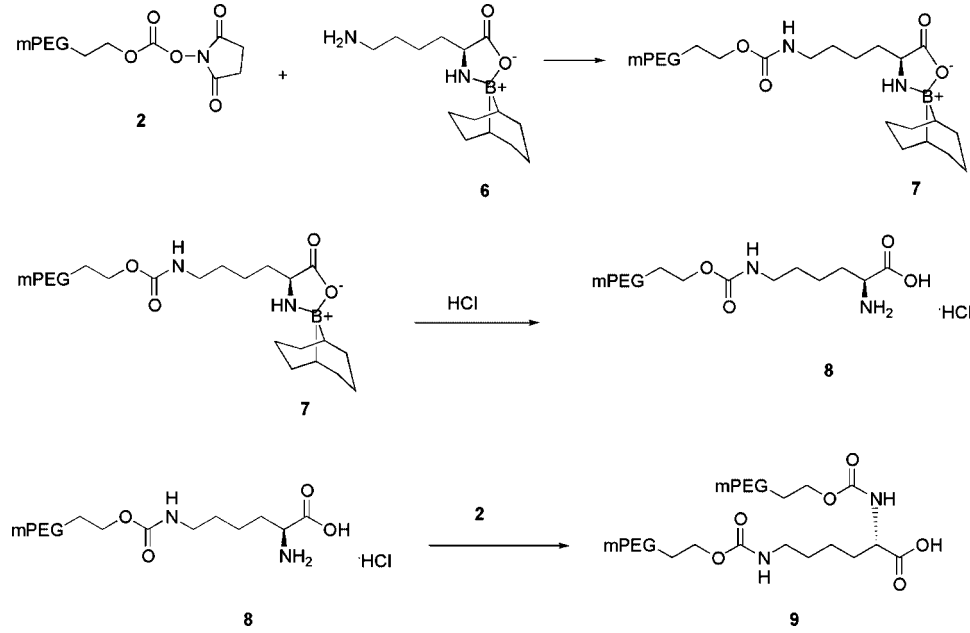
Although 9-BBN-L-lysine **6** has been reported to be insoluble in dichloromethane,[13] we found that when added to solutions of mPEG-SC **2**, homogeneous reaction mixtures are observed. Due to the reactivity of the epsilon nitrogen of 9-BBN-lysine **6**, only molecular solubility of the complexed lysine **6** is required. An excess of 9-BBN-lysine **6** can be used as the PEG polymer **7** can be recrystallized from ethyl acetate and separated from the reagent excess and the byproduct, *N*-hydroxysuccinimide. Acetonitrile is also an useful solvent for this reaction. The reaction can by conveniently monitored by HPLC with evaporative light scattering detection (ELSD) (Figures 1 and 2).

Unmasking of the α-nitrogen of L-lysine was readily achieved by the use of hydrochloric acid in methanol (Figure 3). Additional water was not required to solubilize the mPEG polymer **7**. The course of the hydrolysis could be observed by ELSD-HPLC. Although 9-BBN-lysine **6** has a UV maximum at 205 nm with an extinction coefficient of about 1100 L/mol cm in acetonitrile, reaction monitoring by ELSD-HPLC was found to be much more informative than UV or UV-HPLC.

Azeotropic removal of water from the mPEG-L-lysine **8** was accomplished by the use of toluene and the polymer was considered dry once the distillate had a water content by KF (Karl Fischer titration), which approximated the water content of the starting toluene. This operation left either an oily residue or a stirrable semisolid to which ethyl acetate was added. Large solvent to solute ratios were required so that a filterable suspension would be obtained. The overall yield from mPEG-SC **2** was 67%.

Free-basing of the hydrochloride of mPEG-L-lysine **8** was accomplished by an excess of Hünig's base (diisopropylethylamine, DIEA).[11,12,17−19] An excess was present to ensure complete reaction of the hydrochloride salt and the associated

Scheme 5. Third Generation Synthesis of $(mPEG)_2$-L-lysine 9



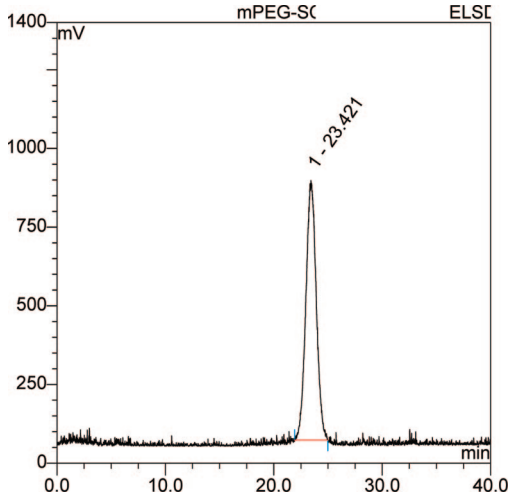


Figure 1. ELSD-HPLC chromatogram of mPEG-SC **2**.

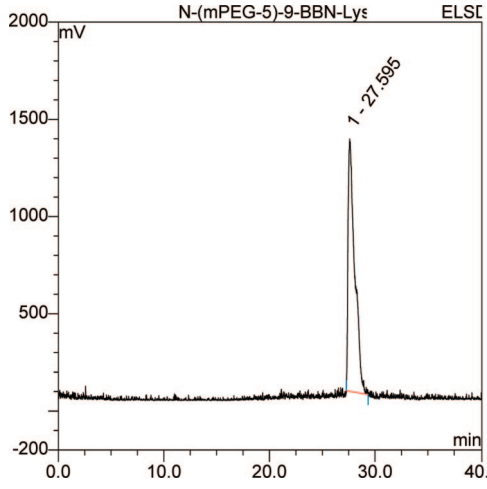


Figure 2. ELSD-HPLC chromatogram of Nϵ-(mPEG-5)-9-BBN-lysine **7**.

formation of the salt of the carboxylic acid. DIEA may also facilitate the coupling reaction by catalysis and *N*-hydroxysuccinimide may form a salt with DIEA. For these reasons, four equivalents of DIEA were present, which meant that an efficient way to remove 4 equiv of diisopropylethylamine hydrochloride from a water soluble polymer was required. The DIEA was converted into DIEA·HCl so as to prevent any racemization that might occur during the solvent exchnage from methylene chloride into ethyl acetate. The amount of DIEA·HCl by weight relative to the polymer is low; however the molar amount of this impurity is high. Because of the two isopropyl groups in DIEA·HCl, this hydrochloride salt has some lipophilicity and it was found that a recrystallization from wet ethyl acetate could separate DIEA·HCl from $(mPEG)_2$-CO_2H **9**.

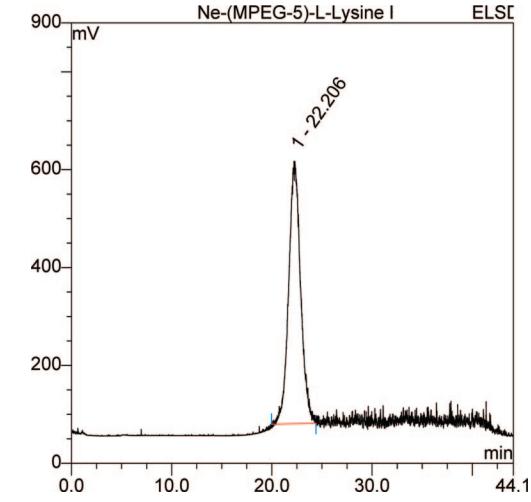


Figure 3. ELSD-HPLC chromatogram of Nϵ-(mPEG-5)-L-lysine HCl **8**.

In the event, mPEG-lysine **8** was reacted with DIEA in methylene chloride at room temperature. mPEG-SC 2 was added and the reaction was monitored by ELSD-HPLC and was complete within several hours at room temperature. Hydrochloric acid was added and the solvent was removed and replaced by ethyl acetate. $(mPEG)_2$-CO_2H **9** was obtained in a 88% yield.

Discussion

In the first reported synthesis of racemic *N*ϵ-monomethoxypoly(ethylene glycol)carbonyl-lysine (**3**), the required chemose-

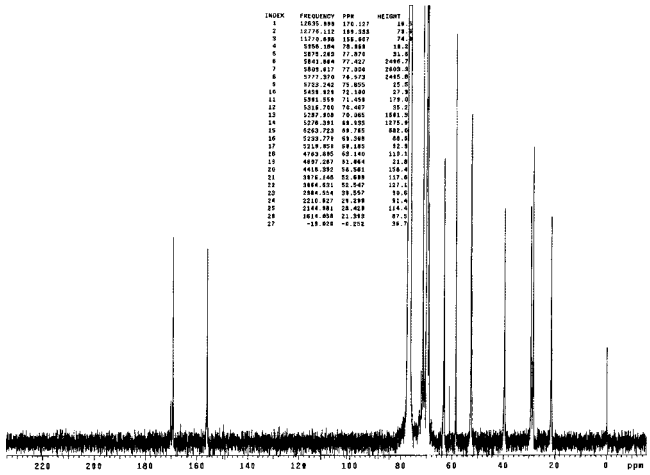


Figure 4. ^{13}C NMR spectrum of Nϵ-(mPEG-5)-L-lysine HCl **8**.

lectivity was partially achieved by the use of an excess of lysine in aqueous medium at a controlled pH of 8.0−8.3.[4] mPEG-*p*-nitrophenyl carbonate was used as the pegylating reagent and the yield of modified amino groups was only 53%. When mPEG *p*-nitrophenyl carbonate was used as the pegylating reagent for the derivatization of the α-amino group of lysine, a 3 day reflux in methylene chloride in the presence of triethylamine was necessary. Such conditions are likely to cause racemization and the procedure also required that an excess of mPEG *p*-nitrophenyl carbonate be used. In order to hydrolyze the reagent excess, the product was exposed to more strongly basic conditions. Because of these shortcomings, the one step procedure (Scheme 2) emerged for the synthesis of symmetrically substituted mPEG reagents.[4]

Because of the insolubility of lysine in organic solvents, water is used as the reaction solvent for the pegylation reaction. This causes hydrolysis of mPEG-SC **2** to mPEG-OH **4** and as such, a chromatographic separation is required to separate mPEG-OH **4** from pegylated lysine derivatives. 9-BBN-L-lysine **6** is ideally suited as a reagent in the coupling with pegylated activated esters as the reagent is freely soluble in organic solvents. The simultaneous protection of the α-amino and α-carboxyl groups means that chemoselective pegylation reactions with respect to the molecular weight of the mPEG-SC **2** reagent are possible with lysine **6** as only the ϵ-nitrogen of lysine will react with mPEG-SC **2**.

Having differentiated the α and ϵ nitrogen of lysine by a protection strategy, a deprotection can unmask the other reactive nitrogen of lysine, the α-amino group. Acidic conditions were employed instead of the use of ethylenediamine as the reagent excesses are more easily separated from the product mPEG-lysine **8**. As shown in Figure 4, the ^{13}C NMR spectrum is correct for this intermediate.

As expected, the α-amino group of mPEG lysine **8** was equally as reactive when combined with mPEG-SC **2** at room temperature in methylene chloride. Although mPEG-L-lysine **8** is a derivative of a hydrochloride salt of lysine, the attachment of the mPEG polymer to the epsilon nitrogen of lysine causes the polymer to be freely soluble in solvents like methylene chloride and acetonitrile. Because of this, the introduction of the second PEG polymer chain can be accomplished in anhydrous organic solvents and the formation of mPEG-OH **4** can be avoided. Equivalent amounts of the activated ester, mPEG-SC **2** can be used. The versatility of this chemistry means that the mPEG-SC **2** reagent can have the same molecular weight as the mPEG polymer or the molecular weights can be different. With these reaction and workup conditions, no racemization of the chiral center of $(mPEG)_2$-L-lysine **9** would be expected.

Conclusion

In conclusion, a practical synthesis of *N*ϵ-monomethoxypoly(ethylene glycol)carbonyl-L-lysine hydrochloride (**8**) is described. This compound is useful as a precursor to chiral and mixed branched pegylated reagents. With the preparation of intermediate **8**, the synthesis of pegylated reagents in which the influence of chirality and branching molecular weight on the properties of the conjugate protein can be ascertained. With respect to the latter characteristic, a lower molecular weight PEG polymer at the α carbon, might increase the reactivity of the pegylated reagent as reduced protection by the PEG polymer at this position might result in increased levels of conjugation.

Experimental Section

All chemicals were used as received. mPEG-SC **2**[20] was a product of internal manufacture and 9-BBN-L-lysine **6** was prepared according to the literature procedure.[13]

The HPLC system consisted of a Thermo Separations degasser, P2000 gradient pump and AS1000 autosampler. The eluent was analyzed by a Shimadzu SPD 10A UV detector connected in series to an Alltech ELSD evaporative light scattering detector. Data collection and analysis was done by Chromeleon software by Dionex with a Dionex UCI-100 interface. A Prosphere HP 300 Å, C18 5 μ column (Alltech) operating at 35 °C was used.[21] Gradient elution was used with initial conditions of 70% water and 30% acetonitrile at a flow rate of 0.8 mL/min. This method was used for mPEG-SC **2** and 9-BBN-L-lysine **7**. For mPEG-L-lysine **8** and (mPEG)2-CO2H **9**, the same conditions were used except that the aqueous mobile phase was 0.1% TFA (v/v). The initial conditions were changed to a final composition of 45% water and 55% acetonitrile over a 20 min period. The ELSD was operated at a drift temperature of 115 °C and a nitrogen flow between 3.02−3.04 SPLM. The exhaust temperature was 56 °C. UV measurements were done on a 8451A diode array spectrophotometer.

Preparation of *N*ϵ-Monomethoxypoly(ethylene glycol)carbonyl-L-lysine Hydrochloride (8). Under an argon atmosphere, 15.43 g (3.0 mmol) of mPEG-SC, 5Kda (**2**), 1.32 g (4.95 mmole) of 9-BBN-L-lysine **6**[13] and 150 mL of anhydrous dichloromethane were combined. The solution was agitated at room temperature for four hours and the course of the reaction was monitored by ELSD-HPLC. The retention time of mPEG-SC **2** was 23.4 min and that of mPEG-9-BBN-L-lysine (**7**) was 27.6 min. 9-BBN-L-lysine **6** was too volatile to be detected by the ELSD and the retention time by UV at 210 nm was approximately 6 min. The solvent was removed under vacuum. After drying at 40−45 °C for 2 h, the residue was recrystallized from 600 mL of ethyl acetate at 40 °C. The suspension was chilled in an ice bath and filtered. Filtration was followed by washing with ethyl acetate. The product was dried at 40−45 °C under vacuum until a constant weight was obtained. There was obtained 15.00 g of mPEG-9-BBN-L-lysine **7** in a 94.5% yield

The mPEG-9-BBN-L-lysine **7** was combined with 150 mL of methanol and 2.5 mL (30 mmol) of concentrated hydrochloric acid was added. The solution was heated to 40−45 °C. By HPLC and after four hours, the deprotection was complete. The methanol was removed under vacuum at 40−45 °C. Toluene (500 mL) was added and the toluene−water azeotrope was removed under vacuum at 40−50 °C. The distillation receiver was emptied and 250 mL of fresh toluene was added to the residue. After evaporation on a rotary evaporator, an aliquot of the distillate was removed and analyzed by KF. The water content of the distillate was 0.00%.

The product was dried at 40−50 °C until a constant weight was obtained and was recrystallized from 600 mL of ethyl acetate. Crystallization at room temperature was followed by cooling in an ice bath. The slurry was filtered and the product was washed with 2 volumes of 100 mL of ethyl acetate. The product was dried at 40−45 °C. There was obtained 10.4 g of *N*ϵ-monomethoxypoly-

(ethylene glycol)carbonyl-L-lysine hydrochloride (**8**) as an off-white solid in a 67% yield from mPEG-SC **2**. The retention time of mPEG-L-lysine **8** was 21.8 min. mPEG-L-lysine **8** had ^{13}C NMR (d_6-DMSO) δ 21.4, 28.4, 29.3, 39.6, 52.5, 58.7, 63.1, 69.2, 69.4, 77.0, 156.0, 169.3

Preparation of *N*α,*N*ϵ-bis(monomethoxypoly(ethylene glycol)carbonyl)-L-lysine (9). Under a blanket of argon was combined mPEG lysine **8** (5.18 g; 1.0 mmol) and 50 mL of anhydrous dichloromethane. DIEA (300 μL; 4.0 mmol) was added and the solution was stirred for 15 min. mPEG-SC, 5Kda (**2**) (5.14 g; 1.0 mmol) was added and the batch was stirred at room temperature for 3 h. The course of the coupling reaction was monitored by ELSD-HPLC. Concentrated hydrochloric acid (0.4 mL) and 0.5 mL of distilled warer were combined and added to the reaction. The methylene chloride was removed under vacuum and the solid was dried to a constant weight at 40−45 °C. The residue was recrystallized from 400 mL of ethyl acetate and was cooled in a wet-ice bath. The solid was filtered and washed with ethyl acetate. After drying, there was obtained 9.1 g of *N*α,*N*ϵ-bis(monomethoxypoly(ethylene glycol)carbonyl)-L-lysine (**9**) as a white solid in a 87.7% yield. The retention time of $(mPEG)_2$-L-lysine **9** was 26.1 min.

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