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Degradable Polyphosphoester-Protein Conjugates: "PPEylation" of Proteins

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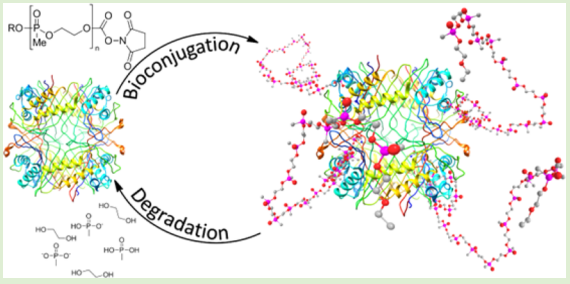
Supporting Information

ABSTRACT: Pharmacokinetic properties determine the efficacy of protein therapeutics. The covalent attachment of poly(ethylene glycol) (PEG) extends the half-life of such biologicals to maintain a therapeutically effective concentration over a prolonged period of time and improves administration and compliance. A major obstacle of these polymer−protein conjugates is the chemical stability of the PEG preventing its metabolism and leading to side effects. Instead, we propose the PPEylation, that is, the conjugation of degradable poly(phosphoester)s (PPE) to proteins, in order to generate fully biodegradable polymer−protein conjugates. The structure of the PPEylated protein conjugates was verified with mass spectrometry and size exclusion chromatography. They were compared to structural analogues, except classical, PEGylated proteins, and exhibit comparable bioactivity, but avoiding any nondegradable polymer in the conjugate. We proved the degradation of the protective polymer shell surrounding the conjugate in aqueous environments at physiological conditions by online triple detection size exclusion chromatography and gel electrophoresis. We believe that this research will provide an attractive alternative for future drug design with implications for the clinical use of biologicals.



INTRODUCTION

Biologics for therapeutic applications are being explored widely to treat serious diseases that are difficult or impossible to address with other drugs. Typical examples for these biotechnological products are monoclonal antibodies, vaccines, hormones, growth and coagulation factors, cytokines, fusion proteins and other enzymes.[1] Since these biologics exhibit rather short half-life times in blood due to renal excretion and degradation there is a strong need to improve their pharmacokinetic properties. Several different strategies have been developed so far to address this problem, typically by increasing the hydrodynamic volume of the therapeutic by coupling a synthetic polymer to it covalently.[2]

Coupling poly(ethylene glycol) to biologics is known as PEGylation and is probably the most prominent and widely used protocol to prolong the blood circulation time and also to lower the immunogenicity of proteins due to the polymer shield surrounding the protein.[3] PEGylated drugs are not recognized by the immune system and exhibit the so-called "stealth" effect. PEGylation was established by Abuchowski in 1977 and the first PEGylated protein drug was approved by the US Food and Drug Administration in 1990, with many other biologics to follow.[4] Although PEG is considered to be safe and well tolerated, the occurrence of renal tubular vacuolization in animal models have raised concerns that a prolonged therapy with PEGylated drugs may lead to an accumulation of PEG in the cytoplasm of kidney cells as the polymer is not biodegradable.[5] In addition, PEG has shown the possibility to form toxic degradation products during storage which could provoke adverse effects.[6] A few degradable PEG-based polymers have been developed to address this problem, e.g., by introduction of redox-responsive disulfide bridges[7] or acid-labile acetals, the latter already tested for bioconjugation.[8]

Continuing replacement therapy with PEGylated biologics; however, may have revealed an important side-effect of this polyether. IgM and IgG anti-PEG antibodies have been observed in healthy donors questioning the reported low immunogenicity of PEG, although interpretation of the reported studies is still under vigorous debate.[9] Moreover, these antibodies may facilitate clearance of PEGylated conjugates, decreasing plasma half-life and thus antagonize the initial purpose of protein modification. For these reasons alternatives for PEG have been developed. Nondegradable polyolefins, e.g., poly(vinylpyrrolidone) (PVP) or copolymers of *N*-(2-hydroxypropyl)methacrylamide (HPMA) have been explored *in vivo* but are either less studied for bioconjugation purposes or have also shown to stimulate antibody production.[10] Poly(2-oxazoline)s (POZ), on the contrary, exhibit a stealth behavior similar to PEG and were successfully tested *in vivo* for many applications, however, risking polymer accumulation if renal excretion is insufficient.[11] Polymer accumulation may be prevented by the usage of degradable

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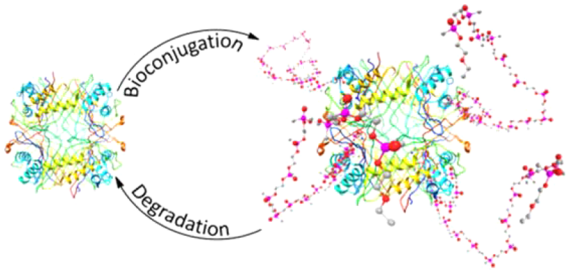
PEG alternatives, e.g., enzymatically degradable poly(amino acid)s ("PASylation") or hydroxyethyl starch ("HESylation"). However, PASylation requires extensive genetic engineering, while HESylation does not allow access to precise conjugates since HES is not as well-defined as PEG or other synthetic biopolymers.[2b,12]

In this study, we report for the first time to have overcome the inherent drawbacks of PEGylation by the living anionic polymerization with selective chain-end functionalization of biodegradable polyphosphoesters (PPE).[13] A straightforward bioconjugation approach with a well-defined, water-soluble, degradable PPE is presented, the PPEylation of proteins, which increases the hydrodynamic radius of the enzyme and remains its enzymatic activity. PPEs are biodegradable and biocompatible polymers, mimicking natural occurring nucleic acids and are therefore predestined for biomedical applications.[13,14] Elaborate drug delivery systems and other biomedical applications have been successfully implemented employing PPEs.[15] We were recently able to prove comparable "stealth" properties of PPEylated and PEGylated nanoparticles.[16] A novel protein-modification technique is accessible with PPEs, as these polymers are susceptible to hydrolysis, i.e., the backbone releases the free protein and nontoxic, excretable products during degradation. Second, preparation of PPEs avoids potentially toxic catalysts, and provides a controlled polymerization with the opportunity to vary the molecular weights, keep polydispersities low and releases a terminal hydroxyl group at the PPE chain end, comparable to PEG derivatives, which allows similar conjugation strategies, i.e., the "PPEylation" of proteins (Scheme 1).

Scheme 1. Covalent Attachment of Poly(phosphoester)s to Proteins: the "PPEylation" of Proteins Produces Fully Degradable Conjugates



In this report, the terminal hydroxyl functionality is reacted with an activated carbonate to prepare an amino-reactive succinimidyl-carbonate (SC). Subsequent conjugation to a model protein, bovine serum albumin (BSA), and the therapeutic enzyme uricase (UC) yields the corresponding PPE-conjugates. In addition, the degradation of the polymeric modifier is evaluated at physiological conditions and the residual enzymatic activity determined.

EXPERIMENTAL SECTION

Materials. Solvents were purchased from Sigma-Aldrich (Germany) and used as received, unless otherwise stated. 2-Methyl-1,3,2-dioxaphospholane 2-oxide (MeEP) was prepared as reported previously.[17] 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) was purchased from Sigma-Aldrich (Germany), distilled from CaH_2 prior to use and stored over molecular sieve (4 Å). 2-(Benzyloxy)ethanol was purchased from ABCR and distilled from sodium prior to use. Acetonitrile (ACN), dichloromethane (DCM), dioxane and toluene over molecular sieve, Dulbecco's phosphate buffered saline (D-PBS), ammonium acetate and sodium tetraborate decahydrate were used as received from Sigma-Aldrich (Germany). Ammonium bicarbonate was used as received from Fisher Scientific. *N,N′*-disuccinimidyl carbonate (DSC) was used as received from Acros Organics (Germany). BSA, *N*-Hdroxysuccinimide (NHS), uric acid and uricase from *Bacillus fastidiosus* were purchased from Sigma-Aldrich (Germany) and used as received. Poly(ethylene glycol) mono methyl ether (M_n = 5000) was purchased from Fluka and postmodified following the procedure reported by Zalipski.[18] Deuterated solvents were purchased from Deutero GmbH (Kastellaun, Germany) and used as received. Ultrapure water with a resistivity of 18.2 MΩ cm^{-1} (Milli-Q, Millipore) was used to prepare buffers.

Instrumentation and Characterization Techniques. Size exclusion chromatography (SEC) was performed with phosphate buffered saline (PBS, 100 mM phosphate, 50 mM sodium chloride, pH 6.5) as eluent. BSA samples were separated over a set of HEMA-Bio columns (40/100/1000) with 10 μm particles with a length of 300 mm and an internal diameter of 8 mm (MZ-Analysentechnik) providing an effective molecular weight range of 2000−3 000 000 at a flow rate of 1.0 mL min^{-1} (Agilent 1260 HPLC). UC samples were separated over a Superdex 200 10/300 GL column with 13 μm particles with a length of 300 mm and an internal diameter of 10 mm (GE Healthcare) providing an effective molecular weight range of 10 000−600 000 at a flow rate of 0.75 mL min^{-1} (Agilent 1260 HPLC). Each sample injection was 50 μL at a protein concentration of 2 mg mL^{-1}. Elution profiles for mass analysis were detected using an ultraviolet−visible detector (280 nm, Agilent 1260), a Wyatt miniDAWN TREOS MALLS detector, a Wyatt ViscoStar II online differential viscometer, and a differential refractometer (Agilent 1260). Using the elution-profile data the weight-averaged molecular mass (M_w) was calculated with Astra 6.1.1 software (Wyatt Technologies) using a d*n*/d*c* of 0.1035 mL g^{-1} for PMeEP (determined by batch measurement in water, SI 4) or 0.135 mL g^{-1} for PEG (American Polymer Standards Corporation).

MALDI-ToF MS measurements were performed on a Shimadzu Axima CFR MALDI-TOF mass spectrometer using sinapinic acid (3-(4-hydroxy-3,5-dimethoxyphenyl)prop-2-enoic acid) as a matrix. A solution (1 mg mL^{-1}) of the samples in a TFA-acidic (0.01 vol %) mixture of H_2O/ACN (50:50) was mixed with a solution of sinapinic acid (10 mg mL^{-1}). After evaporation of the solvent, the sample was transferred into the spectrometer and analyzed.

^1H, ^{13}C, and ^{31}P NMR spectra were acquired on a 300 MHz Bruker system. The temperature was kept at 298.3 K and calibrated with a standard ^1H methanol NMR sample using Topspin 3.0 (Bruker). ^{13}C NMR spectra were referenced internally to solvent signals. ^{31}P NMR spectra were referenced externally to phosphoric acid. The ^{13}C NMR (101 MHz) and ^{31}P NMR (121 MHz) measurements were obtained with a ^1H powergate decoupling method using 30° degree flip angle. 2D ($^1H^{31}P$ HMBC) were measured on a Bruker Avance III 400 NMR spectrometer The spectra were referenced to the residual proton signals of the deuterated solvent ($CDCl_3$ (^1H) = 7.26 ppm; DMSO-d_6 (^1H) = 2.50 ppm). All 1D and 2D spectra were processed with MestReNova 9.0.0−12821.

The DOSY (Diffusion Ordered Spectroscopy) experiments were executed on a Bruker Avance III 400 NMR spectrometer with a 5 mm BBFO ^1H/X *z*-gradient probe and a gradient strength of 5.01 G cm^{-1} A^{-1}. The gradient strength was calibrated with the diffusion coefficient of a sample of ^2H_2O/^1H_2O at a defined temperature and compared with the literature.[19] In this work, the gradient strength was 64 steps from 2% to 95%. The diffusion time d_{20} was optimized to 200 ms at a gradient pulse length of 2.5 s. All measurements were done with a relaxation delay of 1.0 s.

SDS-PAGE was carried out using 8% Tris-HCl gels (Biorad, 1.0 mm, 10 well).

UV measurements were performed with a Jasco V-630 photospectrometer with a Jasco ETC-717 Peltier element.

Synthetic Procedures. *Synthesis of PMeEP-SC.* The monomer MeEP (1.309 g, 10.7 mmol) was placed into a flame-dried Schlenk-tube, dissolved in 1 mL benzene and dried by repeated (three times)

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lyophilization. A stock solution of 2-(benzyloxy)ethanol in dichloromethane (0.2 M) was prepared and 1047 μL (209.4 μmol) were transferred to the monomer solution. The mixture was cooled to 0 °C and the polymerization was started by the rapid addition (1571 μL, 314 μmol) of a dichloromethane stock solution of DBU (0.2 M). The polymerization was terminated after 80 min (~80% conversion as shown before[17]) by the rapid addition of a 10-fold excess of *N*,*N*′-disuccinimidyl carbonate (DSC) dissolved in cold, dry acetonitrile (536 mg in 8 mL ACN). After 30 min the polymer was purified by precipitation in cold diethyl ether and stored until usage at −28 °C. $PMeEP_{42}$−SC ^{1}H NMR (DMSO-d_6, ppm): δ 7.43−7.20 (m, 5H, Ar), 4.52 (s, 2H, Bn−O−$C\underline{H}_2$), 4.39−4.00 (m, 385H, O−$C\underline{H}_2$−$C\underline{H}_2$−O), 2.81 (s, 4H, SC-group), 1.51 (d, *J* = 18 Hz, 135H, P−CH_3). ^{13}C NMR (DMSO-d_6, ppm): δ 169.94 (s, O$\underline{C}$−CH_2−CH_2−$\underline{C}$O), 151.30 (s, O−($\underline{C}$O)−O), 138.21 (Ar), 128.31 (Ar), 127.61 (Ar), 127.53 (Ar), 71.99 (Ar−$\underline{C}H_2$−O), 68.91 (d, *J* = 6.2 Hz, Ar−CH_2−O−$\underline{C}H_2$), 66.76 (d, *J* = 6.4 Hz, Ar−CH_2−O−CH_2−$\underline{C}H_2$), 64.24 (br. s, backbone), 60.36 (d, *J* = 6.5 Hz, $\underline{C}H_2$−O−CO), 25.41 (s, OC−$\underline{C}H_2$−$\underline{C}H_2$−CO), 10.39 (d, *J* = 140.8 Hz, P−CH_3). ^{31}P NMR (DMSO-d_6, ppm): δ 32.0 (backbone), 31.6 (terminal P−CH_3).

Synthesis of PEG-SC. Poly(ethylene glycol) methyl ether (mPEG, M_n 5000, 2.0 g, 400 μmol) was dried under reduced pressure and dissolved in a mixture of 6 mL dry toluene and 2 mL DCM. A solution of 20 wt % phosgene in toluene (2 mL) was added to form the intermediate mPEG-chloroformate. Solvents and residual phosgene were removed under reduced pressure after 12 h. The residue was redissolved in a mixture of 4 mL toluene and 2 mL DCM. A solution of *N*-hydroxysuccinimide (NHS, 69 mg, 600 μmol) and pyridine (32 mg, 400 μmol) in dioxane was added to form the succinimidyl carbonate (SC). After 3 h the solvents were removed under reduced pressure and the residue was recrystallized once from 20 mL ethyl acetate, yielding mPEG-SC (1.37 g, 275 μmol, 69%).^{1}H NMR (DMSO-d_6, ppm): δ 3.51 (br. s, 472H, $C\underline{H}_2$−$C\underline{H}_2$−O), 3.24 (s, 3H, $C\underline{H}_3$−O), 2.81 (s, 4H, SC-group). ^{13}C NMR (DMSO-d_6, ppm): δ 169.90 (s, O$\underline{C}$−CH_2−CH_2−$\underline{C}$O), 151.37 (s, O−($\underline{C}$O)−O), 71.29 (s, $\underline{C}H_2$−O−CH_3), 69.80 (br. s, backbone), 67.69 (s, $\underline{C}H_2$−O−CO), 58.06 (s, O−$\underline{C}H_3$), 25.35 (s, OC−$\underline{C}H_2$−$\underline{C}H_2$−CO).

Synthesis of BSA-PMeEP Conjugate. BSA (5 mg, 75.8 nmol, 2.5 μmol NH_2-groups) was dissolved into 0.5 mL of borate buffer (100 mM, pH 8.5) and added to PMeEP-SC (30 mg, 5 μmol). The mixture was allowed to react at 20 °C for 3 h shaking before repeated dialysis (3x 1 L H_2O, 50 000 MWCO) to remove excess polymer and NHS. Lyophilization yielded the colorless conjugate (6.9 mg, 75.8 nmol, 100%).

Degradation study of BSA-PMeEP Conjugate. BSA-PMeEP (1.5 mg, 16 nmol) was dissolved in buffer (100 mM; ammoniumbicarbonate for pH 9.0, ammonium acetate for pH 7.0 and pH 5.0) and incubated at 37 °C. 100 μL samples were withdrawn and analyzed by MALLS-SEC and SDS-PAGE.

Synthesis of UC-PMeEP Conjugate. UC (5 mg, 38.5 nmol) was dissolved into 1.0 mL of borate buffer (100 mM, pH 8.5) and added to PMeEP-SC (2.1 mg, 385 nmol). The mixture was allowed to react at 20 °C for 1 h shaking before loaded into an Amicon Ultra-15 centrifugal filtration device (MWCO 50 000). Excess polymer and NHS was removed by repeated centrifugal filtration and washing with D-PBS buffer. The efficient removal of the PPE can be proven by aqueous SEC or DOSY NMR spectroscopy. To remove unreacted protein and high molecular weight aggregates the sample was loaded onto a Superdex 200 Increase 10/300 GL column (GE Healthcare) and eluted with D-PBS at 0.75 mL/min (JASCO PU-2086). The flow-through was monitored with a HPLC UV detector (JASCO UV-2075) at 280 nm and the volume containing conjugate was collected for analysis. The collected conjugate was then characterized by SDS-PAGE and aqueous SEC (see Supporting Information). All conjugate containing fractions were stored at −28 °C. The corresponding PEGylated UC conjugates were prepared analogously.

Enzymatic Activity Assay. The UC-conjugate samples were diluted with D-PBS to a final protein concentration of 0.1 mg mL^{-1}. Uric acid (UA) was dissolved in borate buffer (50 mM, pH 9.0) to yield a set of different concentrations (5, 10, 25, 50, 100 nmol mL^{-1}). 300 μL a UA solution was mixed with 30 μL of the UC solution and immediately transferred into a UV spectrometer. The decrease in UV absorbance at 292 nm was measured against time using a 1 mm quartz cuvette at 25 °C for 2 min. The measurement was repeated three times for every concentration.

RESULTS

Synthesis of Polymers and Postmodification. Poly(ethylene methylphosphonate) (PMeEP) has been previously demonstrated by our group to be highly water-soluble, nontoxic, and degradable under physiological conditions.[17] Furthermore, PMeEP is prepared via organobase-catalyzed living anionic ring-opening polymerization (AROP) in unprecedented control over the molecular weight and polydispersity compared to other PPEs reported so far. Due to the stable P−C-bond in the side chain, the polymerization of these cyclic phosphonate monomers can be conducted to full conversion with a minimum degree of backbiting or transesterification.[17,20] The high level of hydration and the availability of defined polymer structures makes PMeEP an ideal candidate for the PPEylation of proteins to compete with the nondegradable gold standard PEG. Both polymers exhibit terminal hydroxyl-functionalities after preparation, requiring ω-postmodification to introduce a protein-reactive moiety. Bioconjugation chemistry allows access to many different functionalities targeting specific amino acids or structural features.[21] The succinimidyl-carbonate (SC) group is, besides activated esters or maleimides, one of the mostly applied protein-reactive groups in PEGylation chemistry, and thus it was chosen as a proof-of-principle to target the ε-amine residues of lysine, one of the most abundant amino acids that is typically located on the surface of biomolecules.[22]

The living anionic polymerization of 2-methyl-1,3,2-dioxaphospholane 2-oxide (MeEP) was initiated with 2-(benzyloxy)ethanol (**1**) and catalyzed by 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU). To the living polymer chains *N*,*N*′-disuccinimidyl carbonate (DSC) was added to functionalize the ω-position of the PPE chain. The protein-reactive PMeEP-SC was purified by precipitation into diethyl ether and obtained in quantitative yields and after full monomer conversion. The number-average molecular weight (M_n) was adjusted to 5.0 kDa by the monomer: initiator ratio and subsequently confirmed by ^{1}H NMR spectroscopy (Supplementary Figure S1). These results were further fortified by multiple laser light scattering (MALLS) SEC measurements in PBS (Figure 1b; note the refractive index increment (d*n*/d*c*) of an unfunctionalized PMeEP was determined previously to be d*n*/d*c* = 0.104 (Supplementary Figure S4). A molecular weight dispersity (*Đ*) of 1.08 was calculated for the sample acknowledging the high control AROP offers. Successful end-group modification was confirmed by ^{1}H DOSY NMR measurements, revealing a single diffusion coefficient of 2.1 × 10^{-6} m^2 s^{-1} for the polymer and the SC group with proton resonances at 2.81 ppm (Supplementary Figure S6).

To compare the novel protein-reactive PPEs with the gold standard PEG, commercially available poly(ethylene glycol) monomethyl ether (mPEG 5.0 kDa) was postmodified employing phosgene to produce the corresponding mPEG-chloroformate which was subsequently reacted with *N*-hydroxysuccinimide (NHS) to form a SC group.[18] This activated mPEG-SC is used as a control to compare PPEylation with the established PEGylation remaining molecular weights

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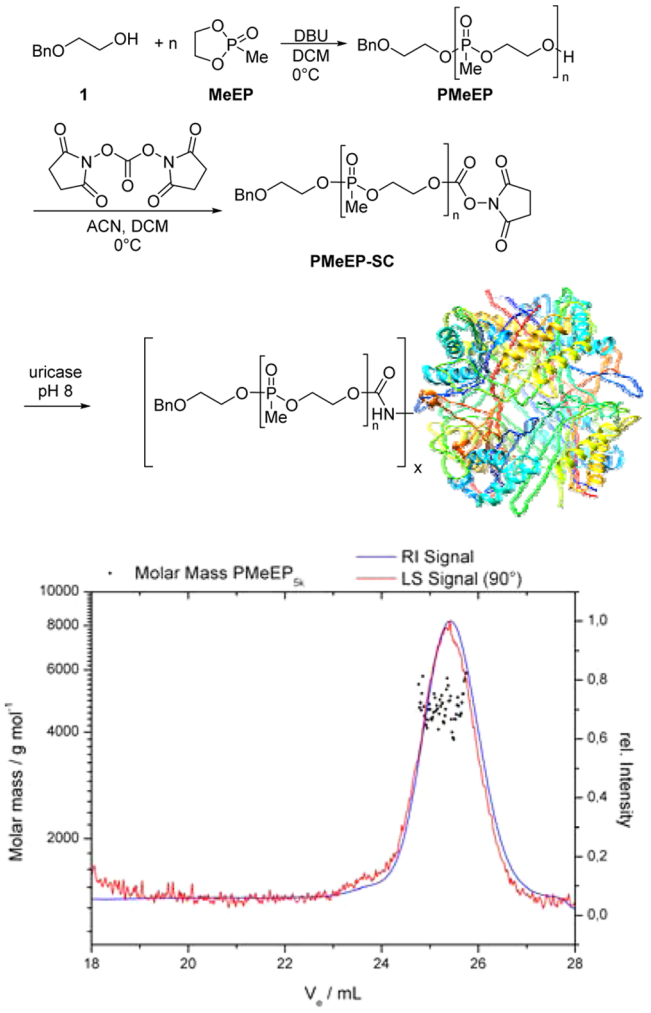

Figure 1. (a) Polymerization of MeEP and ω-postmodification with DSC yielding amino-reactive PMeEP-SC. (b) MALLS-SEC analysis of PMeEP indicating a narrow molecular weight distribution and a molecular weight close to 5000 g mol^{-1}.

constant (characterization detail of PEG-SC can be found in the Supporting Information).

BIOCONJUGATION

To study the conjugation of PMeEP-SC to enzymes, first, bovine serum albumin (BSA) was selected as a model protein, before conjugation to a therapeutically important protein was conducted. The primary structure of BSA consist of 59 lysine residues, with 30−35 being accessible for conjugation.[23] Bioconjugation was performed for 30 min in an aqueous borate buffer (100 mM, pH 9.0) with a 10-fold excess of PMeEP-SC with respect to BSA. The conjugate was purified by dialysis against Milli-Q water and isolated after lyophilization in quantitative yield. Figure 2 shows the aqueous SEC traces of BSA and the conjugate, respectively. The molecular weight was calculated by MALLS using the refractive index (RI) detector as the concentration detector. In addition, the UV absorption at 280 nm was measured.

BSA and its homodimer are separated by the SEC column, so that both molecular weights can be calculated using the LS and RI detector signal. In comparison to the theoretical masses of 66.4 kDa and 132.8 kDa respectively, both masses are slightly underestimated in our setup. After conjugation, all detector signals shift to lower elution volumes, i.e. an increase of the hydrodynamic radii and thus of the molecular weight and indicate the formation of a polymer−protein conjugate. Calculation from the LS and RI signal results in molecular weights of ca. 185 kDa and 91 kDa for the BSA-Dimer conjugate and the BSA-Monomer conjugate, respectively. The average number of polymer chains attached to BSA can be calculated arithmetically from the absolute molecular weight determined by SEC-MALLS to be about 5−6 chains of 5 kDa each per biomolecule. These measurements and calculations were confirmed by MALDI-ToF mass spectrometry (MS) (Figure 3). In comparison to unmodified BSA, the molecular weight increased significantly with a peak maximum at about 91 kDa, verifying the SEC-MALLS measurements.

Degradation. Hydrolytic degradation of the BSA-PPE conjugate was studied in three different buffer systems (pH 5.0, 7.4, 9.0 each 100 mM) incubated at physiological temperature (37 °C) for 18 days. Samples were analyzed via

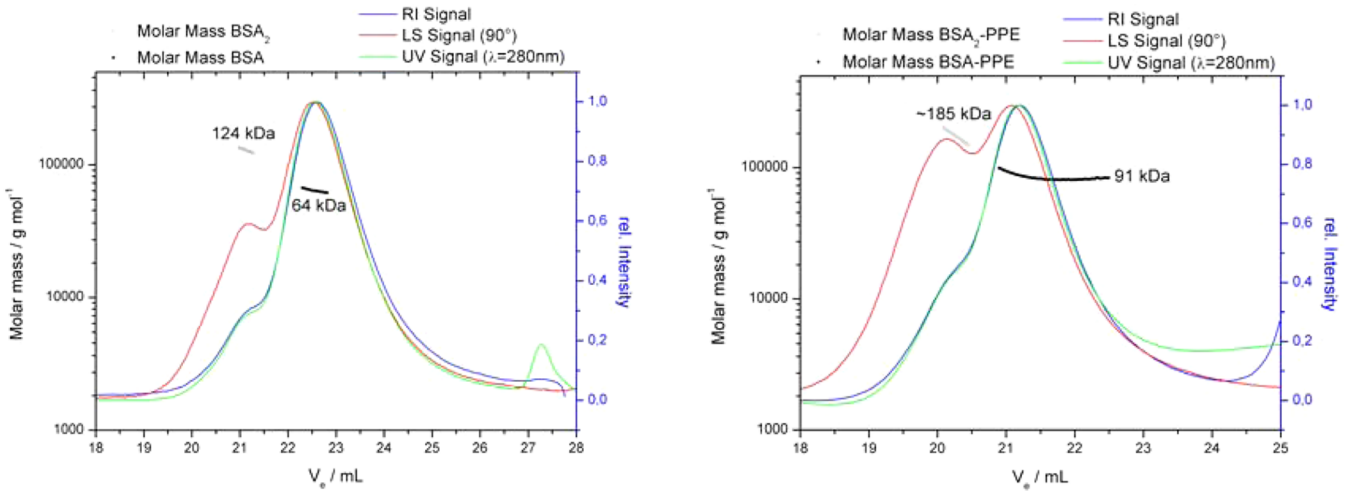

Figure 2. Aqueous SEC traces of BSA (*left*) and the BSA-PPE conjugate (*right*) measured by triple detection (refractive index, light scattering, UV). The molecular weight calculated from LS and RI data is given for the homodimer (124 kDa) and homomonomer (64 kDa) of BSA. After conjugation the molecular weight increases for both species to 185 kDa and 91 kDa, respectively.

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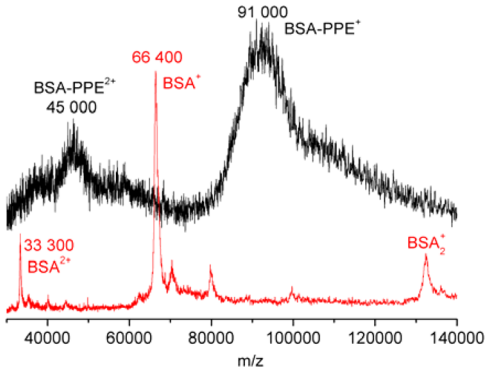

Figure 3. MALDI-ToF MS of unmodified BSA (red) and BSA-PPE conjugate (black). Increasing m/z indicates successful bioconjugation, confirming SEC-MALLS measurements.

sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to visualize the gradual degradation of the PPE-modifier attached to the protein (Figure 4).

The hydrolytic degradation of the BSA-PPE conjugate proceeds faster at elevated pH, as expected for polyesters in general and PPEs in particular. At neutral and acid pH, degradation of the synthetic polymer takes about 3 weeks. The relative molecular weight slowly converges the one of BSA proofing the complete degradability of the PPE-modifier. Accelerated degradation is observed at pH 9.0 within 24 h, because the molecular weight of the cleaved degradation products decreases the hydrodynamic radius of the conjugate dramatically. SDS-PAGE analysis after incubation for 18 days showed a negligible decrease in molecular weight, indicating that the major part of the polymeric modifier was already cleaved. In order to examine the degradation behavior in detail, aqueous SEC-MALLS analysis was performed on the sample incubated at pH 9.0 to determine the absolute molecular weights at different time points (Figure 5).

Continued degradation is detected by SEC-MALLS during prolonged incubation times. The elution volume at the peak maximum increases, which corresponds to a decreasing hydrodynamic radius of the BSA-PPE conjugate during degradation. MALLS allowed calculation of the absolute molecular weight of the degraded conjugate, which is also given in Figure 5. These data confirm that the molecular weight continues to decrease within 8 days until the residual detectable molecular weight is in the range of 65 kDa, which is close to the molecular weight of BSA. After 8 days, the molecular weight does not decrease further, which was also observed in the SDS-PAGE analysis.

In conclusion, hydrolytic degradability of the novel bioconjugates under physiological conditions is assured and confirmed by several valid techniques. This proof-of-principle demonstrates that nontoxic PPEs can be employed for bioconjugation preventing unwanted accumulation of polymer in the body.

Enzymatic Activity. Exploration of PPEs as polymeric modifiers for bioconjugation purposes also involves the conjugation to pharmaceutically relevant enzymes. The enzymatic activity decreases typically after polymer modification due to steric shielding of the active site. This reduction of the activity is typically balanced by longer *in vivo* half-lifes of PEGylated drugs. Drug delivery technologies in general have to cope with the challenges of dose, stability, immunogenicity, and elimination, especially when high dosage applications or chronic administration is desired. Therefore, an enzyme with therapeutic usage was chosen to evaluate the applicability of degradable PPEs for bioconjugation to investigate these issues. Pegloticase (PEGylated porcine-like uricase) is a contemporary

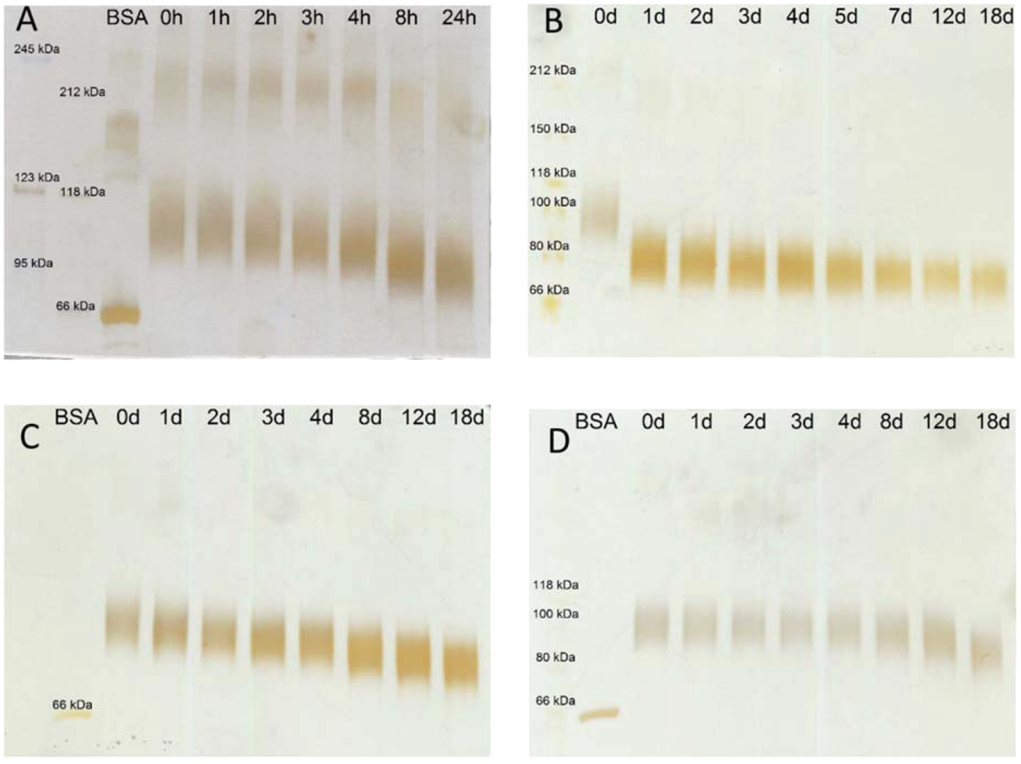

Figure 4. SDS-PAGE visualizing the degradation of BSA-PPE conjugate incubated in aqueous buffer at pH 9.0 (a, b), pH 7.4 (c), and pH 5.0 (d).

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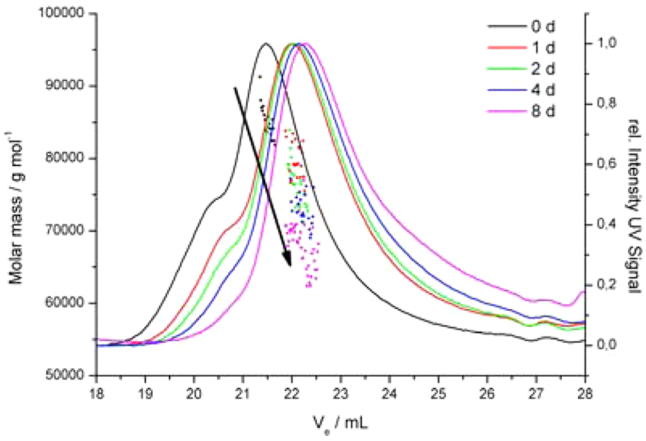

Figure 5. SEC-MALLS analysis of the degradation behavior of the BSA-PPE conjugate incubated at pH 9.0. The molecular weight calculated from MALLS is given as color-coded points representing increasing incubation times. The elugram is represented by the UV signal measured at 280 nm indicating a decrease in hydrodynamic radius (increasing elution volume) with increasing incubation times.

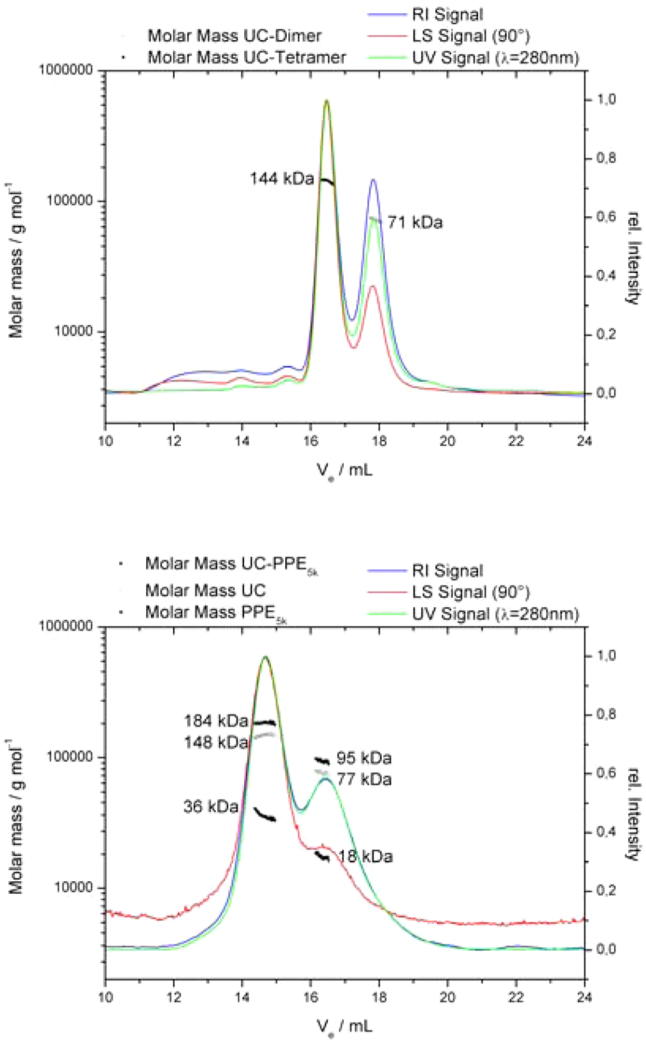

Figure 6. SEC elugrams and molecular weights determined by MALLS of unmodified UC (top) and UC-PPE (bottom).

example of a biologic that is administered for the long-term treatment of severe, treatment-refractory, chronic gout.[24] This

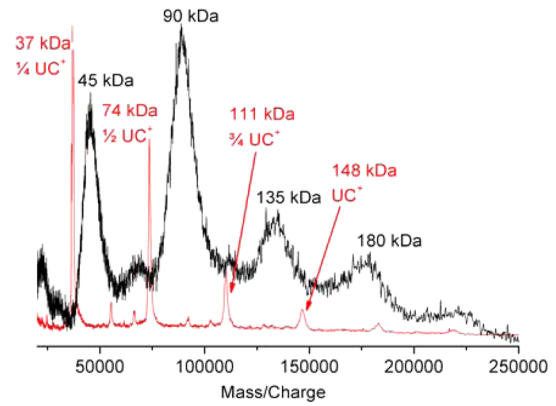

Figure 7. MALDI-ToF MS of UC (red) and UC-PPE (black). UC and its conjugate dissociate during ionization, which gives signals for the monomer (1/4 UC^+), dimer (1/2 UC^+), trimer (3/4 UC^+), and tetramer (UC^+). Increasing mass for all species after conjugation allowed verification of MALLS-SEC measurements.

common arthritis is caused by the deposition of urate crystallites within joints due to hyperuricaemia. The enzyme uricase (urate oxidase, UC) has the ability to convert less-soluble urate to well-soluble allontoin, which can be easily excreted renally. However, missense mutations in the gene encoding the enzyme has led to the absence of UC in humans and some primates.[25] Therefore, PEGylated UC is employed to substitute this deficiency and to help patients in lowering their urate level, preventing arthritic inflammation.

UC exhibits a sophisticated tetrameric form in its natural molecular conformation, which also demonstrates the wide scope of this novel conjugation approach. Conjugates of PMeEP-SC and PEG-SC to UC were prepared in solution. Briefly, a solution of UC was added to the polymer, before purification by centrifugal filtration with Dulbecco's phosphate buffered saline (D-PBS). In this way, excess polymer and NHS were removed completely, with quantitative yield in regards to the protein as determined by UV spectroscopy at 280 nm. In a second purification step, high molecular weight aggregates were removed efficiently by fractionated gel-filtration, lowering the overall yield typically to 50−60% (Supplementary Figure S8).

Characterization of UC-PEG and UC-PPE conjugates was performed by MALLS-SEC and MALDI-ToF MS to determine the molecular weight and the degree of modification. Furthermore, triple detection SEC (online detection of UV absorption, refractive index (RI), and MALLS) allows estimation of the molecular weight of the polymeric modifier attached to the protein. The absolute molecular weight of the conjugate is calculated by the RI and MALLS signal, whereas the UV signal measured at 280 nm is used to calculate the weight contribution of the protein with known extinction coefficient, which was determined beforehand.

Commercially available UC isolated from *Bacillus fastidious* shows superior catalytic capacity compared to fungal UC, but is inactivated in aqueous solutions of low ionic strength.[26] Native intracellular UC is a homotetramer of 144 kDa which dissociates into inactive oligomers (mostly the homodimer) and even the monomer subunit. This dissociation is observed during SEC analysis and is represented by two distinct peaks at 16.5 (homotetramer) and 17.8 mL (homodimer) elution volume (Figure 6a). The molecular weight was calculated from MALLS analysis and is very close to the reported literature value. After conjugation with PEG-SC, the elution

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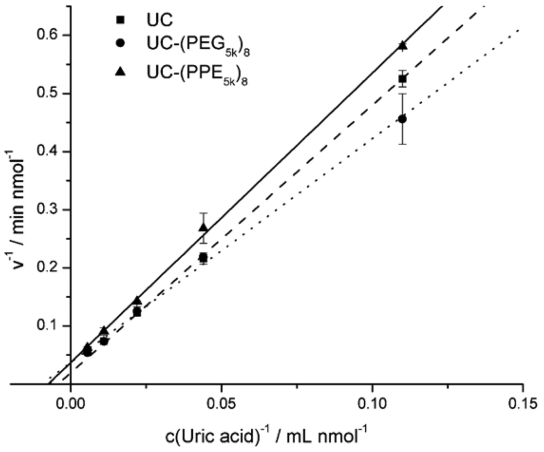

Figure 8. Lineweaver–Burk plot of unmodified UC, PEGylated UC and PPEylated UC. Linear regression allowed calculation of the Michaelis–Menten parameters K_M and v_{max}.

volume decreases to 12.5 mL (Figure S12), indicating a significant increase in the hydrodynamic radius and molecular weight (~190 kDa). Furthermore, dissociation into a PEGylated homodimer was prevented under the conditions employed for analysis, which is attributed to a shielding effect of PEG remaining the subunits in place. In contrast to UC-PEG, the elution volume of the corresponding UC-PPE decreases only to 14.6 (PPEylated homotetramer) and 16.5 mL (PPEylated homodimer), respectively (Figure 6b). Dissociation is not prevented in this case, which might be due to less shielding of the polymeric modifier allowing the subunits to segregate. However, the molecular weight of the UC-PPE conjugate (184 kDa) is comparable to the PEGylated counterpart. The significant difference in the elution volume between the PEGylated and PPEylated enzyme indicates that the PPE does not increase the hydrodynamic volume of the conjugate in the same manner as PEG under these conditions. Both modifiers have a very similar molecular weight and comparable narrow molecular weight distributions. Furthermore, PMeEP is exceptionally well hydrated, which facilitates solubility of the conjugate as it does not show any cloud point temperature in water, similar to PEG.[17] However, it has to be noted, that the chain lengths of both polymers is different as the molecular weight of the repeating units differs by a factor of ca. 3 (MeEP = 122 g mol^{-1}, ethylene oxide = 44 g mol^{-1}), leading to a difference in the overall hydrodynamic radii of the UC-conjugates.

MALDI-ToF MS confirmed the molecular weights calculated from MALLS-SEC. Figure 7 shows the respective mass spectrum of the UC-PPE sample (in black) in comparison to the one of unmodified UC (red). A shift to higher mass is observed for all UC conjugates. In contrast to the SEC measurements above, the intensity of the UC monomer (1/4 UC$^+$) is overrepresented in mass spectrometry, probably due to a higher degree of ionization. The MALDI-ToF mass spectrum of the UC-PEG sample also verifies the MALLS data and furthermore allows distinguishing different conjugation patterns for the PEGylated UC monomer (Supporting Figure S13). The mass difference between the separate signals corresponds to the molecular weight of one PEG chain (5 kDa). Therefore, the average number of PEG chains attached to the tetramer was calculated to be about 8, which proofs the protein conjugate analysis made by triple detection SEC.

Investigation of the residual enzymatic activity of the UC conjugates followed the method described by Mahler.[27] In this assay hydrolysis of uric acid by UC in 50 mM borate buffer (pH 9.0) is monitored by UV at 292 nm. Furthermore, variation of substrate concentration allowed to determine the Michaelis–Menten constant (K_M) and the maximum reaction velocity (v_{max}) at infinite substrate concentration by the method described by Lineweaver and Burk.[28] These characteristic enzymatic constants allow better comparability than to assume a first-order rate equation, which depends on induction periods and other empirical factors.

UC and its conjugates follow conventional Michaelis–Menten kinetics for uric acid as substrate (Figure 8). The values calculated for K_M and v_{max} (Table 1) of the conjugates indicate that the enzymatic activity is lowered after polymer modification (both with PEG or PPE) compared to the native enzyme. As shown above, the number of attached polymer chains to the homotetramer UC and the molecular weight of the modifiers are comparable. Therefore, comparison between the conjugates is justified, if the difference in the hydrodynamic radii of both conjugates is not considered. Both conjugates exhibit similar reaction kinetics at infinite substrate concentration (v_{max}) and similar Michaelis–Menten constants (K_M). No inhibition of the enzyme was observed. The conjugates remained highly active, with a specific activity of ca. 53% relative to the native UC. The catalytic efficiency (v_{max}/K_M) is similar for both conjugates and the native enzyme, which indicates that the substrate (uric acid) diffusion is not limited by the attached modifier and the catalytic center of the enzyme is not hindered. Again, it was shown that PPE is capable of substituting PEG, yielding UC conjugates with similar activities and efficiencies.

CONCLUSIONS

The "PPEylation", i.e., the covalent attachment of poly(phosphoester)s, to enzymes was presented: Covalent conjugation of a degradable, nontoxic poly(phosphoester) (PPE) to two proteins was studied. An amine-reactive poly(ethylene methylphosphonate), PMeEP-SC, was prepared via organobase-catalyzed, living ring-opening polymerization, and subsequent postmodification. Conjugation of the polymer to BSA and UC via urethane bond formation was accomplished in an aqueous solution to produce degradable PPE-conjugates. The conjugates were characterized in detail by MALLS-SEC and MALDI-ToF MS. Degradability in aqueous media was

Table 1. Characterization of Uricase–Polymer Conjugates

sample	MW[a]/kDa	$MW_{Polymer}$[a]/kDa	# chains attached	K_M/nmol mL^{-1}[b]	v_{max}/nmol min^{-1}[b]	v_{max}/K_M/min^{-1}mL^{-1}
UC	144	-	-	235 ± 32	51 ± 7	0.22 ± 0.04
UC-PEG_{5k}	190	42	8	103 ± 16	27 ± 4	0.26 ± 0.06
UC-PPE_{5k}	184	36	7–8	133 ± 20	27 ± 4	0.20 ± 0.04

[a]Determined by MALLS-SEC. [b]Determined by the method described by Lineweaver and Burk.[28]

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Biomacromolecules 2016, 17, 3338–3346

demonstrated for basic, neutral and acid pH. UC conjugates remained the native catalytic efficiency, proving the applicability of this novel polymer-modifier for therapeutic usage. The results together demonstrate that the strategy of conjugating a degradable poly(phosphonate) to UC or other clinically important biologics is valuable as a means for stabilization. During the hydrolysis of the poly(phosphonate), the degradation products prevent accumulation of the synthetic macromolecules in the body and allow for complete renal excretion. This concept will be applied for various proteins and enzymes in the future.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biomac.6b01107.

Additional information, synthetic details, and chemical compound information (PDF)

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Notes

The authors declare no competing financial interest.

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