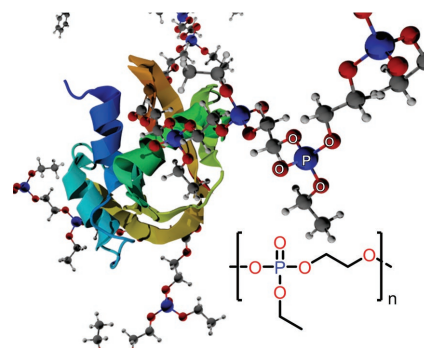


Reversible Bioconjugation: Biodegradable Poly(phosphate)-Protein Conjugates

Tobias Steinbach, Greta Becker, Alina Spiegel, Tamiris Figueiredo, Daniela Russo, Frederik R. Wurm*

Protein-polymer conjugates are widely used to improve the pharmacokinetic properties of therapeutic proteins. Commercially available conjugates employ poly(ethylene glycol) (PEG) as the protective polymer; however, PEG has a number of shortcomings, including non-biodegradability and immunogenicity, that call for the development of alternatives. Here, the synthesis of biodegradable poly(phosphate), that is, poly(ethyl ethylene phosphate) (PEEP), by organo-catalyzed anionic ring-opening polymerization exhibiting dispersity values $\mathcal{D} < 1.3$ is reported. Polymers with molecular weights between 2000 and 33 200 g mol^{-1} are then ω -functionalized with a succinimidyl carbonate group and subsequently conjugated to model proteins. These are the first conjugates based on polyphosphates which degraded upon exposure to phosphodiesterase. As is the case for PEGylated therapeutics, residual *in vitro* activity of the PPEylated conjugates depends on the extent of protein modification. These results suggest that PEEP exhibits the desired properties of a biopolymer for use in next generation, fully degradable drug delivery systems.



1. Introduction

Many biomedical applications employ covalent attachment to synthetic polymers to enhance the efficacy of proteins or other therapeutically active molecules, for

example, hydrophobic drugs.^[1] Hydrophilic polymers such as poly(ethylene glycol) (PEG) improve solubility in aqueous media and provide a steric shield that reduces immunogenicity and increases the effective size of the therapeutic.^[1,2] The resulting hydrodynamic radius (R_h) exceeds the renal filtration limit, delaying clearance from the bloodstream. The “stealth effect” of the PEG layer also prevents recognition by the reticuloendothelial system. This strategy maintains a therapeutically effective concentration over a prolonged period of time, and thus reduces necessary dosage frequencies. The long half-lives accessible by bioconjugation have led to the development of several potent commercially available PEGylated biologics.^[2] PEGylated nanocarriers have also achieved long *in vivo* half-lives.^[3]

Recently, however, PEGylation has raised several concerns. The occurrence of renal tubular vacuolization, caused by the accumulation of PEG in kidney cells, was reported in animal models treated with PEGylated drugs over a prolonged period of time.^[4,5] This observation is attributable to PEG’s non-biodegradability. Furthermore, antibodies against PEG have been detected and shown to increase clearance rates of PEGylated conjugates.^[6] In order

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to address these problems, different technologies have been developed. Reversible PEGylation (rPEGylation) still relies on non-degradable PEG, but ensures cleavage of the polymer chain through a trigger (usually a drop in pH).^[7] However, this technology requires extensive and elaborate coupling chemistry and does not prevent a possible immune response by PEG antibodies. Other polymers have been explored to form pharmacologically active complexes with proteins or drugs.^[1,6,8] Most important are polysaccharides dextran and hydroxyethyl starch (HES), which can be cleaved by enzymes into glucose monomers.^[9] Additionally, synthetic polymers, such as poly(L-lysine), poly(vinyl alcohol), poly(vinylpyrrolidones), poly(oxazolines), and derivatives of poly(acrylic acid) and poly(urethanes) have been proposed for bioconjugation.^[1,10]

Poly(phosphate)s (PPEs) have not been employed for extensive bioconjugation thus far, despite their unique properties and similarity to naturally occurring nucleic and teichoic acids.^[11] This class of polyesters degrades hydrolytically and enzymatically into small molecular weight phosphate esters and alcohols. PPEs prepared by anionic ring-opening polymerization have shown high water solubility and, like PEG, provide immediate access to a terminal hydroxyl functionality that can be modified with a protein-reactive moiety, for example, a succinimidyl carbonate (SC) group.^[12] Here, we describe the synthesis of protein-reactive PPEs and the modification of several proteins (bovine serum albumin (BSA), catalase) by covalent conjugation with a linear PPE, a biodegradable and biocompatible hydrophilic polymer, that is, PPEylation. PPEylation produces fully biodegradable polymer-protein conjugates.^[12] Recently, PPEylated nanoparticles have also been shown to have similar stealth behavior to PEGylated nanocarriers *in vitro*.^[13] The PPEylated protein conjugates exhibit comparable bioactivity to their PEGylated counterparts and exhibit degradation of the protective polymer in aqueous media. This research provides a viable PEG alternative that will influence future conjugation strategies, and presents general implications for the use of biologicals in clinical applications.

2. Experimental Section

2.1. Materials

Solvents were purchased from Acros Organics, Sigma Aldrich, or Fluka and used as received, unless otherwise stated. Phosphorus trichloride was purchased from Sigma Aldrich. Ethanol and ethylene glycol were purchased from Sigma Aldrich and dried before use (distillation from sodium and stored over molecular sieves). Phosphodiesterase I from *Crotalus adamanteus* venom was used for the enzymatic degradation. All other chemicals were ordered from Sigma Aldrich and used as received. 2-Ethoxy-2-oxo-1,3,2-dioxaphospholane (EEP) was synthesized according to literature.^[14,15]

2.2. General Polymerization Procedure

The polymerization was carried out in 25 mL Schlenk tubes. The tube was flamed-dried under reduced pressure, and purged with argon three times prior to use. In a typical polymerization, the monomer, for example, EEP (2.056 g, 13.5 mmol, 33 eq.), was introduced into the tube with a syringe. 2-(Benzyloxy)ethanol (62 mg; 0.41 mmol, 1.0 eq., distilled and stored over molecular sieves 4 Å) was added to the monomer with a syringe. A stock solution of an organic base, for example, 1,5,7-triazabicyclo[4.4.0]undec-5-ene (TBD) (19 mg mL⁻¹, 136 nmol mL⁻¹), was prepared by dissolving the base in dichloromethane (DCM). The polymerization was initiated at -20 °C (or 0 °C, respectively) by addition of 1 mL (0.33 eq.) of the stock solution to the monomer/initiator mixture. After the solution had been stirred at -20 °C for 10 min, the polymerization was terminated using a solution of acetic acid in DCM (100 mg mL⁻¹). The product was purified by repeated precipitation into cold diethyl ether. The desired polymer (1.79 g, 84%) was dried under reduced pressure.

Example of a representative NMR spectrum: PEEP₃₃. ¹H NMR (DMSO-*d*₆): δ 7.40 (m, 5H, Ar), 4.89 (t, 1H, P-O-CH₂-CH₂-OH, *J* 5.5 Hz), 4.52 (s, 2H, Ar-CH₂-O), 4.27–3.27 (m, 196H, O-CH₂-CH₂-O and O-CH₂-CH₃), 1.26 (td, 97H, O-CH₂-CH₃, *J* 7.1, 1.0 Hz). ¹³C NMR (DMSO-*d*₆): δ 128.43 (Ar), 128.38 (Ar), 127.82 (Ar), 68.42 (Ar-CH₂-O), 66.07 (O-CH₂-CH₂-O), 63.74 (O-CH₂-CH₃), 15.82 (O-CH₂-CH₃). ³¹P NMR (DMSO-*d*₆): δ -0.97, -1.12, -1.23.

2.3. Activation of PEEP-OH

2.3.1. Phosgene Activation

The activation with phosgene should be performed in a well-ventilated hood by experienced staff wearing protective clothing!

The respective polymer (1.66 g, 320 μmol, 1 eq.) was dissolved in dry acetonitrile (5 mL) and transferred into a 25 mL Schlenk tube. The polymer was dried by azeotropic removal of the solvent and redissolved in 5.3 mL dry dichloromethane. A 20 wt% solution of phosgene in toluene (1 mL, excess) was transferred into the Schlenk tube. Excess phosgene and solvents were removed at reduced pressure after 12 h (phosgene was inactivated by condensation in isopropyl alcohol). The dry activated polymer was redissolved in a mixture of 700 μL dichloromethane and 1.4 mL toluene. A stock solution of *N*-hydroxysuccinimide (NHS) (110 mg, 0.96 mmol) and dry pyridine (59 mg, 0.75 mmol) in 1.2 mL dichloromethane and 2.4 mL toluene was prepared. The NHS stock solution (1.8 mL, 480 nmol, 1.5 eq.) was added to the activated polymer and stirred overnight. The desired polymer was obtained quantitatively by precipitation in diethyl ether and dried under reduced pressure. The NHS activated polymer, PEEP-SC, was stored at -28 °C until usage.

PEEP₃₃-SC. ¹H NMR (DMSO-*d*₆): δ 7.40 (m, 5H, Ar), 4.52 (s, 2H, Ar-CH₂-O), 4.27–3.27 (m, 214H, O-CH₂-CH₂-O and O-CH₂-CH₃), 2.81 (s, 4H, O=C-CH₂-CH₂-C=O), 1.26 (t, 106H, O-CH₂-CH₃, *J* 7.0 Hz).

2.3.2. Activation with *N,N'*-Disuccinimidyl Carbonate (DSC)

The polymer (1.02 g, 329 μmol, 1 eq.) was dissolved in 6 mL benzene and transferred into a 25 mL Schlenk tube and dried

by lyophilization. A solution of *N,N'*-disuccinimidyl carbonate (85.6 mg, 329 μmol , 1 eq.) and pyridine (26 mg, 329 μmol , 1 eq.) in 5 mL dry acetonitrile was added to the dry polymer and stirred overnight. The activated polymer was obtained by repeated precipitation in diethyl ether and dried under reduced pressure (Yield: 778 mg, 76%). The NHS activated polymer, PEEP-SC, was stored at $-28\text{ }^\circ\text{C}$ until usage.

PEEP₃₀-SC. ^1H NMR (CDCl_3): δ 7.37 (m, 5H, Ar), 5.08 (d, 2H, *J* 6.0 Hz), 4.30–4.00 (m, 176H, $\text{O}-\text{CH}_2-\text{CH}_2-\text{O}$ and $\text{O}-\text{CH}_2-\text{CH}_3$), 2.84 (s, 4H, $\text{O}=\text{C}-\text{CH}_2-\text{CH}_2-\text{C}=\text{O}$), 1.34 (t, 90H, $\text{O}-\text{CH}_2-\text{CH}_3$, *J* 6.0 Hz).

2.3.3. Polymer-Protein Conjugation

The protein (BSA or catalase) was dissolved in PBS at pH 8.0. The respective amount of the NHS-activated polymer (either PEG-SC or PEEP-SC) was added to the protein solution dropwise. The reaction was allowed to stir at room temperature for 4 h and then dialyzed against water ($\text{MWCO} = 50\,000\text{ g mol}^{-1}$) over a period of 24 h. The conjugates were then lyophilized, yields typically quantitative (with respect to protein).

2.3.4. Size-Exclusion Chromatography (SEC)

SEC measurements were performed in DMF (containing 0.25 g L^{-1} of lithium bromide as an additive) with an Agilent 1100 Series as an integrated instrument, including a PSS HEMA column ($10^6/10^5/10^4\text{ g mol}^{-1}$), a UV (254 nm), and a refractive index (RI) detector. Calibration was carried out using poly(ethylene glycol) standards provided by Polymer Standards Service.

2.3.5. Cytotoxicity Assay

The effect of polyphosphates on the viability of a human cervical cancer cell line (HeLa) was measured with a commercial fluorescence assay PrestoBlue (Life Technologies, Germany). The assay is based on the reduction of non-fluorescent resazurin into fluorescent resorufin by metabolic active cells.^[16] HeLa cells were cultured in Dulbecco's modified eagle medium (DMEM), supplemented with 10% FCS, 100 units penicillin, 100 mg mL^{-1} streptomycin, and $2 \times 10^{-3}\text{ M}$ L-glutamine (all from Invitrogen, Germany). Cells were grown in a humidified incubator at $37\text{ }^\circ\text{C}$ and 5% CO_2 . For determination of cell viability, HeLa cells were seeded at a density of 15 000 cells cm^{-2} in 96-well plates (black, opaque-walled, Corning, Netherlands). Polyphosphate was dissolved in sterile water (10 mg mL^{-1} , Ampuwa, pH 7.4, Fresenius Kabi, Germany) and the indicated concentrations were produced by serial dilution in cell culture medium (DMEM, 10% FCS). After 24 h, the culture medium was replaced by the polyphosphate supplemented medium (200 μL , DMEM, 10% FCS) or medium without compound (DMEM, 10% FCS) as a specific control for 100% cell viability. The cells were treated for 48 h and the number of viable cells was determined by the PrestoBlue assay following the manufacturer's instructions. Fluorescence was detected with a plate reader (Infinite M1000, Tecan, Germany) at excitation wavelength 560 nm ($\pm 10\text{ nm}$) and emission wavelength 590 nm ($\pm 10\text{ nm}$) using i-control software (Tecan, Germany). The values

represent the mean \pm SD of six replicates and were plotted relative to the untreated cells.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 8% Tris-HCl gels (Biorad, 1.0 mm, 10 well).

MALDI-ToF mass spectrometry was conducted with a Shimadzu Axima CFR plus MALDI-ToF mass spectrometer equipped with a nitrogen laser at a wavelength of 337 nm and a pulse length of 3 ns. Data processing was performed using Kompact v2.4.3 software. Sinapinic acid was used as a matrix and silver triflate was added to facilitate ionization. Good results were obtained for samples prepared from H_2O /acetonitrile/TFA (50:50:0.005) solution by mixing matrix (10 mg mL^{-1}), polymer-protein conjugate (1 mg mL^{-1}), and salt (10 mg mL^{-1}) in a volume ratio of 1:1:1. A volume of 0.9 μL sample solution was deposited on the MALDI target and allowed to dry at room temperature for 0.5 h prior to the measurement. The samples were measured either in linear or reflective mode.

2.3.6. Catalase Assay

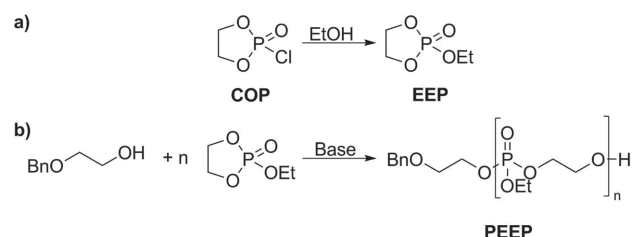
Catalase activity was determined according to literature.^[17] Briefly, 100 μL aliquots of free and conjugated enzyme (0.01 or 0.1 mg mL^{-1}) were added to 0.05% (v/v) of hydrogen peroxide in 0.01 M phosphate buffer pH 7.0. The solution was immediately transferred into a 1 cm cuvette and the hydrolysis of the substrate was measured by UV-vis absorption spectroscopy for 2 min at 240 nm. The residual activity of the conjugates was calculated as reported in literature and the residual activity given in % compared to the unmodified enzyme.

3. Results and Discussion

Poly(ethylene glycol) is frequently used as a synthetic modifier for therapeutic peptides and proteins to enhance the pharmaceutical properties of the biologics. In order to compare PEGylated and PPEylated conjugates, both polymers have been conjugated to model proteins.

3.1. Monomer Synthesis

The anionic ring-opening polymerization (AROP) of phosphates^[18] and phosphonates^[19,20] requires strained five-membered rings, as shown by Penczek and co-workers.^[21–24] Phosphate monomers are easily accessible via the commercially available precursor COP (2-chloro-2-oxo-1,3,2-dioxaphospholane), which can also be prepared in a straightforward manner following literature procedures.^[10,14] Simple condensation of a (functional) alcohol with COP yields the corresponding monomer in one step, as demonstrated in some elegant examples by other groups.^[25–28] In this way, we prepared phosphate monomer bearing a methoxy ethoxy (EEP) group (Scheme 1a), which produces water-soluble polymers; the successful synthesis can be confirmed by ^{31}P NMR spectroscopy with the typical chemical shift of 16.8 ppm (for EEP) for strained cyclic



Scheme 1. a) Preparation of 2-ethoxy-2-oxo-1,3,2-dioxaphospholane. b) Anionic ring-opening polymerization of the monomer initiated by a primary alcohol and catalyzed by an organic base.

phosphoesters. Other substituents, for example, isopropoxy, phenoxy, or higher aliphatic groups, are increasingly hydrophobic and produce polymers with poor water solubility or a temperature-dependent phase transition (e.g., a lower critical solution temperature (LCST))^[29] below body temperature.

3.2. Polymer Synthesis

Controlled AROP of 2-ethoxy-2-oxo-1,3,2-dioxaphospholane was conducted in DCM and catalyzed by TBD (Scheme 1b). Initiator concentration, temperature, and duration of the polymerization were varied depending on the desired molecular weight (Table 1, Scheme 1).

As in our previous studies, 2-(benzyloxy)ethanol was used as an initiator to enable calculation of the molecular weight of the polymer by ¹H NMR spectroscopy.^[13,20,30] Aromatic signals at ≈7.5 ppm were compared with the signals of the polyphosphate backbone at 4 ppm (see the Supporting Information for details). Furthermore, this initiator is a primary aliphatic alcohol, whose reactivity toward the nucleophilic phosphate monomer should be similar to that of the living chain end during the polymerization. ³¹P NMR spectroscopy reveals the efficient

polymerization with the typical chemical shifts of phosphotriesters at 1 ppm.

TBD (pK_a: 26.0 in acetonitrile^[31]) was employed to prepare a library of well-defined PEEP polymers, ranging from ≈2000 to 33 200 g mol⁻¹. Transesterification reactions can be suppressed efficiently at lower temperatures, yielding defined structures with the aromatic initiator in the α-position and a terminal hydroxyl functionality. The terminal hydroxyl gives access to postfunctionalization strategies to prepare protein-reactive polyphosphates.

Bioconjugation to drugs and proteins requires the synthetic polymer to be biocompatible and non-toxic. In order to verify this crucial requirement for the synthesized PEEP polymers, in vitro cytotoxicity against HeLa up to a concentration of 600 μg mL⁻¹ was investigated. During this cytotoxicity assay, metabolic activity was measured as the ATP content of living cells after 48 h of treatment in comparison with untreated cells (Figure S4, Supporting Information, depicts the excellent biocompatibility of PEEP as no adverse effects on the relative cell viability can be observed).

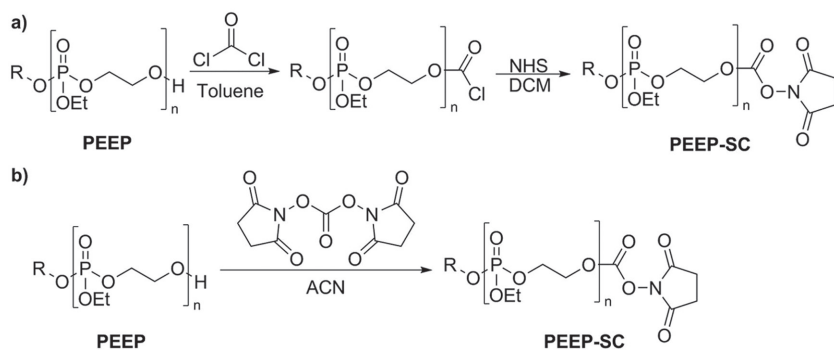
3.3. Functionalization of PEEP

In order to prepare PEEP for conjugation to accessible amine groups (e.g., from lysine residues) on the surface of a protein, the well-known and reliable SC ester group was selected as a linker. Activation of the hydroxyl terminated PEEP was accomplished by following procedures previously described in literature for other polymers (e.g., mPEG-OH,^[32] poly(oxazoline)s^[33]). One possible procedure involves the intermediate formation of a chloroformate via phosgene treatment of the corresponding polymer, followed by a condensation reaction with NHS to produce the desired NHS-activated polymer

Table 1. Characterization data of PEEP prepared in this study.

#	$[M]_0/[I]_0$	$[M]_0$ [mol L ⁻¹]	Time [min]	T [°C]	Conv ^{a)} [%]	M_n^{theo} [g mol ⁻¹]	$M_n^{\text{b)}$ [g mol ⁻¹]	$M_n^{\text{c)}$ [g mol ⁻¹]	$M_w/M_n^{\text{c)}$
PEEP a	13	6.8	10	-20	100	2000	2100	1400	1.19
PEEP b	20	6.8	10	-20	100	3000	3100	1800	1.10
PEEP c	26	6.8	15	-20	100	4000	3900	2200	1.18
PEEP d	33	6.8	10	-20	100	5000	5200	2800	1.14
PEEP e	42	6.6	10	-20	100	6400	6500	3400	1.07
PEEP f	56	6.6	15	-20	100	8500	8700	4000	1.09
PEEP g	66	6.2	5	0	97	9700	10 200	4800	1.53
PEEP h	114	6.5	60	-20	100	17 300	16 600	8500	1.57
PEEP i	227	6.7	60	-20	100	34 500	33 200	18 900	1.55

^{a)}Determined by weight; ^{b)}Number average molecular weight (M_n) determined by ¹H NMR; ^{c)}Molecular weight dispersity (M_w/M_n) determined via SEC in DMF versus PEG standards.



Scheme 2. NHS Activation of PEEP a) via its chloroformate and b) by *N,N'*-disuccinimidyl carbonate.

(Scheme 2a). In order to avoid the usage of phosgene, activation of PEEP-OH is also possible using the commercially available DSC, which is soluble at low concentrations in acetonitrile (Scheme 2b). Both strategies yield the desired amine-reactive PEEP-SC in very good yields.

The successful introduction of the succinimidyl carbonate group was verified by ^1H NMR spectroscopy as the succinimidyl ethylene protons correspond to a distinct signal at 2.8 ppm (see the Supporting Information).^[13] Residual NHS (and NHS released due to hydrolysis) may be present in the corresponding spectra at 2.65 ppm, and does not hamper the subsequent conjugation step. It is essential that the activated polymer is stored under inert gas at low temperatures to prevent unwanted hydrolysis and possible transesterification reactions (best storage conditions are below $-50\text{ }^\circ\text{C}$, at which the polymers remained unaltered for at least 12 months).

The degree of SC substitution was determined by the reaction of a known amount of PEEP-SC with glycylglycine (Gly-Gly). Unreacted Gly-Gly was then reacted with a solution of 2,4,6-trinitrobenzene sulfonic acid (TNBS) to form a UV active complex. The relative concentration was determined spectroscopically by comparison of the absorbance of this solution (A) with that of a standard solution of Gly-Gly treated with TNBS (solution B). The degree of SC substitution of the polymer was calculated using the following formula

$$\text{Activation (\%)} = 100 - \left(\frac{\text{Abs}_A}{\text{Abs}_B} \right) \times 100$$

Typical degrees of activation for PEEP-SC determined by this method are $>90\%$ for PEEP and $\approx 82\%$ for PEG.

3.4. Hydrolysis and Reactivity of PEEP-SC

Investigation of the rates of hydrolysis and aminolysis of the protein-reactive polymer is important because bioconjugation is usually performed in aqueous media at elevated pH. Hydrolysis of the SC group can therefore

compete with aminolysis by the amine residues of the biomolecule, preventing or at least lowering the conjugation efficiency. In order to measure the rate of hydrolysis in pure solvent and the reactivity against free amine groups (Gly-Gly), the activated polymer was dissolved in dioxane and treated with borate buffer 0.1 M, pH 8.5 or a buffered glycyl-glycine solution (1.0 eq.), respectively, at room temperature. The release of *N*-hydroxysuccinimide was monitored by measuring an increase in absorbance at 260 nm. The hydrolysis/aminolysis of the SC group follows a first-order ordinary differential equation because the concentration of the nucleophile can be assumed constant and is therefore part of the decay constant k

$$\frac{dN_{\text{SC}}}{dt} = -k \cdot N_{\text{SC}}$$

where N_{SC} refers to the number of functionalized PEEP molecules. The solution of this differential equation gives

$$N_{\text{SC}} = N_{\text{SC},0} \cdot e^{-kt}$$

Since N , the number of SC groups which have not been hydrolyzed, cannot be determined spectroscopically, the equation can be rewritten to calculate the number of NHS molecules released during the process

$$N_{\text{NHS}} = N_{\text{NHS},\infty} \cdot (1 - e^{-kt})$$

Application of the Beer–Lambert law

$$E_{\lambda} = \epsilon_{\lambda} \cdot c \cdot d \sim N$$

gives the following equation

$$E_{260\text{nm}} = E_{260\text{nm},\text{max}} \cdot (1 - e^{-kt})$$

This gives a linear relationship after rearrangement which allows for the determination of k (and an integration constant A) and therefore the half-life $t_{1/2}$

$$\ln \left(1 - \frac{E_{260\text{nm}}}{E_{260\text{nm},\text{max}}} \right) = k \cdot t + A$$

$$t_{1/2} = \frac{\ln 2}{k}$$

The kinetic investigation of protein-reactive PEEP-SC revealed values comparable to those measured for established PEG-SC derivatives that are typically

■ **Table 2.** Hydrolysis and reactivity rates of PEEP-SC and PEG-SC.

#	$t(\text{hyd})_{1/2}^{\text{a)}$ [s]	$t(\text{gly})_{1/2}^{\text{b)}$ [s]
mPEG ₁₁₃ -SC	375	245
PEEP ₁₂₀ -SC	400	180

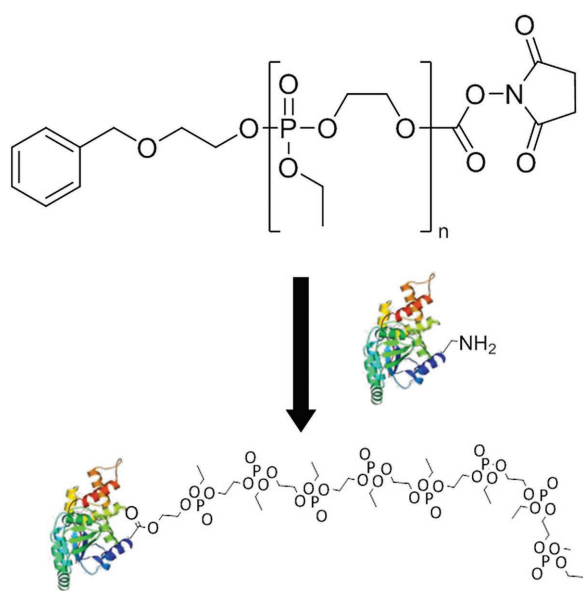
^{a)}Hydrolysis at pH 8.5; ^{b)}Reactivity with Gly-Gly at pH 8.5.

employed for bioconjugation. The reaction in the presence of glycyl-glycine was twice as fast as the corresponding hydrolysis (Table 2). This acceleration can only be attributed to aminolysis by the amine nucleophiles of the model peptide. The effect of the alkaline aqueous environment on the premature release of NHS cannot be ruled out, but does not seem to prevent efficient conjugation to available amines.

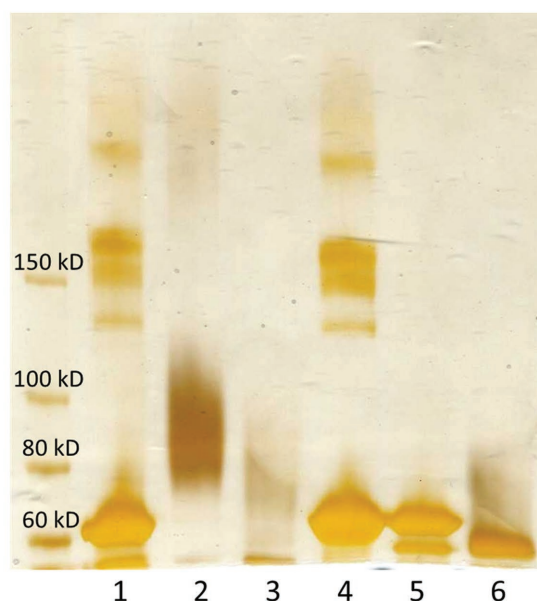
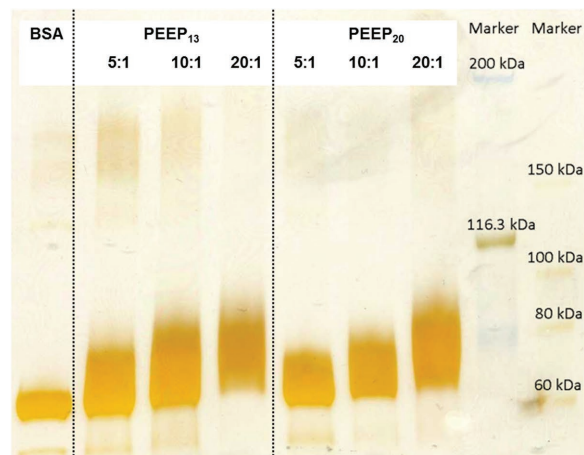
3.5. Bioconjugation

The protein-reactive polyphosphates were linked to the amine residues of several model proteins. BSA acts as a well-established model protein for both for conjugation and degradation as it can be easily monitored by various methods. Different amounts of NHS-activated PEEP polymer with molecular weights 2000 and 3000 g mol⁻¹ were coupled to BSA (molar ratio PEEP:BSA 1:5, 1:10, 1:20, Scheme 3).

In addition, SDS-PAGE gel electrophoresis reveals an upward shift of the apparent molecular weight band corresponding to BSA in accordance with the PEEP:BSA ratio utilized in the conjugation reaction (Figure S5, Supporting



■ **Scheme 3.** PPEylation of lysine residues of proteins via NHS-activated PEEP.



■ **Figure 1.** (Top) SDS-PAGE of PPE-BSA conjugates. (Bottom) SDS-PAGE of degradation of PPE-BSA conjugates: (1) BSA untreated, (2) conjugate, (3) conjugate after treatment with phosphodiesterase, (4) BSA in PBS pH 8.5, (5) BSA + phosphodiesterase, and (6) phosphodiesterase.

Information, and Figure 1). Degradation of the polymer-protein conjugates by phosphatase is also depicted by SDS-PAGE (Figure 1, lane 3).

BSA acts as a convenient model protein; however, it is important to understand the effect of polymer conjugation on enzymatic activity. We selected catalase as a model enzyme, which was modified with one or two equivalents of PEEP (with respect to the available lysine groups, PEEP with a molecular weight 3000 g mol⁻¹) and tested for residual enzymatic activity. PEGylated enzymes typically exhibit reduced enzymatic activity, compared to their unmodified counterparts.^[34] Both PPEylated conjugates showed reduced enzymatic activity of 23%

(PEEP₂₀:catalase 1:1) and 18% (PEEP₂₀:catalase 2:1), respectively, similar to that of PEGylated analogues with 22% (PEG₄₄ catalase 1:1) and 16% (PEG₄₄ catalase 2:1) that have also been reported in literature.^[33] This suggests that PEEP is a potential biodegradable alternative to well-known PEGylation strategies.

It is also important to study the impact of PPEylation on the structure and folding of the protein, since changes in structure can negatively influence therapeutic activity. To this end, measurements of protein secondary and tertiary structures, as affected by the presence of polymer chains linked to the surface, were performed at room temperature using circular dichroism and fluorescence spectroscopy. PPEylated BSA with protein:polymer ratios of 1:5, 1:10, and 1:20 were analyzed (PEEP₂₀ with $M_n = 3000 \text{ g mol}^{-1}$).

Circular dichroism measurements were performed in the *far* (180–260 nm) and *near* (250–350 nm) UV region to investigate secondary and tertiary structure, respectively (Figure 2). BSA concentration was kept constant

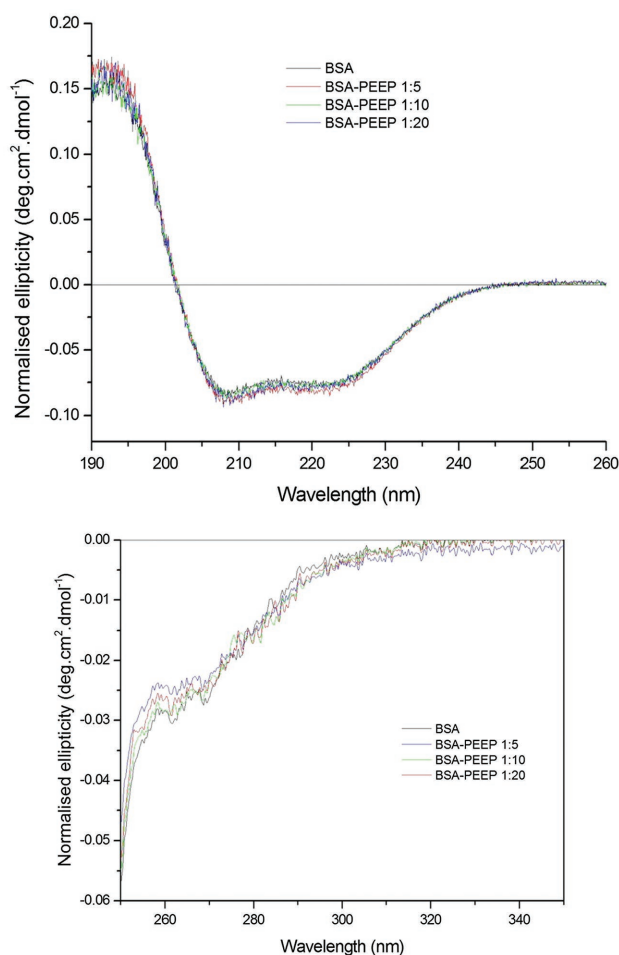


Figure 2. (Top) Near UV-CD spectra of native BSA and BSA-PEEP conjugates at room temperature. (Bottom) Far UV-CD spectra of native BSA and BSA-PEEP conjugates at room temperature.

among test samples of the investigated conjugates ($c = 0.18 \text{ mg mL}^{-1}$ or $2.7 \times 10^{-6} \text{ M}$ for the far-UV CD and 2 mg mL^{-1} for the near-UV CD). The far-UV CD spectra of native BSA and all conjugated BSA compounds superimpose, suggesting that PPEylation does not significantly affect the secondary structure of the protein, even when up to 20 polymer chains are attached to the surface. Furthermore, the similarity of the near-UV CD spectra of native BSA and its conjugates indicates that tertiary structure also remains unaffected after conjugation. There are small deviations in the region 255–270 nm, but none significant enough to suggest that the native structure of the protein was altered.

Further investigations of tertiary structure were performed with standard fluorescence emission measurements (Figure 3). BSA concentrations were kept constant among test samples (0.046 mg mL^{-1}) and emission spectra were recorded at room temperature between 280 and 550 nm after excitation at 280 nm. The spectra show that PPEylation produces a blue shift, as a function of polymer content, of the maximum emission wavelength. As the amount of polymer attached to the protein increases, fluorescence intensity decreases and the blue shift becomes more pronounced. This shift toward lower wavelengths suggests a change in the environment around the fluorophores in the protein rather than an unfolding process (red shift). The screening effect of a shell-like arrangement of polymer at the protein surface likely explains the observed behavior.

Finally, to complete our characterization of BSA-PEEP, we performed static and dynamic light scattering measurements to determine the hydrodynamic radius (R_H), polydispersity index, and diffusion coefficient of a conjugate. Measurements were conducted using 1 mg mL^{-1}

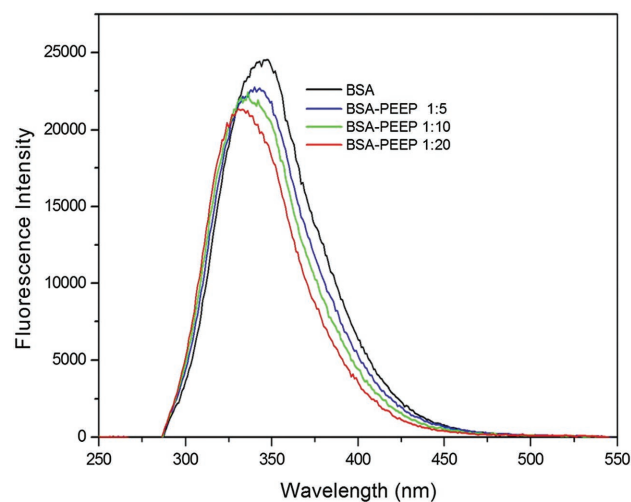
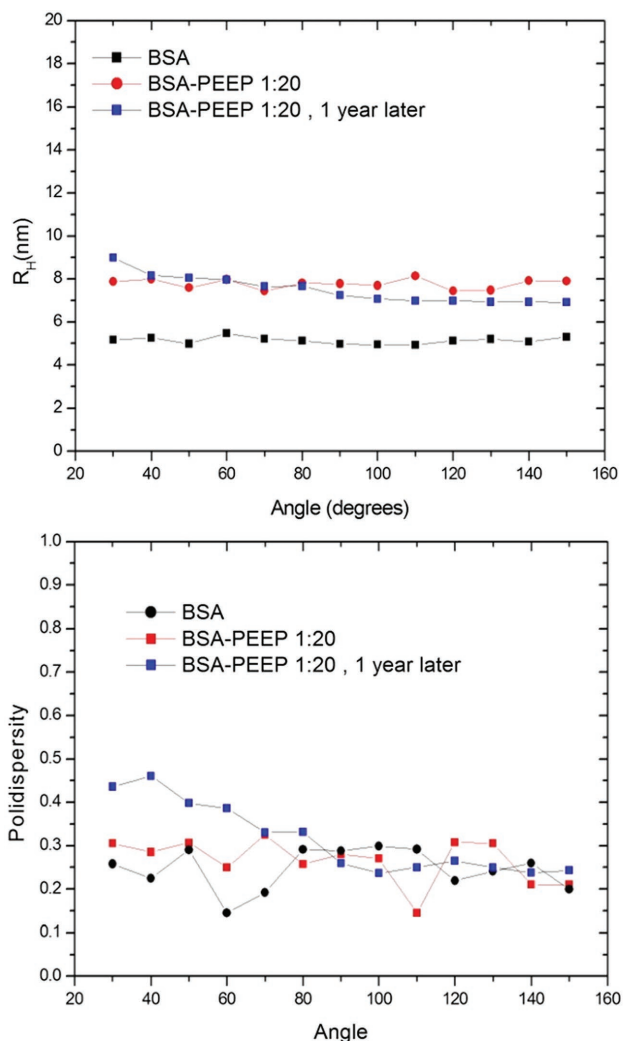


Figure 3. Emission fluorescence spectra of native BSA and BSA-PEEP conjugates, as recorded at room temperature.



■ Figure 4. DLS data on BSA and BSA-PPE conjugates.

aqueous solutions (in BSA content) of BSA and 1:20 BSA-PEEP₂₀ over the range of scattering angles between 30° and 150° at room temperature. Measurements at multiple angles allowed for the detection of potential aggregates. Figure 4 plots hydrodynamic radius and polydispersity index as a function of scattering angle; consistent with literature we find that the R_H of BSA is ≈ 5 nm, while the conjugated BSA-PEEP 1:20 has a higher R_H , equal to 8 nm, which is a typical value for PEGylated proteins. Given the CD and fluorescence results, which establish that the protein remains in its native state after conjugation, these results imply that a 3 nm polymer shell is created around the protein. Continued investigations will include a detailed analysis of polymer configuration performed with more insightful techniques. For both BSA and BSA-PEEP, R_H is independent of scattering angle, indicating that there are no aggregates, and average polydispersity is 25%.

Diffusion coefficients were calculated based on static light scattering measurements and found to be equal to $0.5 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ for native BSA and $0.31 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ for the conjugated protein. The BSA diffusion coefficient is consistent with the literature value ($0.6 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$).^[35] The lower diffusion coefficient of the conjugate reflects its increased size and mass relative to unmodified protein, and further supports the absence of aggregation. Additionally, storage of the fully biodegradable conjugate was briefly investigated by light scattering (see Figure 4). After one year of storage at 4 °C, no aggregation was observed. Additional long-term storage analyses are underway.

4. Conclusions

In this report, we describe the first fully degradable polyphosphate (PEEP)-protein conjugates. Several well-defined, protein reactive polyphosphates were synthesized and coupled by NHS-chemistry to model proteins catalase and bovine serum albumin. Degradation of the polymer was confirmed by SDS-PAGE analysis, and the resulting PPEylated protein conjugates exhibited enzymatic activity comparable to that of their PEGylated counterparts. These results together demonstrate that conjugation to degradable poly(phosphate)s is a valuable strategy for the stabilization of clinically important proteins. As the polymer degrades, enzymatically and hydrolytically, the modified protein regains its activity, while cleavage of the polymer into unobjectionable products prevents the accumulation of the synthetic macromolecule in the cells.

Supporting Information

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

Acknowledgements: F.R.W. thanks the Deutsche Forschungsgemeinschaft (DFG, WU 750/6-1) for funding. T.S. and F.R.W. are grateful to the Max Planck Graduate Center with the Johannes Gutenberg-Universität Mainz (MPGC) for support. T.S. is a recipient of a fellowship through funding of the Excellence Initiative (DFG/GSC 266) in the context of the graduate school of excellence "MAINZ" (Materials Science in Mainz). This work used the platforms of the Grenoble Instruct center (ISBG; UMS 3518 CNRS-CEA-UJF-EMBL) with support from FRISBI (ANR-10-INSB-05-02) and GRAL (ANR-10-LABX-49-01) within the Grenoble Partnership for Structural Biology (PSB).

Received: September 1, 2016; Revised: October 9, 2016;
Published online: ; DOI: 10.1002/mabi.201600377

Keywords: biodegradable polymer; PEGylation; poly(phosphoester)s; protein-polymer conjugates

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