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Polymerization of Diaspirin Crosslinked Hemoglobin (DCLHb) with PEG Activated with Benzenesulfonate Bearing Electron-Withdrawing Groups

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Abstract: The hemoglobin-based oxygen carrier solution, DCLHb, was polymerized with novel polyethylene glycol polymerization reagents to provide a solution of monomers to tetramers of hemoglobin molecules. The polyethylene glycol reagents **4** and **5** were designed to be selective toward nucleophilic displacement of the substituted benzenesulfonates by the sulfhydryl groups of β93 Cys of DCLHb. © 1999 Elsevier Science Ltd. All rights reserved.

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INTRODUCTION

Many investigators have hypothesized that the ideal blood substitute is a hemoglobin solution composed of hemoglobin molecules with an average molecular weight of 150,000 to 250,000 Daltons (hemoglobin dimer to tetramer) and negligible hemoglobins having either low (e.g., 64,000 Daltons) or high (e.g., more than 400,000 Daltons) molecular weights.[1,2] The fact that hemoglobin modification reagents are often nonspecific and will react with any of the forty-four available primary amine groups and the two reactive sulfhydryl groups on hemoglobin leads to hemoglobin mixtures with undesirable molecular weight distributions. The only known method for obtaining hemoglobin compositions having the desired molecular weight characteristics involves exhaustive diafiltration and/or chromatographic separation to remove hemoglobin molecules outside the desired molecular weight range.

Since there are only two reactive sulfhydryl groups on hemoglobin and hemoglobin-based oxygen carriers, such as Diaspirin Crosslinked Hemoglobin (DCLHb, molecular weight of 64,000 Daltons),[3] thiol specific polymerization reagents may provide the necessary control to furnish hemoglobin solutions with the ideal molecular weight distribution. We have studied a number of bis(maleimide) crosslinking reagents for the polymerization of DCLHb[4]. Even though the maleimide group reacts preferentially with thiols, the group can also react with the primary amino groups of hemoglobin which leads to mixtures of polymerized DCLHb with molecules outside the desired molecular weight range. In this manuscript, we describe the preparation of polyethylene glycol polymerization reagents with selectivity toward nucleophilic attack by sulfhydryl groups and their effectiveness in the polymerization of DCLHb.

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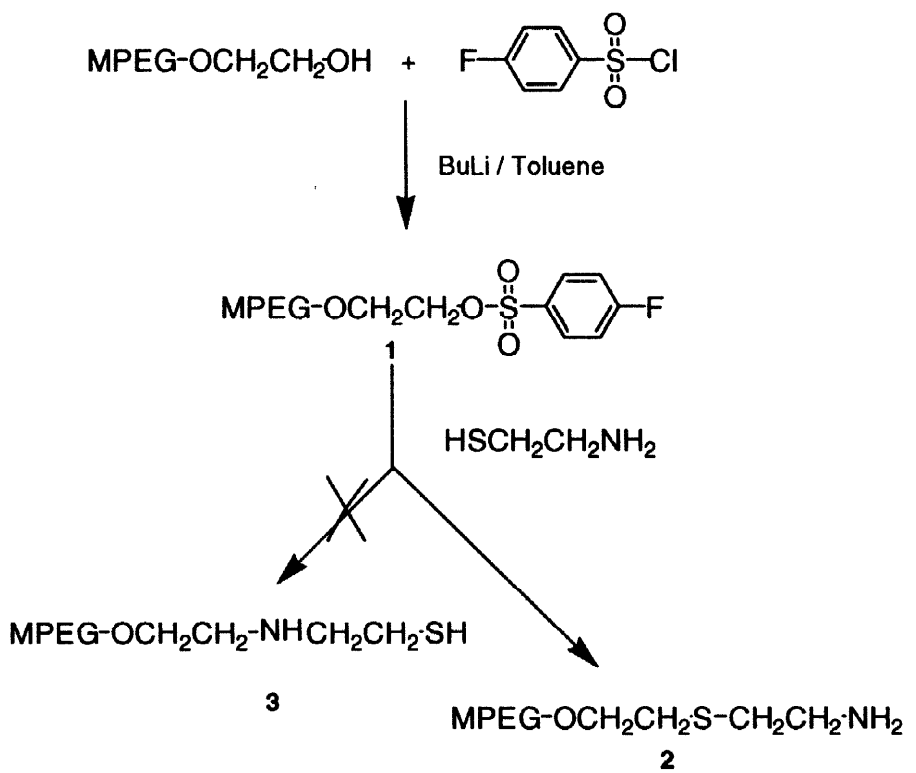
RESULTS and DISCUSSION

During our survey of activated poly(ethylene glycol) methyl ether (MPEG) reagents for protein modification, we prepared the 4-fluorobenzenesulfonate ester of MPEG (**1**). Our initial approach to activate MPEG with 4-fluorobenzenesulfonyl chloride was to complete the conversion of MPEG to **1** in the presence of triethylamine and 4-(dimethylamino)pyridine (DMAP). However, this procedure was not amenable to scale-up due to the tedious chromatographic purification and the use of the highly toxic DMAP. Our second approach eliminated these shortcomings. Butyllithium has been used to convert alcohols to lithium alcoholates that react rapidly with acid halides to give esters.[5] Thus, the conversion of MPEG by butyllithium to the corresponding lithium alcoholate and subsequent reaction with 4-fluorobenzenesulfonyl chloride in tetrahydrofuran or toluene gave **1** and lithium chloride, which could be removed from the reaction mixture by simple filtration. Furthermore, the synthesis of **1** was complete in about an hour and the crude product could be recrystallized to provide pure **1** in a good yield on a large scale.

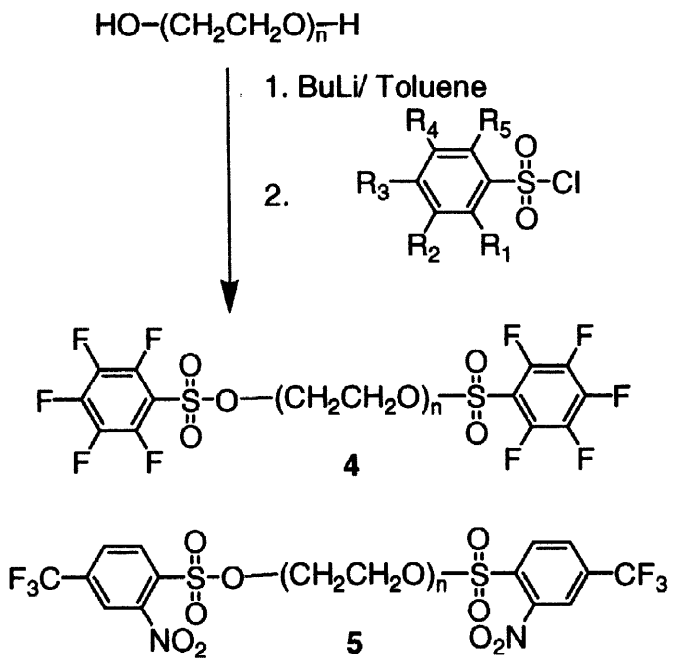


Scheme I.

The reaction of **1** with five molar equivalents of 2-aminoethanethiol in the presence of sodium methoxide in methanol at room temperature overnight gave a single MPEG-containing derivative (**2**) with an amino end group (Scheme 1). This result was not expected, since the sulfhydryl group and the amino group could displace

the 4-fluorobenzenesulfonyl group resulting in the formation of **2** and **3**, respectively. The absence of a MPEG-containing product with an active thiol group indicated that the 4-fluorobenzenesulfonyl ester of **1** was preferentially displaced by sulfhydryl groups.

However, attempts to surface modify DCLHb with **1** failed to give MPEG modified hemoglobin. This finding was unexpected, since Chang *et al.*[6] have reported that enzymes or antibodies can be covalently attached to a solid phase that had hydroxy groups activated via 4-fluorobenzenesulfonate esters. We reasoned that since DCLHb did not react with **1**, a single fluorine substituent on the benzenesulfonyl group was not sufficiently electron withdrawing to activate the ester toward nucleophilic displacement by sulfhydryl groups (β93 Cys) on the surface of DCLHb. Polysubstitution of the aromatic ring with additional electron-withdrawing groups should activate the benzenesulfonyl group toward displacement by the β93Cys sulfhydryl groups of DCLHb. In addition, activation of both ends of PEG would provide a sulfhydryl specific polymerization reagent with which a polyhemoglobin with the desired MW range could be prepared. To this end, the bis(polysubstituted-benzenesulfonate ester) polyethylene glycols **4** and **5** were prepared.



Scheme II.

Our initial attempt at the preparation of **4** by treatment of PEG (average molecular weight of 3400 Daltons) with pentafluorobenzenesulfonyl chloride in the presence of triethylamine and 4-(dimethylamino)-pyridine in chloroform failed. The product was a mixture of PEG mono- and bis(pentafluorobenzenesulfonate)

which required tedious purification to isolate pure **4**. However, PEG bis(benzenesulfonates) **4** and **5** were obtained in excellent yields if the PEG was converted to the corresponding bis(lithium salt) by treatment with butyllithium, followed by treatment with the appropriate substituted benzenesulfonyl chloride in toluene (Scheme II). Lithium chloride, the by-product of the reaction, is insoluble in toluene. Thus, the use of toluene allowed the products to be purified easily by simple filtration and subsequent crystallization from a suitable solvent. Under these conditions, the bis(benzenesulfonate esters) of PEG **4** and **5** were obtained in good yield and high purity.

The specificity toward nucleophilic displacement of the benzenesulfonyl esters of **4** and **5** by sulfhydryl groups was assessed by the reaction of these derivatives with ten molar equivalents of 2-aminoethanethiol in the presence of sodium methoxide in methanol at room temperature. The products from the reaction were characterized by thin-layer chromatographic separation followed by visualization with iodine or ultraviolet light (all products were visualized) or with a ninhydrin reagent (only primary amines were detected). If the same products were visualized by both techniques, then the ester reacted specifically with thiols. By this method, **4** gave predominantly PEG-bis(amine). In contrast, **5** gave several major products. Therefore, the pentafluorobenzenesulfonyl esters are specific for nucleophilic substitution by sulfhydryl groups, whereas, 2-nitro-4-trifluoromethylbenzenesulfonyl esters are non-specific.

Compounds **4** and **5** were tested as polymerization reagents for DCLHb to determine if a sulfhydryl specific reagent, such as **4**, would have an effect on the polyDCLHb product profile . A typical polymerization of DCLHb with **4** was performed with deoxyDCLHb and various molar ratios of **4** in 0.1 M borate buffer, pH 9.3 under nitrogen, at room temperature overnight. The product composition was detected by Size Exclusion Chromatography (SEC) (Figure 1). Direct determination of the extent of PEG-modification of polyDCLHb was accomplished by NMR spectrometry.[7] NMR analysis provided a direct determination of the PEG content of the hemoglobin polymer by virtue of the fact that PEG gave a single, sharp resonance in a ^{1}HNMR or a ^{13}CNMR spectrum at chemical shifts of 3.4 and 70.4 ppm, respectively, at 5^{0}C. There is no DCLHb interference in these regions of the spectra. Experimental results are summarized in Table 1.

The experimental data indicate the following. a) Compound **4** was an effective reagent for the polymerization of DCLHb. b) Reaction of 3 to 13 molar equivalents of **4** with DCLHb gave a mixture of oligomers of DCLHb. In general, the SEC profiles of these products consisted of three peaks. It is known that incorporation of PEG on the surface of a protein will change the retention time of the protein on SEC due to the change on the hydrodynamic properties of the modified protein. Peak I (peak maximum at 19.5 minutes) is unmodified DCLHb. Peak II (peak maximum at 17.8 minutes) is a dimer of DCLHb with a molecular weight of approximately 128,000 Daltons as determined from a protein molecular weight standard curve. The retention time of this peak remained constant with an increase in the molar ratio of **4** used for polymerization.

However, the retention time of Peak III (peak maximum at 15.5-16.8 minutes) decreased with an increase in the number of moles of **4** used in the polymerization of DCLHb. Therefore, Peak III is a mixture of trimers, tetramers of DCLHb and the corresponding surface modified counterparts. c) The steady decrease in the number of reactive thiols with an increase in the molar ratio of **4** to DCLHb indicates that this reagent reacts selectively with the thiols (β93 Cys) of DCLHb. An approximately 50 % decrease in the number of reactive thiols was observed when as few as nine equivalents of **4** was used. d) The P_{50} and cooperativity ($n_{40\text{-}60}$) values of this class of polyDCLHb are in the range of 24-26 mm Hg and 1.8-2.2, respectively.

Table 1: Polymerization of DCLHb with **4**.

Molar Ratio **4** / DCLHb	SEC Profile[a]			Number of Titratable Thiols	P_{50}, mmHg (n)	Molar Ratio[b] PEG / DCLHb
	% Peak 1	% Peak 2	% Peak 3			
0 (DCLHb)	100 (19.5)	-	-	1.9	29 (2.6)	0
3	50 (19.5)	50(17.8)	(shoulder)	1.5	26 (2.2)	-
5	40 (19.5)	60(17.8)	(shoulder)	1.4	25(2.2)	-
7	28 (19.5)	28(17.8)	42(16.8)	1.2	25(1.8)	-
9	20 (19.5)	23(17.8)	57(16.7)	1	24(1.7)	8
11	19 (19.5)	22(17.8)	59(16.6)	1	24(1.8)	9
13	19 (19.5)	(shoulder)	81(15.5)	0.9	24(1.8)	11

[a] The numbers in parentheses are the retention in minutes of the corresponding peak maximum. The percent composition is the ratio of peak areas, as defined by dropping perpendicular lines to the baseline at nadirs between peak maxima, to the total area of all DCLHb-related responses. [b]Determined by NMR.

When the products of the polymerization were analyzed by SDS-PAGE, conditions in which DCLHb will dissociate into the α-α chain (molecular weight of 32 kDa) and the β chains (molecular weight of 16 kDa), protein bands having molecular weights between 21.5 and 31 kDa were observed. This indicates that the β-chains of DCLHb were modified by **4** , as would be expected if the sites of modification were the two reactive cysteine residues at the β93 positions. In addition, a series of indistinct bands between 31 and 97.4 kDa were observed when an excess amount of **4** was used. The determination of the PEG content of the resulting polyDCLHb by ^{1}HNMR and ^{13}CNMR (Table 1) indicated that 85-90 % of the moles of **4** that were used for

the polymerization were attached to DCLHb. This suggests the products with a shorter retention time were surface modified with PEG. Thus, under the polymerization conditions employed the benzene-sulfonate group of **4** could be displaced not only by the sulfhydryl groups of β93 Cys but also by the primary amino groups on the surface of DCLHb.

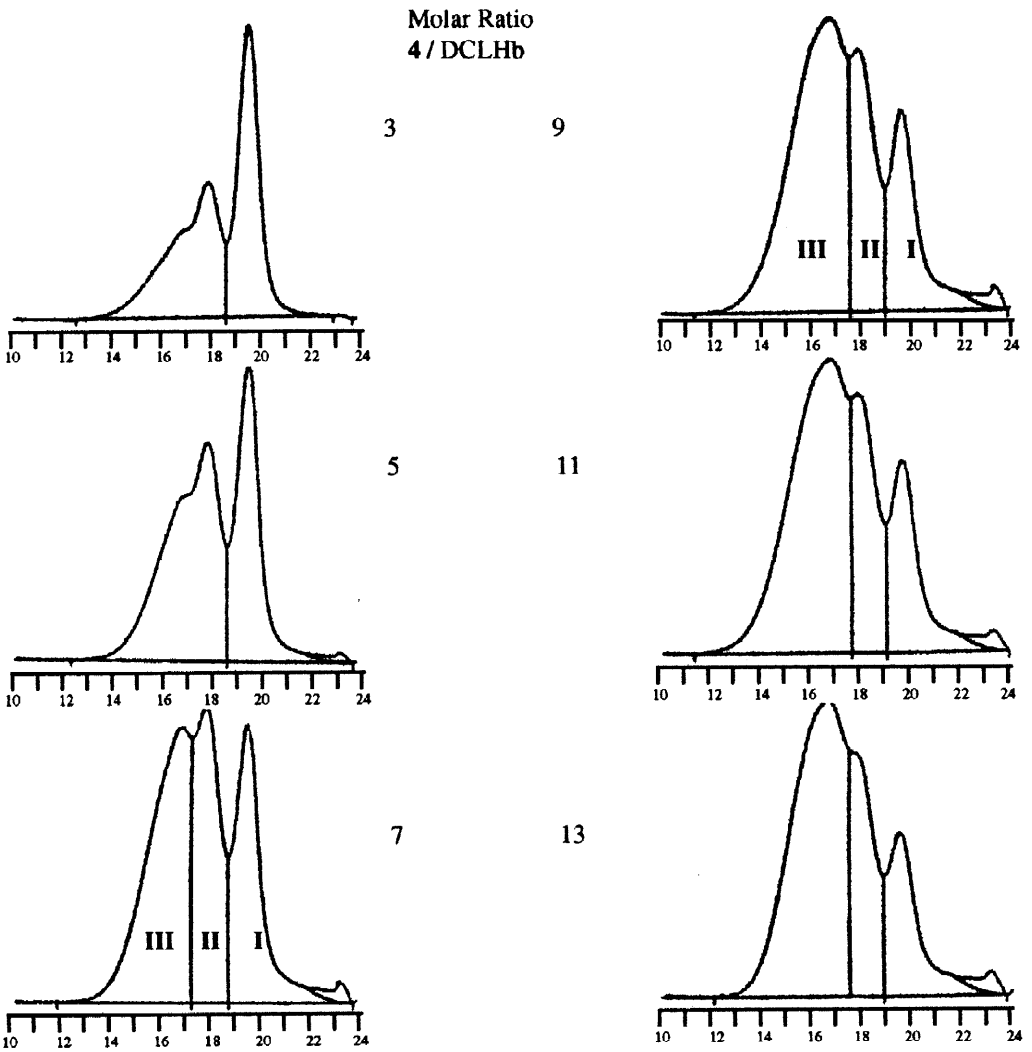


Figure 1. SEC Profiles of DCLHb polymerized with 4.

Polymerization of DCLHb with **5** was performed by the same procedure as was described for **4**. Experimental results are presented in Table 2. The data indicate that: a) **5** polymerizes DCLHb to oligomers, but much less effectively than does **4**. b) As was true with **4**, polymerization of DCLHb with **5** gave DCLHb

oligomers consisting mainly of dimers to tetramers of DCLHb and the corresponding surface modified counterparts. c) **5** also reacted with the thiols of DCLHb, as indicated by the decrease in the number of reactive thiols in polyDCLHb as the concentration of **5** was increased. d) SDS-PAGE analysis of the polyDCLHb showed that the products were also surface modified with PEG. e) The P_{50} and cooperativity values were in the useful ranges of 24-25 mm Hg and 1.9-2.0, respectively.

Table 2: Polymerization of DCLHb with **5**.

Molar Ratio **5** / DCLHb	SEC Profile[a]			Number of Titratable Thiols	P_{50}, mmHg (n)
	% Peak 1	% Peak 2	% Peak 3		
0 (DCLHb)	100 (19.5)	-	-	1.9	29 (2.6)
5	48 (19.5)	22(18.0)	29(sh)	1.6	25 (2.1)
7	39 (19.5)	24(18.0)	37(17.5)	1.5	25(2.0)
9	32 (19.5)	23(18.0)	42(17.0)	1.4	24(1.9)
11	25 (19.5)	22(18.0)	52(16.9)	1.3	23(2.0)
13	20 (19.5)	18(18.0)	62(16.7)	1.3	24(1.9)
15	16 (19.5)	17(17.9)	66(16.1)	1.1	24(2.0)

[a] The numbers in parentheses are the retention in minutes of the corresponding peak maximum. The percent compositions is the ratio of peak areas, as defined by dropping perpendicular lines to the baseline at nadirs between peak maxima, to the total area of all DCLHb-related responses.

In summary, the polymerization of DCLHb with **4** or **5** demonstrated that electron-withdrawing groups on the benzenesulfonate leaving group activates these groups for displacement by thiol nucleophiles. The polyDCLHb's consisted largely of dimers, trimers, tetramers and their surface modified counterparts of DCLHb resulting from reaction of the β93 Cys thiols and surface amino groups of DCLHb. Thus, the specificity toward nucleophilic displacement by sulfhydryl groups of the pentafluorobenzenesulfonate ester was diminished under the conditions used for the polymerization of DCLHb. Both polymerizations of DCLHb with **4** and **5** provided polyDCLHb solutions with suitable of P_{50} values (24-26 mm Hg) and cooperativity values (1.8-2.0) for hemoglobin-based oxygen carrying solutions.

EXPERIMENTAL

Materials. Diaspirin crosslinked hemoglobin (DCLHb) and Plasmalyte A were obtained from Baxter Healthcare Corporation. MPEG, PEG, 4-fluorobenzenesulfonyl chloride, pentafluorobenzenesulfonyl chloride,

2-nitro-4-trifluoromethylbenzenesulfonyl chloride, and 2-aminoethanethiol hydrochloride were purchased from Aldrich. All solvents used were reagents of HPLC grade.

Analytical Methods. Size exclusion chromatography (SEC) was performed using TSK G4000SW and TSK G3000SW columns (7.5 x 300 mm, TosoHaas) connected in series. Analytes were eluted at 1 mL/min. with a binary mobile phase (50 mM phosphate, pH 6.5 / 2-propanol, 9:1 by volume) and detected at 280 nm. TLC was performed on Whatman 250 μm silica gel TLC plates; eluent: $CHCl_3$ / MeOH / NH_4OH, 10:1.5:0.08, by volume; products were visualized with iodine, UV light or ninhydrin reagent. Oxygen equilibrium curves were recorded by reoxygenation of nitrogen equilibrated hemoglobin solution in 0.1 M Bis-Tris buffer (pH 7.4 at 37°C) in the spectral cuvette of Hemox Analyzer (TCS Medical Products Co, Huntington Valley, PA). The oxygen pressure at which hemoglobin is half saturated (P_{50}) and the subunit cooperativity ($n_{40\text{-}60}$) were calculated from the oxygen equilibrium curves. The concentration of hemoglobin was determined by a multiwavelength absorbance technique. Measured absorbance values at the three wavelengths (630, 576, and 560 nm) were used in conjunction with literature molar extinction coefficients[8] to solve three simultaneous equations that calculate the concentration of oxyHb, deoxyHb and metHb. Total hemoglobin was calculated by summation of the concentrations of the components. Reactive thiols of polyDCLHb were determined by the method of Neis *et al.*[9] Melting points were taken on a Fisher-Johns melting point apparatus and are uncorrected.

NMR spectra were recorded on a Bruker 270 MHZ or a Bruker 300 MHZ spectrometer. ^1H and ^{13}C chemical shifts were measured relative to partially deuterated solvent peaks but are reported relative to tetramethylsilane. For NMR analysis, polyDCLHb solutions (7 g/dL, 0.5 mL) were diluted with D_2O (0.2 mL) and then transferred to NMR tubes, which were placed in the instrument probe at 5°C for data acquisition. The intensity of the PEG-derived singlet at 3.4 ppm for ^1H-NMR and 70.4 ppm for ^{13}C-NMR was measured. A standard curve was established by determining the intensity of the PEG-derived NMR response for DCLHb solutions to which known quantities of PEG had been added before identical dilutions. Extrapolation of the value of the signal intensity of the polyDCLHb from the standard curve enabled estimation of the concentration of PEG in the test sample.

Poly(ethylene glycol) Methyl Ether 4-Fluorobenzenesulfonate (1).

Method 1: Poly(ethylene glycol) methyl ether (MW 2000, 10.0 g, 5.3 mmol) , triethylamine (1.85 mL, 13.3 mmol) and DMAP (1.6 g, 1.6 mmol) were combined in chloroform (50 mL). 4-Fluorobenzenesulfonyl chloride (1.6 g, 7.95 mmol) in chloroform (10 mL) was added in one portion. The solution was stirred overnight at room temperature. The solvent was removed and the residue was dissolved in chloroform (50 mL) and passed through a pad of silica gel. The pad was washed with chloroform (3 × 200 mL) and chloroform:methanol (200 mL:5 mL). The washes were combined and evaporated to an oil. The oil was dissolved in chloroform (15 mL)

and ethyl ether (300 mL) was added to precipitate the product. After 30 min, the solid was collected and dried under vacuum to give 5.5 g (42 %) of **1**. Mp = 46-48° C. ^{1}H-NMR ($CDCl_3$) δ 3.34 (s, 3H); 3.50 (m, 179H); 4.06 (t, 2H, J = 4.8 Hz); 7.13 (t, 2H, J = 8.5 Hz); 7.81 (m, 2H). ^{13}C-NMR ($CDCl_3$) δ 58.9; 68.5; 69.5; 70.4; 71.8 ; 1165 (d, J=23.4 Hz); 130.7 (d, J=9.8 Hz); 132.0 (d, J=3.8 Hz), 165.6 (d, J=255.9 Hz).

Method 2: Poly(ethylene glycol) methyl ether (MW 2000, 500.0 g, 0.25 mol) was dissolved in toluene (1.5 L) with warming. The solution was allowed to cool to room temperature and a solution of butyllithium (2.5 M solution in hexane; 110 mL) in toluene (50 mL) was added dropwise. After 30 min, 4-fluorobenzenesulfonyl chloride (73.0 g, 0.375 mol) in toluene (50 mL) was added in one portion. The mixture was stirred for 2 h and then filtered through Celite 541. The filtrate was evaporated to a semi-solid which was dissolved in chloroform (200 mL). Ethyl ether (3 L) was added to the solution and the mixture kept overnight at room temperature. The solid was collected and dried under vacuum to give 395.5 g (64 %) of **1**.

α-Methyl-ω-(2-aminoethylthio)-poly(oxyethylene) (2).

Sodium methoxide (121.8 g, 2.250 mol) was dissolved in methanol (8 L). 2-Aminoethylthiol.HCl (127.8 g, 1.125 mol) was added as a solid. Once a solution was obtained, **1** (450.1 g, 0.225 mol) was added. The solution was stirred for 3 h and the solvent was removed by evaporation. The residue was combined with chloroform (500 mL) and the mixture was evaporated to a solid. Chloroform (2 L) was added to the solid and the resulting mixture was filtered though Celite 521. The filtrate was passed through silica gel and the latter was washed with chloroform (2 L). The filtrate was evaporated to an oil and the residue was dissolved in chloroform (500 mL). The product was precipitated by the addition of ethyl ether (4.5-5 L). The solid was collected and washed with ethyl ether (500 mL). A second crop was obtained from the filtrate. The crops were combined to provide 405 g (83 %) of **2**. Mp = 52-54° C. ^{1}H-NMR ($CDCl_3$) δ 2.28 (broad s, 2H); 2.56 (t, 2H, J = 6.4 Hz); 2.60 (t, 2H, J = 6.7 Hz); 2.79 (t, 2H, J = 6.4 Hz); 3.25 (s, 3H); 3.41-3.60 (m, 242H). ^{13}C-NMR ($CDCl_3$) δ 30.9; 35.9; 40.9; 58.7; 61.3; 69.9; 70.3; 70.8; 71.6.

Poly(ethylene glycol) Bis (Pentafluorobenzenesulfonate) (4).

A stirred suspension of polyethylene glycol (MW 3400, 10.2 g, 3 mmol) in toluene (100 mL) was gently heated to dissolve the solid. The solution was allowed to cool to room temperature. To this solution was added dropwise a solution of butyllithium in hexane (2.5 M, 3.6 mL, 9 mmol) under nitrogen. After addition, stirring was continued for 5 min and the solution became viscous. A solution of pentafluorobenzene-sulfonyl chloride (6.398 g, 24 mmol) in toluene (20 mL) was added dropwise to the PEG solution over 10 min. After the addition, the mixture was stirred for a further 2 h and filtered through Celite 521 to remove LiCl. The filtrate was evaporated to dryness and the residue was crystallized from chloroform-ethyl ether to give 9.15 g (79 %) of **4**. Mp = 46-50° C. ^{1}H-NMR ($CDCl_3$) δ 3.51- 3.74 (m, 360H); 4.25 (t, 4H, J = 4.78 Hz). ^{13}C-NMR ($CDCl_3$) δ 42.5; 68.1; 70.1; 70.9; 71.9 120.1 (m); 134.2 (m); 138.7 (m); 142.0 (m); 143.0 (m).

Poly(ethylene glycol) Bis (2-Nitro-4-trifluoromethylbenzenesulfonate) (5).

The reaction of PEG (MW 3400, 10.2 g, 3 mmol) and 2-nitro-4-trifluoromethylbenzenesulfonyl chloride (3.475 g, 12 mmol) in the presence of butyllithium (7.8 mmol) was performed by the procedure described for **4.** The desired product **5** was obtained by crystallization of the crude product from chloroform-ethyl ether to give 9.0 g (77 %) of final product. Mp = 49-51° C. ^{1}H-NMR ($CDCl_3$) δ 3.59 (m, 388H); 4.43 (t, 4H, J = 4.5 Hz); 7.67-8.41 (m, 6H). ^{13}C-NMR ($CDCl_3$) δ 42.5; 68.2; 70.1; 70.9; 71.9; 118.2; 119.9-121.8 (m); 127.4; 127.5; 129.1; 130.8-132.9 (m); 138.4-138.9 (m); 147.9.

Polymerization of DCLHb with 4 or 5.

A typical polymerization of DCLHb with **4** was performed as follows: A solution of DCLHb (15 g/dL) in 0.1 M borate, pH 9.3 was deoxygenated by successive vacuum /nitrogen cycles for 1.5 hours at room temperature. The solution of **4** or **5** in deoxygenated water was added, and the reaction mixture was stirred under nitrogen at room temperature overnight. The progress of the reaction was monitored by SEC which indicated that the polymerization was complete in 24 hours. The reaction mixture was dialyzed against Plasma Lyte™ A to give a final product, which was characterized by SEC and NMR. Experimental results are summarized in Table 1-2 and Figure 1-2.

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