SUPPORTING INFORMATION

Role of the Methoxy Group in Immune Responses to mPEG–Protein Conjugates

Merry R. Sherman,* L. David Williams, Monika A. Sobczyk, Shawnya J. Michaels and Mark G. P. Saifer

Mountain View Pharmaceuticals, Inc., 3475 Edison Way, Suite S, Menlo Park, California 94025-1821, United States

*To whom correspondence should be addressed:

Email: sherman@mvpharm.com
Phone: 650-365-5515 x224
Fax: 650-365-5525

Bioconjugate Chemistry Manuscript ID: bc200551b

#	List of Figures	Page
S1	Size-exclusion Chromatography (SEC) of 20 kDa mPEG-Aldehyde and HO-PEG-Aldehyde	3
S2	Reversed-phase Chromatography of Reaction Mixtures for the Synthesis of 10 kDa HO-PEG-NPC and *t*-BuO-PEG-NPC, and of Purified HO-PEG-NPC	4
S3	SEC of Porcine Superoxide Dismutase (SOD) and of SOD Conjugates with 10 kDa mPEG, HO-PEG and *t*-BuO-PEG	6
S4	SEC of Human Interferon-α, of Reaction Mixtures with 20 kDa mPEG-Aldehyde and HO-PEG-Aldehyde, and of Purified PEG_1- and PEG_2-Interferon-α Conjugates	7
S5	SEC of Human Erythropoietin (EPO) and of HO-PEG_1-EPO and HO-PEG_2-EPO Synthesized with 30 kDa HO-PEG-Aldehyde	8
S6	SEC of Porcine Uricase and of Conjugates with 10 kDa mPEG and HO-PEG	10
S7	SEC of Human Serum Albumin and of Conjugates with 10 kDa mPEG, HO-PEG and *t*-BuO-PEG	11

EXPERIMENTAL PROCEDURES

Chromatography. Mono Q® HR 10/10, Superdex 200™ 10/300 GL and PD-10 columns were from GE Healthcare Life Sciences (Piscataway, NJ). Reversed-phase (RP) chromatography was performed on Jupiter® C4 300Å columns from Phenomenex (Torrance, CA). A Toyopearl™ MD-G SP-650S column used for cation-exchange chromatography and a TSK-G5000PW$_{XL}$ column (7.8 x 300 mm) used for size-exclusion chromatography (SEC) were from Tosoh Bioscience (King of Prussia, PA). Amberchrom™ CG-300m (Rohm and Haas Co., Philadelphia, PA) was purchased from Sigma-Aldrich and was packed into a 100 mL HR 16/50 column (GE Healthcare).

Analytical chromatography was performed with an HPLC system from Thermo Scientific (now Thermo Fisher Scientific), which included a P2000 pump, an AS3000 autosampler, a UV2000 UV detector, a Varian Star 9040 refractive index (RI) detector and Agilent EZChrom Elite data acquisition and analysis software. Size-exclusion chromatography of proteins and PEG-protein conjugates was usually performed on a Superdex 200 column at a flow rate of 0.5 mL/min in 10 mM sodium carbonate buffer, pH 10.1, containing 100 mM NaCl. Protein concentrations were determined from the areas of peaks detected at 214 nm, relative to that of a standard solution of bovine serum albumin.

For chromatographic peaks containing PEG but not protein, PEG concentrations were calculated from the peak areas of the RI signal, relative to that of a standard solution of 20 kDa methoxyPEG (mPEG). Size-exclusion chromatographic analyses of 20 kDa mPEG-aldehyde and 20 kDa HO-PEG-aldehyde (both purchased from NOF Corporation) are shown in Figure S1. The leading shoulders, with retention times of about 24 min (indicated by asterisks*), contain less than 7% of the total PEG in each sample. They include mixtures of c. 40 kDa PEG diols, which are unreactive, and the products of aldol condensation of the 20 kDa PEG aldehydes. Reversed-phase chromatography of proteins and PEG-protein conjugates was usually performed on a Jupiter C4 column at a flow rate of 1 mL/min with linear gradients from 20% to 65% (v/v) acetonitrile (ACN) containing 0.1% (v/v) TFA. The elution profiles were monitored by UV absorbance. Samples of *p*-nitrophenylcarbonate derivatives of PEG (NPC-PEGs) were analyzed using mildly acidic buffers to minimize hydrolysis, and the eluates were monitored at the absorbance maximum of 271 nm for NPC. For analyses of NPC-PEGs by SEC, the buffer contained 150 mM NaCl in 20 mM sodium acetate, pH 4.6. For RP chromatography of NPC-PEGs, the elution buffers contained 1 mM HCl instead of 0.1% TFA.

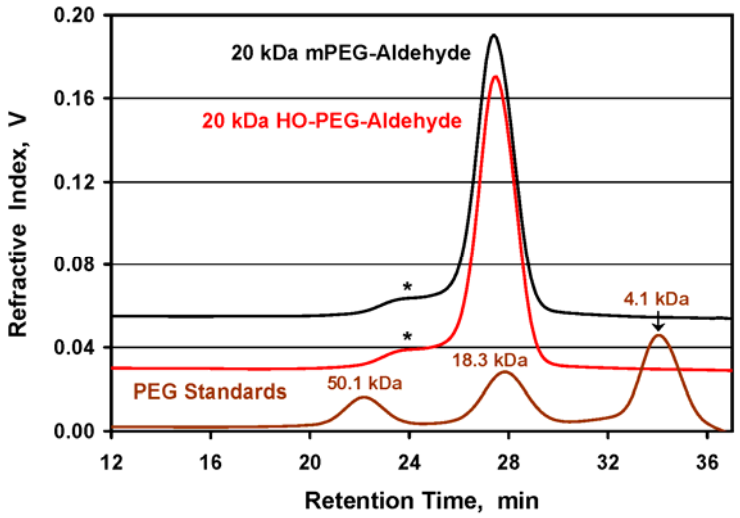

Figure S1. Size-exclusion chromatography of 20 kDa mPEG-aldehyde and 20 kDa HO-PEG-aldehyde (purchased from NOF Corporation) on a Superdex 200 column in 10 mM sodium carbonate buffer, pH 10.1, containing 100 mM NaCl. Three PEG standards from Polymer Laboratories, with the indicated molecular weights, were analyzed under the same conditions. The chromatograms for both mPEG- and HO-PEG-aldehydes have a leading shoulder (designated by *) with a calculated molecular weight of about 40 kDa, which comprises about 7% of the total refractive index signal in each sample.

Synthesis of HO-PEG-NPC by Partial Derivatization of PEG Diol. PEG diol (10 kDa, NOF) was dried under vacuum, and 5 g was dissolved in 15 mL of ACN with warming and mixing in a screw-cap glass tube. To the clear solution, 0.9 mL of 0.82 M *p*-nitrophenyl-chloroformate (pNP-COCl, Aldrich) in ACN was added and mixed. After the addition of 0.16 mL of pyridine (Aldrich), the solution was mixed again and incubated overnight at room temperature. The reaction was quenched by being stirred into a mixture of 58 mL of cold water plus 2 mL of 1 M HCl. The cold solution was filtered through a 0.2 µm filter to remove a white precipitate of dinitrophenyl carbonate. Reversed-phase chromatography on a Jupiter C4 column (4.6 x 150 mm) at 1 mL/min with a dogleg gradient of 20%-38%-50% (v/v) ACN resolved four peaks of absorbance at the following retention times: c. 6.7 min (*p*-nitrophenol), c. 10.9 min (HO-PEG-NPC), c. 12.4 min (diNPC-PEG) and c. 15.1 min (dinitrophenyl carbonate, which hydrolyzed within 2 days to *p*-nitrophenol and carbonate). The peaks of UV absorbance at 10.9 and 12.4 min had similar areas, indicating that the molar ratio of HO-PEG-NPC to diNPC-PEG was c. 2:1. Figure S2A shows the RP chromatographic analyses of the reaction mixtures for the synthesis of HO-PEG-NPC and *t*-BuO-PEG-NPC. Figure S2B shows the RP analysis of the purified HO-PEG-NPC.

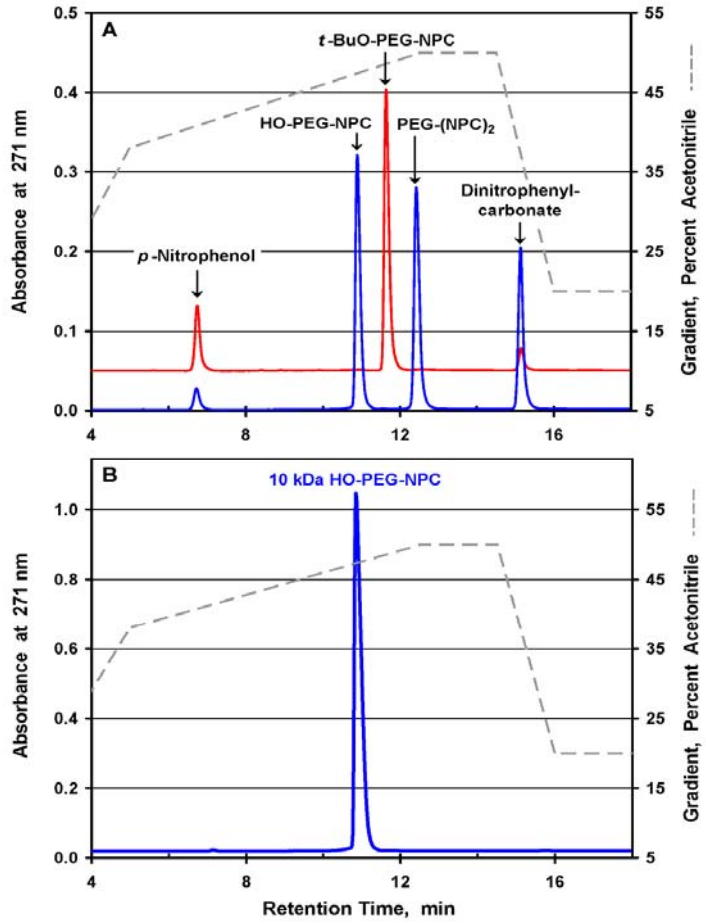

Figure S2. Analytical reversed-phase chromatography of reaction mixtures for synthesis of 10 kDa HO-PEG-NPC and *t*-BuO-PEG-NPC (A) and of purified HO-PEG-NPC (B) on a Jupiter C4 column (4.6 x 150 mm), with a dog-leg gradient of 20% to 38% to 50% (v/v) acetonitrile containing 1 mM HCl. Absorbance was monitored at 271 nm, the absorbance maximum of the *p*-nitrophenylcarbonate group.

The quenched and filtered solution was fractionated in two portions on a 100 mL column of Amberchrom CG-300m, HR 16/50, at 2 mL/min with a linear gradient of 30% to 80% (v/v) ACN containing 1 mM HCl. Fractions were collected and aliquots were analyzed on the Jupiter C4 column, as described above. Fractions containing at least 99% pure HO-PEG-NPC were combined. The ACN was removed under vacuum and the aqueous solution was freeze-dried. The dry, activated PEG was stored desiccated at or below -20 °C.

Synthesis of *t*-ButoxyPEG-NPC. Six hundred milligrams of 10 kDa *t*-butoxyPEG (*t*-BuO-PEG) was dried under vacuum, dissolved in 2 mL ACN, mixed with 48 mg of pNP-COCl (a 4-fold molar excess) and then mixed with 54 µL of pyridine (an 11-fold molar excess over PEG). Analytical RP chromatography of the reaction mixture is shown in Figure S2A. After incubation overnight at room temperature, the reaction was quenched in 13 mL cold water plus

0.7 mL of 1 M HCl. The solution was filtered through a 0.2 µm filter, stored at 4 °C overnight, and fractionated in two portions on a 20 mL Jupiter C4 column (10 x 250 mm) with a linear 100 mL gradient of 20% to 50% (v/v) ACN containing 1 mM HCl. Aliquots of the fractions across the peak were analyzed on a Jupiter C4 column (4.6 x 150 mm). Fractions containing at least 99% pure *t*-BuO-PEG-NPC were pooled. The ACN was removed under vacuum and the aqueous solution was freeze-dried. The dry, activated PEG was stored desiccated at or below -20 °C.

Synthesis of Conjugates of SOD with *t*-ButoxyPEG, mPEG and HO-PEG. Cu-Zn superoxide dismutase from porcine liver (SOD, 0.333 mL of 60 mg/mL) was mixed with 10 kDa *t*-BuO-PEG-NPC (0.276 mL of 200 mg/mL in 1 mM HCl). The reaction was started by the addition of two 13.6 µL portions of 0.5 M Na_2CO_3 at an interval of 6 min. After incubation overnight at 4 °C, the reaction mixture was dialyzed for 4 h against 150 mL of 10 mM sodium carbonate buffer, pH 10.1, containing 100 mM NaCl, in a Spectra/Por 1 dialysis bag. It was then dialyzed overnight against 700 mL of 5 mM Tris-HCl buffer, pH 8.1. The dialyzed reaction mixture was loaded onto a Mono Q HR10/10 column and eluted with a linear 10 mL gradient of 0 to 0.6 M NaCl in 5 mM Tris-HCl buffer, pH 8.1. The peak that was eluted between 18.7 and 21.6 mL was collected, and aliquots were analyzed by SEC on a Superdex 200 column. The average number of PEG molecules per 16 kDa subunit of SOD was calculated to be 2.5, using the method of Kunitani et al. [(1991) *J. Chromatogr. 588*, 125-137].

Samples of mPEG-SOD and HO-PEG-SOD were prepared in a similar manner, using 10 kDa mPEG-NPC (Shearwater Polymers) or 10 kDa HO-PEG-NPC (synthesized by partial derivatization of PEG diol with pNP-COCl and purified by RP chromatography on an Amberchrom column, as described above). Like the *t*-BuO-PEG conjugates of SOD, the mPEG and HO-PEG conjugates had an average of between 2 and 3 molecules of PEG per 16 kDa subunit of SOD. Figure S3 shows the absorbance tracings from SEC analyses of three representative PEG-SOD preparations. Using the method of Kunitani et al. [(1991) *J. Chromatogr. 588*, 125-137], with the RI tracings from the same chromatographic analyses (RI not shown), the extent of PEGylation of the conjugate with *t*-BuO-PEG (c. 2.5 PEGs/SOD subunit) was calculated to be slightly less than the mean for the illustrated mPEG-SOD and HO-PEG-SOD conjugates (c. 3 PEGs/SOD subunit).

Synthesis of mPEG and HO-PEG Conjugates of Interferon-α. Recombinant human interferon-α-2b (IFN-α, 24.6 mL, 1.37 mg/mL) was mixed with 2.5 mL of phosphate-acetate buffer (191 mM Na_2HPO_4, 229 mM HOAc, 29 mM NaOH) to bring the pH to 6.2. This solution was mixed with 2.2 mL of 100 mg/mL 20 kDa mPEG-propionaldehyde (NOF) in 1 mM HCl and

refrigerated. The reaction was started by adding 1 mL of a 1/11 dilution in 95% ethanol of 8 M borane-pyridine, to a final borane-pyridine concentration of 24 mM. After incubation at 4 °C for 5 days, the reaction mixture was dialyzed against 20 mM sodium acetate buffer, pH 4.6, in a Spectra/Por 1 dialysis bag.

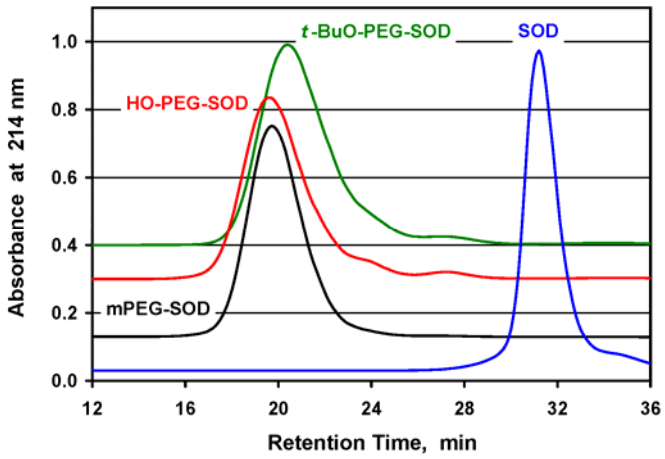

Figure S3. Size-exclusion chromatography of superoxide dismutase (SOD) and of conjugates with 10 kDa HO-PEG, mPEG and *t*-BuO-PEG containing an average of c. 2-3 molecules of PEG per SOD subunit. The Superdex 200 column was eluted with 10 mM sodium carbonate buffer, pH 10.1, containing 100 mM NaCl at a flow rate of 0.5 mL/min.

The dialyzed reaction mixture was loaded onto a Toyopearl MD-G SP-650 cation-exchange column (1 x 6.8 cm) and eluted at 0.5 mL/min with a linear gradient of 0 to 0.4 M NaCl in 20 mM sodium acetate buffer, pH 4.6. The fractions eluting between 18.7 and 21.6 mL were collected and aliquots were analyzed by SEC. The fractions containing predominantly mono-mPEG-IFN-α ($mPEG_1$-IFN-α) or di-mPEG-IFN-α ($mPEG_2$-IFN-α) were pooled separately and were combined with the corresponding fractions from a duplicate reaction and fractionation. Each pool was further fractionated by RP chromatography on a Jupiter C4 column. Aliquots of the fractions were analyzed by SEC on a Superdex 200 10/300 GL column and the more homogeneous fractions were pooled, dialyzed against phosphate-buffered saline, pH 7.4 (PBS), and the preparations of 20 kDa $mPEG_1$-IFN-α and 20 kDa $mPEG_2$-IFN-α were adjusted to a protein concentration of 1 mg/mL.

The synthesis, purification and analysis of HO-PEG conjugates of recombinant human IFN-α were performed in the same manner as for the mPEG conjugates described above, except that the PEGylation reagent was 20 kDa HO-PEG-propionaldehyde (NOF). The products were solutions in PBS of 20 kDa $HO\text{-}PEG_1$-IFN-α and 20 kDa $HO\text{-}PEG_2$-IFN-α in which the protein concentrations were 1 mg/mL. Figure S4 shows the SEC analyses of the two reaction mixtures

(with mPEG- and HO-PEG-aldehydes) and the four purified conjugates with IFN-α ($mPEG_1$, $mPEG_2$, $HO\text{-}PEG_1$ and $HO\text{-}PEG_2$).

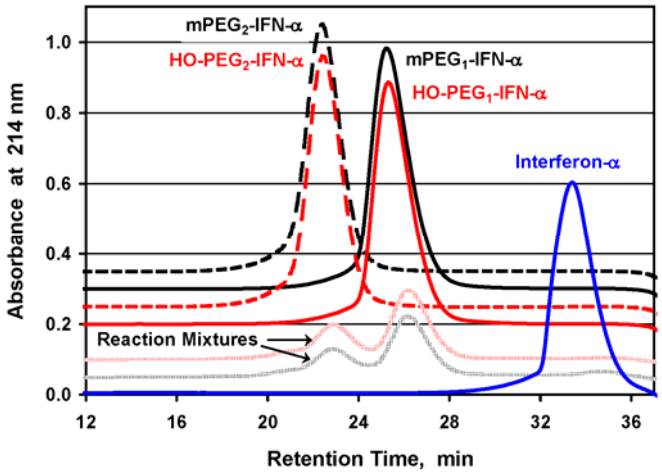

Figure S4. Size-exclusion chromatography of interferon-α starting material, reaction mixtures with mPEG or HO-PEG, and four purified conjugates. The Superdex 200 column was run as in Figures S1 and S3. The ratio of absorbance at 214 nm to refractive index (not shown) was used to confirm the composition of the four conjugates, two of which contained a single molecule of 20 kDa mPEG or HO-PEG, and the other two contained two molecules of 20 kDa mPEG or HO-PEG.

Synthesis of HO-PEG Conjugates of Erythropoietin. Recombinant human erythropoietin-α (EPO, CYT-201) contained 0.6 mg as protein per 1 mg of glycosylated EPO. The lyophilized powder also contained sodium citrate and NaCl. Thirty milligrams of 30 kDa HO-PEG-aldehyde (NOF) in 0.5 mL of 6 mM citric acid were mixed with 1.2 mg of EPO as protein in 1 mL of water to give 1.5 mL of solution with a pH of 6. The PEGylation reaction was started by adding 0.16 mL of a 1/32 dilution in 95% ethanol of 8 M borane-pyridine, to a final borane-pyridine concentration of 25 mM. After incubation for 11 days at 4 °C, the reaction mixture was exchanged into 20 mM Tris-HCl buffer, pH 8.1, by passage through a PD-10 desalting column (GE Healthcare). The buffer-exchanged reaction mixture was divided into five portions of 0.65 mL, which were fractionated by SEC in the same Tris-HCl buffer through a Superdex 200 10/300 GL column (GE Healthcare) plus a TSK-G5000PW_{XL} column (7.8 x 300 mm) in series. The eight fractions of 0.5 mL that were eluted between 17.5 and 21.5 mL from all five SEC runs were combined, loaded onto a Jupiter C4 column (4.6 x150 mm) and eluted with a gradient of 20% to 65% (v/v) ACN in 0.1% TFA. Aliquots of the 0.5 mL fractions from the RP column were analyzed by SDS-PAGE. Fractions containing predominantly monoPEG-EPO, which were eluted between 18 and 20 mL, were pooled, concentrated under vacuum to 0.1 mL, and again fractionated by SEC, this time on a Superdex 200 column in 20 mM

sodium acetate buffer containing 1 M NaCl, pH 4.4. The monoPEG-EPO peak, which was eluted between 9.5 and 10.9 mL, was dialyzed into 20 mM sodium acetate buffer containing 100 mM NaCl, pH 4.4. The final preparation contained 0.1 mg/mL protein, based on its absorbance at 214 nm. Size-exclusion chromatographic analyses of HO-PEG$_1$-EPO and HO-PEG$_2$-EPO show that the monoPEG conjugate was well resolved from any diPEG conjugate that might have been produced in the reaction mixture, as shown in Figure S5.

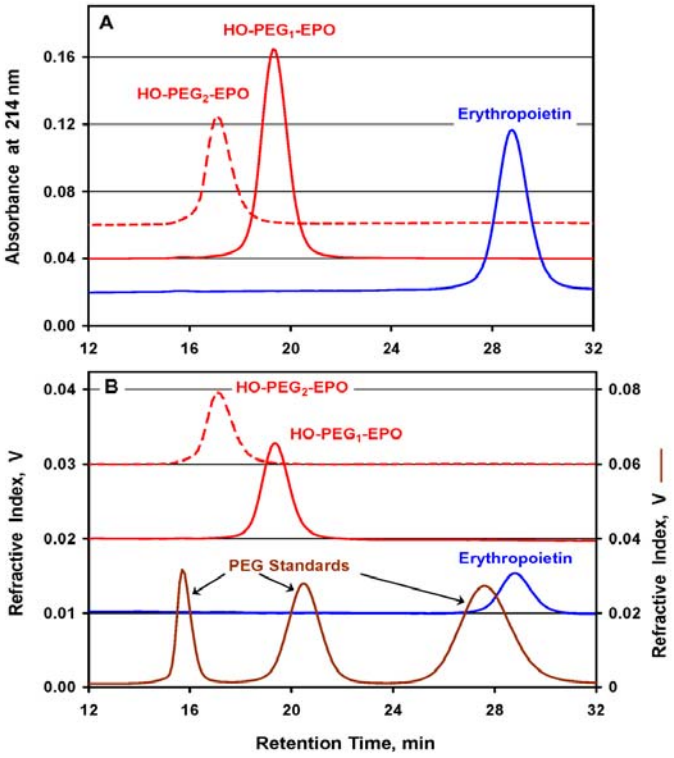

Figure S5. Size-exclusion chromatographic analyses of erythropoietin and of two conjugates synthesized by reductive alkylation with 30 kDa HO-PEG-aldehyde. The unmodified erythropoietin and the monoPEG and diPEG conjugates were all well resolved from each other on this Superdex 200 column, which was eluted at a flow rate of 0.5 mL/min, with 20 mM Tris buffer, pH 8.0, containing 100 mM NaCl. The retention times of the refractive index signals were corrected for the lag of 0.3 min between the absorbance detector and the RI detector. The PEG standards had molecular weights of 219.3, 32.6 and 10 kDa, respectively.

Synthesis of mPEG and HO-PEG Conjugates of Porcine Uricase. Recombinant porcine uricase (81 mg in 66 mL) was concentrated by ion-exchange chromatography on a Mono Q HR10/10 column and elution with a linear 100 mL gradient of 0 to 0.6 M NaCl in 0.1 M sodium carbonate buffer, pH 10.2. Fractions of 4 mL were collected and aliquots were analyzed by SEC on a Superdex 200 column. The main peak started at 60 mL into the 100 mL gradient of the ion-exchange column. The first two fractions of this peak, which had protein concentrations of 4.9 and 9.2 mg/mL, respectively, contained protein that was >95% homogeneous tetrameric uricase.

These fractions were used as the substrates for PEGylation. A 93 mg/mL solution of 10 kDa mPEG-NPC (SunBio) was prepared by dissolving 133 mg in 1.325 mL of 1 mM HCl. A 1.8 mL portion of each fraction was mixed with vortexing into 0.24 mL of NPC-PEG solution and incubated overnight at 4 °C. After SEC analysis of aliquots, another 0.22 mL of NPC-PEG solution was mixed with vortexing into the more concentrated reaction mixture (containing c. 8.1 mg/mL protein) to bring the PEG-to-protein ratio in both conjugates to about 2 molecules of PEG per uricase subunit. After incubation for three more days at 4 °C, the two reactions were combined and ultrafiltered against PBS in a Miniplate with a 100 kDa cutoff membrane (Millipore, Billerica, MA). Size-exclusion chromatographic analyses of aliquots of the 17 mL retentate showed that the product contained an average of 2.3 molecules of PEG per uricase subunit, 1.2 mg/mL of uricase protein and 0.1 mg/mL of free 10 kDa PEG.

A solution of 10 kDa HO-PEG-NPC was prepared to match the absorbance at 271 nm of the 93 mg/mL solution of mPEG-NPC prepared as described above. The same procedure was then used to prepare a solution of HO-PEG-uricase in PBS, matching the composition and concentration of the mPEG-uricase prepared as described above, except for the presence in the conjugate of an average of 2.3 molecules of HO-PEG, instead of mPEG, per uricase subunit. Representative chromatograms of the porcine uricase used as starting material and of the mPEG and HO-PEG conjugates of uricase on a TSK-G5000PW$_{XL}$ column are shown in Figure S6.

Synthesis of mPEG, HO-PEG and *t*-ButoxyPEG Conjugates of Human Serum Albumin. A 100 mg/mL solution of 10 kDa mPEG-NPC (Shearwater Polymers) was prepared by dissolving 179.5 mg in 1.65 mL of 1 mM HCl. Human serum albumin (18.75 mg in 75 µL) was added and 0.134 mL of 1 M Na_2CO_3 was added dropwise while stirring, to neutralize the acid formed by the reaction. The mixture was incubated overnight at 4 °C and exchanged into 10 mM Tris-HCl buffer, pH 8.1, by passage through a 9.1 mL PD-10 desalting column (GE Healthcare). The buffer-exchanged solution of mPEG-albumin was loaded onto a Mono Q HR10/10 column and eluted at 0.5 mL/min with a linear 10 mL gradient of 0 to 0.6 M NaCl in 10 mM Tris-HCl buffer, pH 8.1 (between 12 and 22 mL), followed by 10 mL of 0.6 M NaCl in the same Tris-HCl buffer. The peak eluting between 18.7 and 24.7 mL was collected as 1 mL fractions, and aliquots were analyzed by SEC on a Superdex 200 10/300 GL column. The leading edge of the peak (the first two fractions) was combined with a similarly prepared and fractionated pool and dialyzed against water in a Spectra/Por 1 dialysis bag.

A 100 mg/mL solution of 10 kDa HO-PEG-NPC (made by RP chromatographic purification of partially derivatized 10 kDa PEG diol, as described above) was prepared by dissolving 101.6 mg

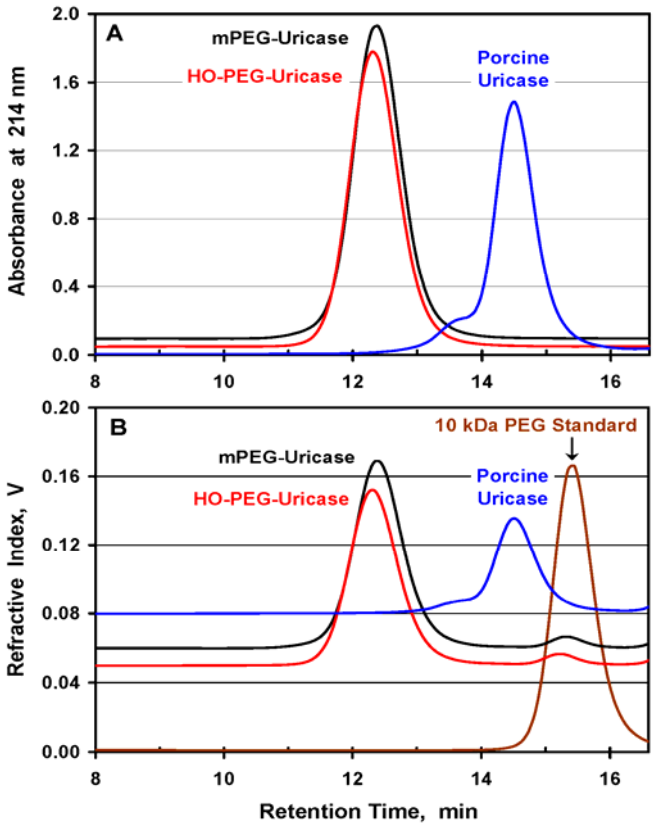

Figure S6. Size-exclusion chromatography of porcine uricase and of conjugates containing an average of c. 2-3 molecules of 10 kDa mPEG or 10 kDa HO-PEG per uricase subunit on a TSK G-5000PW$_{XL}$ column in 10 mM sodium carbonate buffer, pH 10.1, containing 100 mM NaCl, at a flow rate of 0.6 mL/min. The retention times of the refractive index signals (panel B) were corrected for the 0.25 minute lag between the absorbance detector and the RI detector.

in 0.92 mL of 1 mM HCl. Human serum albumin (12.5 mg in 50 µL) was added and 0.075 mL of 1 M Na_2CO_3 was added dropwise while stirring. The mixture was incubated overnight at 4 °C and exchanged into 10 mM Tris-HCl buffer, pH 8.1, by dialysis. The buffer-exchanged solution of HO-PEG-albumin was loaded onto a Mono Q HR10/10 column and eluted at 0.5 mL/min with a linear 10 mL gradient of 0 to 0.6 M NaCl in 10 mM Tris-HCl buffer, pH 8.1 (between 12 and 22 mL), followed by 10 mL of 0.6 M NaCl in the same Tris-HCl buffer. The peak eluting between 18.5 and 25 mL was collected as fractions, and aliquots were analyzed by SEC on a Superdex 200 column. The leading edge of the peak was combined with a similarly prepared and fractionated pool and dialyzed against water at 4 °C in a Spectra/Por 1 dialysis bag.

For the synthesis of *t*-BuO-PEG-albumin, the PEGylation reagent consisted of 360 mg of 10 kDa *t*-BuO-PEG-NPC that had been purified by RP chromatography (see Figure S2) and was dissolved in 1.5 mL of 1 mM HCl. A 0.433 mL portion of the resultant 200 mg/mL solution was mixed with 25 mg of albumin in 100 µL and 20 µL of 1 M Na_2CO_3 was added while the solution

was mixed with a vortex mixer. The reaction mixture was incubated overnight at 4 °C and dialyzed into 5 mM Tris-HCl buffer, pH 8.1. The dialyzed solution of *t*-BuO-PEG-albumin was divided into two portions that were loaded onto a Mono Q HR10/10 column and eluted at 0.5 mL/min with a linear gradient of 0 to 0.6 M NaCl in 10 mM Tris-HCl buffer, pH 8.1 (between 12 and 22 mL). Aliquots of fractions from the two runs were analyzed by SEC on a Superdex 200 column and the material eluting between 17 and 24 mL was pooled and dialyzed against water in a Spectra/Por 1 dialysis bag.

Analyses of the SEC elution profiles by the method of Kunitani et al. [(1991) *J. Chromatogr. 588*, 125-137] showed that various preparations of mPEG-albumin, HO-PEG-albumin and *t*-BuO-PEG-albumin contained an average of between 17 and 22 molecules of 10 kDa PEG per molecule of albumin. Representative chromatograms from which these calculations were made are shown in Figure S7.

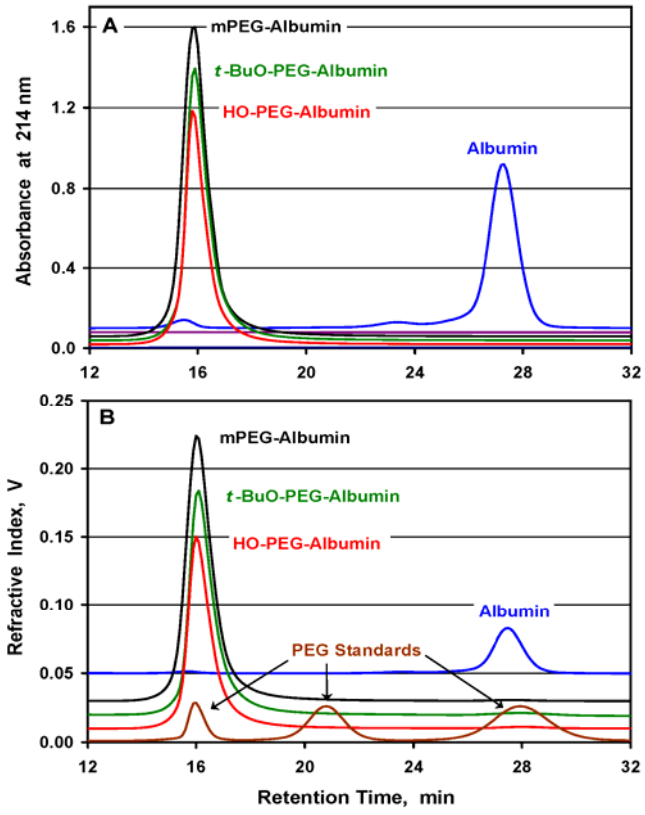

Figure S7. Size-exclusion chromatograms of human serum albumin and conjugates with 10 kDa mPEG, *t*-BuO-PEG and HO-PEG, each of which contained an average of c. 20 molecules of PEG per molecule of albumin. The samples were analyzed on a Superdex 200 column in 10 mM sodium carbonate buffer, pH 10.1, containing 100 mM NaCl, at a flow rate of 0.5 mL/min. The retention times of the refractive index signals were corrected for the lag of 0.3 min between the absorbance detector and the RI detector. As in Figure S5, the PEG standards had molecular weights of 219.3, 32.6 and 10 kDa.