Simple Synthesis of Aldehyde and Carboxylic Acid Terminated Methoxypoly (ethylene glycol)

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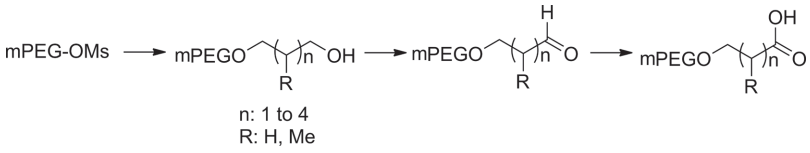
Dedicated to the memories of Professor Ricardo J. A. Grau and Professor María I. Cabrera

mPEG-aldehydes and activated mPEG-carboxylic acids on which the polymer and the reactive functional group are separated by aliphatic chains of different lengths, are well known reagents of widespread application in PEGylation technology. The syntheses of these functional polymers are not easy and usually the preparation of each of these compounds requires a very specific set of reagents and experimental conditions. In fact, a general method for their preparation is, still nowadays, unavailable. In this work a simple synthetic method is developed for preparation of mPEG-aldehydes and carboxylic acids with excellent yields. The key step of the method involves a substitution reaction of mPEG-mesylate with mono-alkoxides of symmetrical diols to afford hydroxyl-terminated polymers, which are converted to the aldehydes and acids by mild oxidation reactions.



1. Introduction

Over the last 30 years it has been well documented that the chemical conjugation to methoxypoly(ethylene glycol) (mPEG) is a powerful tool for the improvement of physicochemical and pharmaceutical properties of therapeutically important peptides, proteins, and biomacromolecules.[1]

Although various different strategies have been developed, the site-selective conjugation of terminal $-NH_2$ and the acylation of ubiquitoulsy present ε-NH_2 of lysine amino acids, still nowadays remain as very important approaches to achieve peptide and protein PEGylation.

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Site selective PEGylation by reductive amination using mPEG-acetaldehyde or mPEG-propionaldehye **1a** (n = 1, R = H, Scheme 1), in the presence of adequate reducing reagents, is a well-known strategy that has found wide application in bioconjugation.[2,3] However, **1a** is preferred over mPEG-acetaldehyde since the latter is unstable and dimerizes by aldol condensation, affording usually low yields of conjugates. It is worth to mention that **1a** of 20 kDa is used for the conjugation of human G-CSF (Neulasta). Related important reagents include mPEG-(2-methyl)-propionaldehyde **1b** (n = 1, R = Me) and mPEG-butyraldehyde **1c** (n = 2, R = H, Scheme 1). For example, **1b** is used in the conjugation of interferon β-1a (Plegridy).[4] In connection, the more stable diethyl-acetals of mPEG-acetaldehyde and propionaldehyde are also known and useful reagents.[5]

PEGylation of $-NH_2$ using activated carboxylic acid esters **2**, such as mPEG-succinic, acetic, propionic, and butyric *N*-hydrosuccinimidyl esters, are well known acylation reactions. In these reagents, the polymer is connected to the activated carboxyl group by means of a linker. It has been demonstrated that the nature and the length of the linker can have a profound effect not only

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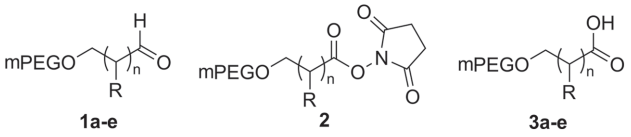


Scheme 1. mPEG-aldehydes, mPEG-carboxylic acids and activated mPEG-carboxylic acids.

in the reactivity of the activated polymer, but also in the stability of the conjugate.[1b]

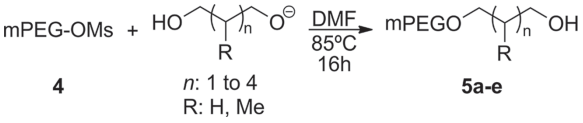
Although structurally simple, the syntheses of these widely used mPEG-(aliphatic chain)-aldehydes, and key mPEG-(aliphatic chain)-carboxylic acids, are usually not facile and have a rather limited scope. Moreover, very specific sets of reaction conditions and reagents are required for the synthesis of each of these activated mPEGs, limiting the number of PEGylation reagents which can usually be evaluated in a given conjugation reaction and the development of new mPEG reagents. In addition, given their utility for the PEGylation of therapeutically relevant proteins, most of the methods for their preparation have been published in the patent literature.

In this work we have developed a simple and general synthetic strategy for the preparation mPEG-aldehydes and key mPEG-carboxylic acids bearing aliphatic linkers of different lengths (n = 1 to 4, in compounds **1** and **3**, Scheme 1). The key step for these preparations involves the nucleophilic substitution reaction of mPEG-mesylates (mPEG-OMs) with adequately selected symmetric alkoxides to afford mPEG-(aliphatic chain) alcohols, which were readily oxidized to the corresponding aldehydes under mild experimental conditions. Furthermore, the aldehydes were cleanly converted to the carboxylic acids by oxidation with iodine in alkaline medium. Some selected acids were transformed into the activated *N*-hydroxysuccinimide (NHS) esters. mPEG-aldehydes **1** and selected NHS esters **2** were tested in the PEGylation of epoetin α as model protein.

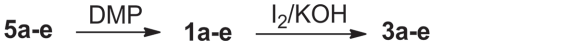
2. Results and Discussion

Aldehyde **1a**, used for the site-selective conjugation,[6] has been prepared by nucleophilic substitution reaction of potassium mPEG alkoxide with 3-chloropropanaldehyde diethyl acetal followed by deprotection (around 50% yield).[7] In a detailed kinetic study, Su and co-workers showed that 15 equivalents of the substrate in boiling dioxane or toluene are required to obtain the protected aldehyde with quantitative yield.[8] Other method for the synthesis of **1a** has been published in the patent literature.[9] Aldehyde **1b** has been obtained by reaction of mPEG sodium alkoxide with 3-bromo-2-methylpropene followed by hydroboration and oxidation with Dess–Martin periodinane (DMP).[4] Two methods for the synthesis of mPEG-butyraldehyde **1c** have been published in the patent literature.[10] It should be noted that most of these transformations involve the initial substitution reaction of nucleophilic mPEG alkoxides with adequately substituted electrophilic acceptors. In connection, mPEG-halides, tosylates, and mesylates are well known electrophilic compounds that have been used mainly for the preparation of more elaborated mPEG derivatives.[11] mPEG-OMs are preferred over tosylates and halides since they are more stable, but still reactive enough to be used as electrophilic counterparts in nucleophilic substitution reactions.

Being interested in the development of new PEGylation methods, we turned our attention to mPEG-aldehyde **1a**.[12] Thus, we evaluated the synthesis of **1a** of 20 kDa by the published method of Su.[8] Although the published conditions were carefully followed, after several attempts we were able to obtain the aldehyde **1a** with 45% yield (^{1}H-NMR and spectrophotometric assay).[8] Similar results were obtained when we evaluated 4-chlorobutyraldehyde diethyl acetal as substrate for the synthesis of **1c**.[10a] Challenged by these results we decided to explore the development of a new simple and general synthetic protocol that allows the preparation of not only **1a**, but also of other related mPEG-aldehydes bearing aliphatic linkers of different lengths. In contrast with previously published methods, the approach proposed herein involves the initial reaction of electrophilic mPEG-OMs **4** with the monoalkoxides of symmetric diols, which might afford key mPEG-(aliphatic chain) hydroxyl polymers **5** (Equation (1)). DMP oxidation of **5** should afford the required aldehydes **1**, which could be converted to carboxylic acids **3** under mild reaction conditions (Equation (2)).



(1)



(2)

mPEG-OH of 12, 20, and 30 kDa were chosen as starting materials since they are usually the molecular weights of choice in modern PEGylation technology. Thus, starting from mPEG of 20 kDa we prepared mesylate **4** by slightly modified literature procedure and used this compound in the nucleophilic substitution reaction with the sodium alkoxide of butanol (20 equivalents), in DMF at 85 °C and for 16 h.[13] ^{1}H-NMR analysis of the product showed complete disappearance of peaks at 3.10 ppm (O—SO_2—CH_3) and at 4.41–4.38 ppm region (OCH_2CH_2—OSO_2CH_3), and appearance of a set of signals in aliphatic region corresponding to the butyl group. Integration of the peaks at 1.61–1.62 ppm (m, 2H, m-PEG-$OCH_2CH_2CH_2CH_3$) and at 3.39 ppm (terminal CH_3-O) revealed complete conversion

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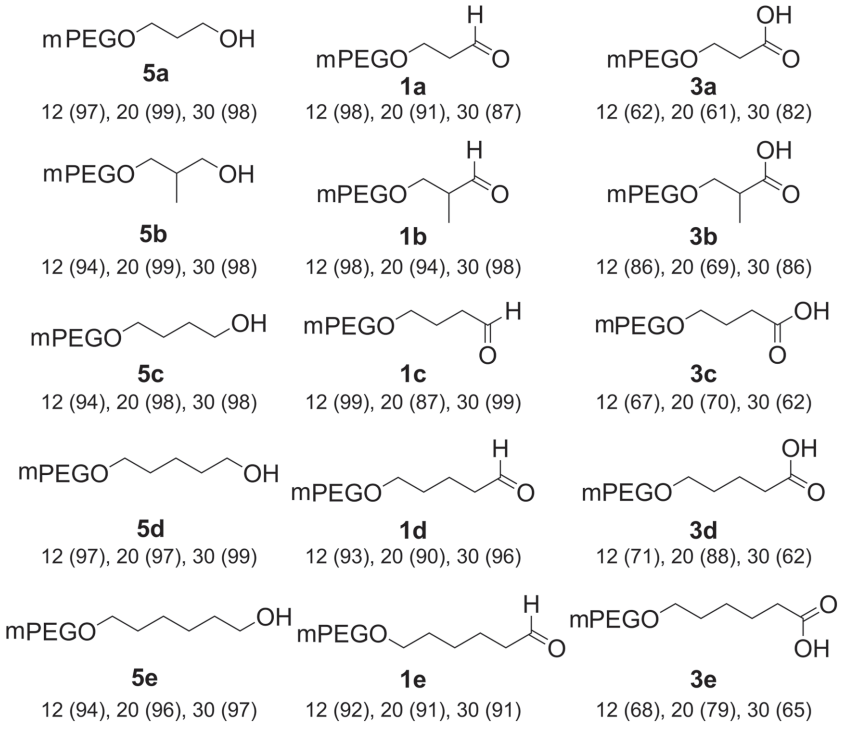

Scheme 2. mPEG alcohols **5**, aldehydes **1** and key carboxylic acids **3**. MW in kDa (yields based on recovered polymer). Purity was checker by ^{1}H-NMR.

of **4** to the corresponding mPEG-butyl ether. Prompted by this promising result we then evaluated the substitution reaction of **4** with sodium mono-alkoxide of 1,3-propanediol as nucleophile. We assumed that the use of adequate molar ratios of **4**, symmetric diol and base (60% NaH in mineral oil) might allow the selective preparation of the key hydroxyl polymer **5a** (Equation (1)), which would be amenable for further synthetic manipulations. Accordingly, 20 equivalents of 1,3-propanediol were treated with 10 equivalents of 60% NaH and the alkoxide was reacted with 1 equivalent of **4** of 20 kDa. Integration of NMR peaks indicated complete conversion of the substrate to hydroxyl polymer **5a** (Scheme 2).

Finally, mPEG-propanol **5a** was converted to the required aldehyde **1a** in high yield by Dess–Martin oxidation (Scheme 2). Similarly, excellent results were obtained when **4** of 12 and 30 kDa were used as substrates (Scheme 2).

The more sterically hindered 2-methylpropane-1,3-diol also performed well in the substitution reaction. In fact, good isolated and NMR yields of hydroxyl derivatives **5b** were obtained from **4** of 12, 20, and 30 kDa (Scheme 2). The DMP oxidation of **5b** afforded high yields aldehydes **1b** (Scheme 2).[4]

Other symmetric 1-n diols (1,4-butanediol, 1,5-pentanediol, and 1,6-hexanediol) worked well in the synthesis strategy and in all cases complete conversion of mesylates **4** to the corresponding hydroxyl-terminated polymers **5c–e** were obtained (Scheme 2). Furthermore, alcohols **5c–e** were cleanly transformed into the required aldehydes **1c–e** by Dess–Martin oxidation (Scheme 2). mPEG-butyraldehyde **1c** of 20 kDa was further characterized by gCOSY (see Supporting Information).

To further extend the scope of this study, aldehydes **1a–e** of 12, 20, and 30 kDa were evaluated in the bioconjugation of Epoetin α and the results of these conjugations compared with those obtained using a commercial sample of **1a** of 20 kDa and with mPEG-acetaldehyde of 12, 20, and 30 kDa. mPEG-acetaldehydes were obtained with 87–98% yields by Dess–Martin oxidation of mPEG-OH. Thus, the reductive amination of Epoetin α using 6 equivalents of aldehydes **1a–e** and $NaCNBH_3$ as reducing reagent (pH 5.0) afforded, in all cases, the required mPEG-Epoetin α conjugates. SEC-HPLC and SDS-PAGE analysis revealed that the conjugates were obtained with 16–32% yield under these un-optimized experimental conditions (see Supporting Information). Noteworthy, SDS-PAGE and SEC-HPLC (ELSD detection) of aldehydes **1a–e** indicated that polymer dimerization, as well as polymer chain degradation, did not occur under the experimental conditions used for the substitution and oxidation reactions. Furthermore, the in house prepared **1a** of 20 kDa afforded PEGylated Epoetin α with 24% yield. This result is comparable with that obtained using a commercial sample of **1a** under identical experimental conditions (30%, SEC-HPLC). As expected, lower yields of PEGylation were obtained with mPEG-acetaldehydes.

mPEG-succinic, acetic, propionic, and butyric *N*-hydrosuccinimidyl esters, obtained by Steglich esterification of the parent acids, are particularly important reagents that have found application in the conjugation of various different proteins.[14] Although structurally simple, the preparation of key mPEG-carboxylic acids **3** is usually not facile, and most of the methods for their syntheses have been patented.[1] For example, mPEG propionic acid **3a** (Scheme 2) has been prepared by conjugate addition of mPEG alkoxide to various Michael acceptors, followed by hydrolysis in strongly basic conditions.[15–19]

On the other hand, mPEG-butyric acid **3c** has been prepared by a nucleophilic substitution reaction using diethylmalonate anion and mPEG-mesylate **4** as substrate, followed by hydrolysis and decarboxylation.[20] Other syntheses of mPEG-butyric acid and related compounds have been published.[21,22]

Given the usefulness of mPEG-acids, and taking into account the lack of a general and facile method for their preparation, we decided to evaluate the syntheses of **3a–e** by oxidation of aldehydes **1a–e**. In fact, aldehydes **1** seem to be the natural precursors for the preparation of acids **3**. It is well known that strong metal oxidants are not recommended for the oxidation of mPEG-OH or mPEG derivatives, since they usually lead to undesirable polymer chain degradation. Thus, chromium and manganese-based oxidants were not evaluated.[23,24] On the other hand, it has been reported that mPEG-acetaldehyde, obtained upon reaction of mPEG-OH with MnO_2, can be converted to mPEG-acetic acid using H_2O_2 in neutral conditions.[25] However, when we evaluated the H_2O_2 oxidation of **1a** of 20 kDa very low yields of **3a** were obtained, as shown by ^{1}H-NMR analysis. Furthermore, very low yields were found in the oxidation of **1a** using H_2O_2/HCl in the presence of hydroxylamine.[26] The oxidation of aldehydes to the corresponding carboxylic acids or esters by molecular iodine in alkaline medium is a well-known reaction that has been exploited to some extent in synthetic organic chemistry.[27] Thus, after some experimentation we were delighted to find that the mild oxidation reaction devised by Yamada et al. performed well for the conversion of aldehyde **1a** (20 kDa) to mPEG-propionic acid **3a** (Scheme 2).[28] ^{1}H-NMR spectrum of the oxidation product showed disappearance of aldehyde signals, and appearance of a triplet at 2.45 ppm, corresponding to the C*H*$_2$—C(O)—OH. The same experimental conditions were employed for the oxidation of **1a** of 12 and 30 kDa (Scheme 2) and for the preparation of mPEG-(2-methyl)-propionic (**3b**), butyric (**3c**), pentanoic (**3d**), and hexanoic (**3e**) acid starting from the corresponding aldehydes **1b–e** (Scheme 2). mPEG-butyric acid **3c** of 20 kDa was further characterized by gCOSY, gHSQC, and gHMBC (see Supporting Information). SDS-PAGE and SEC-HPLC (ELSD detection) analyses of acids **3a–e** indicated that polymer chain degradation did not occur during the mild oxidation.

Finally, some selected carboxylic acids (**3c** (30 kDa), **3d** (20 kDa)) were converted to NHS esters following well known methodology.[29] In all cases, high yields of activation were achieved, demonstrated by a published spectrophotometric assay.[29] The activated esters were evaluated in the PEGylation of Epoetin α as model protein. Thus, PEGylation of Epoetin α using two equivalents of activated esters of **3c** and **3d** in borate buffer pH 8.0 afforded the required mono-conjugates with good yields, as shown by SEC-HPLC analysis (49.6% and 49.7% respectively, see Supporting Information). Noteworthy, a 53.6% yield of mPEG-Epoetin α conjugate was obtained when a commercial sample of activated **3c** (30 kDa) was employed under identical experimental conditions.

3. Conclusions

In this work we have developed a simple and general method for the synthesis of mPEG-adehydes and carboxylic acids bearing aliphatic linkers of different lengths. The method relays on a key substitution reaction using mono-alkoxides of symmetric diols as nucleophiles and mPEG-mesylates as electrophiles. The substitution reaction affords hydroxyl-terminated polymers, which are amenable for further oxidative synthetic manipulation. The required aldehydes and acids were obtained with good to excellent yields. The method probed to be versatile since all symmetric alcohols, bearing aliphatic chains of different lengths, performed well in the substitution and oxidation reactions. Some selected acids were converted to the NHS esters and these compounds, together with the aldehydes, were screened in the PEGylation of Epoetin α, and the results compared with those obtained using commercially available reagents, further supporting the potential of the method presented here.

4. Experimental Section

4.1. General Methods

mPEG-OH of 12, 20, and 30 kDa were purchased to JenKem Technology (Allem, USA). 1,3-propanediol, 1,4-butanediol, 1,5-pentanediol, and 1,6-hexanediol, NaH (60% in mineral oil) and DMP were all commercially available and used as received from the supplier. DMF was distilled under vacuum and stored over molecular sieves (4 Å). Methylene chloride was distilled from P_2O_5 and stored over molecular sieves (4 Å). All other reagents and solvents were used as received from the suppliers. Epoetin α was obtained from Zelltek (Santa Fe, Argentina).

^{1}H-NMR experiments were performed in $CDCl_3$ or DMSO-d_6 in a Bruker Avance II 300 MHz spectrometer and referenced to the residual solvent signal.

Polyacrilamide gel electrophoresis (SDS-PAGE) was performed according to the methods of Laemmli.[30] After running, gels were rinsed with distilled water and placed in a 5% barium chloride solution. The gels were maintained for 10 min with gentle mixing, and then they were rinsed again, and placed in a 0.1 N KI_3 solution for 5–10 min. In the case of proteins or PEG-protein conjugates, polyacrilamide gels were run, and standard staining procedures with Coomasie Brilliant Blue were followed.

HPLC analysis of the PEGylation reactions was performed in a Shimadzu Prominence Serie LC20A chormatograph, using a TOSOH TSK gel G3000 SW column (7.5 mm ID × 30 cm, 10 μm). The elution was isocratic with AcONa buffer 45×10^{-3} M–0.2 M NaCl 10% EtOH pH 5.2 with UV detection at 220 nm.

HPLC analysis of mPEG acids **3c** (30 kDa), **3d** (20 kDa), and commercial **3d** (30 kda) was performed in a Shimadzu Prominence Serie LC20A chromatograph, using a Shodex IEQ-QA825 column (8.0 mm ID × 75 mm, 12 μm), with ELSD detection (Shimadzu ELSD LTII). The gradient elution was performed using

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water-ammonium acetate 4×10^{-3} M (0% buffer to 100% buffer in 20 min).

NMR spectra, as well as chromatograms and SDS-PAGE analyses, are provided in the Supporting Information accompanying this article.

4.2. Synthesis mPEG-Mesylates 4 (CH_3-$(OCH_2CH_2)_n$-OSO_2-CH_3)

mPEG-mesylates **4** of 12, 20, and 30 kDa were obtained by slightly modified literature procedure as follows: in a dry 50 mL round bottomed flask equipped with magnetic stirring and a rubber septum were added 0.1 mmol of mPEG-OH (1.2000 g, 2.0000 g, or 3.0000 g of mPEG-OH of 12, 20, and 30 kDa respectively) and 15.0 mL of dry methylene chloride. The mixture was cooled in an ice bath and 77 μL (1.0 mmol) of methanesulfonyl chloride were added. Triethylamine (0.418 mL, 3.0 mmol) was added over 1 h. The reaction was maintained with stirring in the ice bath for 2 h and then at room temperature overnight. Water was added (50 mL) and the organic phase was separated. The aqueous phase was extracted with methylene chloride (3 × 50 mL). The combined organic extract was washed with water (5 × 50 mL), dried with Na_2SO_4 and evaporated under reduced pressure. The solid was transferred to two 50 mL Falcon tubes and dissolved in a minimum amount of methylene chloride (≈5 mL) and precipitated with diethyl ether (≈40 mL). The tubes were centrifuged for 10 min at 4000 rpm. The ethereal phases were separated, the process was repeated two additional times and the solids were dried under vacuum. Following this method high yields of **4** were obtained for all mPEG-OH (>90%).

4.3. Synthesis of mPEG-(Aliphatic Chain) Hydroxyl Polymers 5

In a dry 25 mL Schlenk flask equipped with magnetic stirring and a rubber septum were added with a syringe 3.0 mL of dry DMF and the diol under nitrogen (0.5 mmol, 20 equivalents), followed by the addition of 10 mg of 60% NaH (0.25 mmol, 10 equivalents). The reaction was stirred for 30 min at room temperature. mPEG-mesylate **4** (0.025 mmol, 1 equivalent) was then added and the reaction was heated at 85 °C with stirring for 16 h. After cooling to room temperature, water was added, and the aqueous phase was extracted three times with methylene chloride (30 mL each). The combined organic extract was dried (Na_2SO_4), filtered, and solvent was evaporated under reduced pressure. The solid was transferred to a 50 mL Falcon tube and dissolved in a minimum amount of methylene chloride (≈5 mL) and then precipitated with diethyl ether (≈40 mL). The tube was centrifuged for 10 min at 4000 rpm. The ethereal phase was separated, the process was repeated two additional times and the solid was dried under vacuum. Following this method high yields of **5** were obtained for all mPEG-(aliphatic chain)-hydroxyl polymers (Scheme 2).

4.4. Oxidation of mPEG-(Aliphatic Chain) Hydroxyl Polymers 5 to mPEG-(Aliphatic Chain) Aldehydes 1

In 50 mL Falcon tube, equipped with magnetic stirring, containing 0.025 mmol of mPEG-hydroxyl polymer **5** were added 5.0 mL of dry methylene chloride and 50 mg of DMP (0.112 mmol). The reaction was stirred for 3 h at room temperature and 1.0 mL of saturated solution of $NaHCO_3$ and 1.0 mL of saturated solution of $Na_2S_2O_3$ was added. The mixture was stirred for 1 additional hour.[4] Water and methylene chloride (20 mL each) were added and the organic phase was separated. The aqueous solution was extracted with methylene chloride (2 × 20 mL) and the combined organic extract was dried (Na_2SO_4) and the solvent was evaporated under reduced pressure. The solid was transferred to a 50 mL Falcon tube and dissolved in a minimum amount of methylene chloride (≈5 mL) and then precipitated with diethyl ether (≈40 mL). The tube was centrifuged for 10 min at 4000 rpm. The ethereal phase was separated, the process was repeated two additional times and the solid was dried under vacuum. Following this method high yields of aldehydes **1** were obtained (Scheme 2).

4.5. Oxidation of mPEG-(Aliphatic Chain) Aldehydes 1 to mPEG-(Aliphatic Chain) Acids 3

In 50 mL Falcon tube, equipped with magnetic stirring, containing 0.025 mmol of mPEG-aldehyde **1** were added 3 mL of acetonitrile and 3 mL of water. The mixture was cooled in an ice bath and 459 μL of a 43.1 mg mL^{-1} solution of I_2 in acetonitrile (19.8 mg of I_2, 0.078 mmol) and 161 μL of a 54.4 mg mL^{-1} solution of KOH in water (8.8 mg of KOH, 0.156 mmol) were added. The solution was maintained with stirring in the ice bath for 2 h and 62 μL of the KOH solution (3.4 mg of KOH, 0.061 mmol) were further added and the mixture was allowed to proceed for 1 h. After that 1 mL of saturated solution of $Na_2S_2O_3$ was added and the mixture was stirred for 1 h. 1 mL of H_2SO_4 solution (0.2 M), water (20 mL) and methylene chloride (30 mL) were added, the organic phase was separated and extracted with methylene chloride (2 × 30 mL). The combined organic extract was dried (Na_2SO_4) and the solvent was evaporated under reduced pressure. The solid was transferred to a 50 mL Falcon tube and dissolved in a minimum amount of methylene chloride (≈5 mL) and then precipitated with diethyl ether (≈40 mL). The tube was centrifuged for 10 min at 4000 rpm. The ethereal phase was separated, the process was repeated two additional times and the solid was dried under vacuum. Following this method good yields of carboxylic acids **3** were obtained (Scheme 2).

4.6. Activation of mPEG-(Aliphatic Chain) Carboxylic Acids 3 to the *N*-Hydroxysuccinimidyl Esters

Activation of **3** was accomplished by a slight modification of a known method as follows:[31,32] NHS (0.062 mmol) was dissolved in 2 mL of anhydrous dichloromethane and 2 mL of anhydrous THF under nitrogen atmosphere and was kept under stirring in an ice bath. The selected acid **3** (0.0206 mmol) and Dicyclohexylcarbodiimide (DCC, 0.041 mmol, 8.5 mg) were added under nitrogen. The solution was stirred for 2 h in the ice bath, 0.02 mmol, and DCC (4.1 mg) was then added and the reaction mixture was stirred for 30 min in the ice bath and then maintained for 16 h at 4 °C without stirring. Dry diethyl ether was added to the reaction tube (30 mL), and the precipitated product

was separated by centrifugation (10 min, 4000 rpm). The solid was washed with dry diethyl ether (30 mL), dried and re-dissolved in 2 mL of acetonitrile. Acetic acid (35 μL) was added and the solution was kept 1 h under stirring at room temperature. The upper phase was separated by centrifugation and the product was precipitated with dry diethyl ether, separated by centrifugation and once more washed and precipitated. The obtained solid was dried at reduced pressure (5 mmHg) until a constant weight (yields 75–90%). The activations were achieved in 98% yield, as shown by a known spectrophotometric assay.[33]

4.7. PEGylation of Epoetin α Using Aldehydes 1a–e of 12, 20, and 30 kDa

PEGylation of Epoetin α with aldehydes **1a–e** was performed as follows: to 1.0 mL of Epoetin α solution (1.0 mg mL^{-1}) in phosphate buffer 0.1 M pH 5.0, containing 5% sacarose and 20×10^{-3} M $NaBH_3CN$ at 4 °C, were added 6 equivalent of the required mPEG-aldehyde. The reaction was gently mixed and maintained at the same temperature for 24 h without stirring. The conjugation was quenched by addition of enough acetic acid as to reach pH 4.0 and then analyzed by SEC-HPLC and SDS-PAGE.

4.8. PEGylation of Epoetin α Using NHS Esters of Acids 3c and 3d

PEGylation of Epoetin α with NHS esters of mPEG-acids **3c** (30 kDa) and **3d** (20 kDa) was performed as follows: to 1.0 mL of Epoetin α solution (2.0 mg mL^{-1}) in borate buffer 0.1 M pH 8.0 at 4 °C, were added two equivalents of the activated ester. The reaction was maintained at the same temperature with stirring for 3 h. The conjugation was quenched by addition of enough acetic acid as to reach pH 4.0 and then analyzed by SEC-HPLC and SDS-PAGE. For comparison purposes the conjugation was also run using a commercial sample of NHS ester of **3c** (30 kDa).

Supporting Information

Supporting Information is available from Wiley Online Library or from the author.

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