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Fully Degradable Hydrophilic Polyals for Protein Modification

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Abstract

Modification of proteins with hydrophilic polymers is an effective strategy for regulation of protein pharmacokinetics. However, conjugates of slowly or nonbiodegradable materials, such as poly(ethylene glycol), are known to cause long-lasting cell vacuolization, in particular in renal epithelium. Conjugates of more degradable polymers, e.g., polysaccharides, have a significant risk of immunotoxicity. Polymers that combine complete degradability, long circulation in vivo, and low immuno and chemical toxicity would be most beneficial as protein conjugate components. This study explores new fully biodegradable hydrophilic polymers, hydrophilic polyals. They are nontoxic, stable at physiological conditions, and undergo proton-catalyzed hydrolysis at lysosomal pH. The model enzyme–polyal conjugates were prepared with 61–98% yield using conventional and novel conjugation techniques and retained 90–95% of specific activity. The model conjugates showed a significant prolongation of protein circulation in rodents, with a 5-fold reduction in the renal accumulation. The data suggests that hydrophilic polyals may be useful in designing protein conjugates with improved properties.

Introduction

Protein modification with water soluble polymers is a widely used strategy for regulation of protein pharmacokinetics. In view of the increasing number of protein drug candidates, especially truncated proteins and unglycosylated nonmammalian expression products, the significance of this strategy is expected to grow.

Selection of an optimal polymer component is an important step in the development of macromolecular therapeutics. To date, the choice of materials remains limited; none of the available materials combines all desirable features, such as negligible reactivity with biological milieu, low immuno and chemical toxicity, complete biodegradability of the main chain, and sufficient technological flexibility. This provides a strong rationale for developing new polymers with improved pharmaceutical qualities.

Biodegradation of macromolecular therapeutics is an important but incompletely studied issue, even for most widely used polymers. For example, there is a potential risk that extended clinical use of conjugates containing non- or slowly biodegradable polymer

fragments can lead to long-term cell vacuolization¹ and overload, development of lysosomal disease syndrome,² and, at higher doses, to other pathological metabolic alterations.³ The predominant clearance route of relatively large (>7–8 nm) long circulating conjugates, regardless of the size of the polymer component, is through uptake by cells (mostly in RES, but also in other tissues) followed by intracellular degradation and metabolization. Reducing the molecular weight of the polymer component, e.g., to 30–40 kDa, which has been used to enable renal clearance of small molecule drug conjugates,⁴ is not a feasible solution for protein conjugates or other large constructs. Conjugates degrading upon cell uptake with release of smaller but still nonbiodegradable fragments, such as PEG telomers with degradable linkages between PEG blocks,⁵ would also be unlikely to fully solve the problem, because no efficient cellular mechanisms to transport such fragments back to the extracellular space have been identified. Development of completely biodegradable polymers, preferably degrading with formation of nontoxic, readily clearable, or metabolizable products, appears to be the only radical solution of the long-term intracellular deposition problem. The type of protein–polymer linkage and the degree of polymer modification can also alter both conjugate degradability and biological properties.⁶ This necessitates combining polymer backbone structures and conjugation strategies that would not interfere with biological functions of the protein component or (where applicable) would not adversely alter protein properties upon release from the conjugate. A combination of a macromolecular material and a cross-linking reagent that ensures sufficient conjugate stability in the normal extracellular environment and an acceptable rate of conjugate disintegration upon uptake by cells would be most beneficial.

Hydrophilic fully degradable polyals, e.g., poly[1-hydroxymethylethylene hydroxymethyl-formal] (PHF), were developed in our laboratory as acyclic mimetics of polysaccharides.^{7,8} These materials, which can be prepared synthetically and by exhaustive lateral cleavage of some polysaccharides, were shown to be nonbioreactive, nontoxic, fully degradable, and thus potentially feasible in various pharmaceutical applications.^{9–11} Polyals contain pH-sensitive acetal groups within the main chain, which provides the desired combination of polymer stability at the extracellular physiological pH = 7–7.5 and destabilization in acidic environments characteristic for the intracellular vesicular compartments where polymers end up upon uptake by cells (endocytosis, phagocytosis).²⁸

This report further expands the scope of potential applications for hydrophilic polyals and demonstrates the suitability of these materials for preparation of fully degradable protein conjugates with preservation of protein functionality. A hydrophilic polycetal (PHF) is used to obtain and characterize conjugates of well-known model proteases, trypsin and R-chymotrypsin. Conjugation techniques include the use of new bifunctional coupling reagents containing an aminoxy (*O*-hydroxylamino) group; these reagents were also developed in our laboratory and specifically tailored for conjugations involving aldehyde-bearing molecular modules in aqueous media. The main model protein of this study, trypsin, was selected as a relatively small protein with readily measurable activity and fast blood clearance, well characterized in immobilization reactions involving various soluble and solid carriers and conjugation techniques.

Experimental Section

Materials

Bovine pancreatic trypsin (EC 3.4.21.4) Type III, α -chymotrypsin (EC 3.4.21.1) Type II, *N*- α -benzoyl-L-arginine ethyl ester (BAEE), acetyltyrosine ethyl ester (ATEE), and dextran B-512, $M_n = 188\,000$ Da (M_n : number-average molecular weight) were obtained from Sigma Chemical Company (St Louis, MO). Sodium borohydride, sodium cyanoborohydride, sodium metaperiodate, 1-[3-(dimethylamino)propyl-3-ethylcarbodiimide hydrochloride (EDC), diethylenetriaminepentaacetic acid (DTPA), 4-(dimethylamino)pyridine (DMAP), and succinic anhydride were from Aldrich (St Louis, MO). [^{111}In]InCl₃ was from Perkin-Elmer Life Sciences (Boston, MA). Anhydrous pyridine, ethyl alcohol, and other solvents were obtained from Sigma-Aldrich and used without further purification.

Equipment and Methods

Size exclusion chromatography in aqueous media was carried out using a Varian-Prostar HPLC system equipped with a BIO-RAD model 1755 refractive index detector and an LDC/Milton Roy Spectro-Monitor 3000 UV detector. HPSEC columns, Biosil SEC-125 and Biosil SEC-400 (BIO-RAD), and a low pressure Superose-6 column (Pharmacia), were used for studying molecular weights and molecular weight distributions of polymers and polymer–protein conjugates. SEC column calibration was performed using protein standards and broad molecular weight dextran standards. Unless otherwise stated, elution was performed isocratically in 50 mM pH = 7.0 phosphate buffer with 0.9% NaCl. ^1H and ^{13}C NMR were carried out on Varian Mercury-300, Bruker DPX-300, and Bruker Aspect 3000 NMR spectrometers using the solvent peak as the reference standard. The molecular weights were expressed as *effective* M_w and M_n (the weight-averaged and number-averaged molecular weights as defined for linear polymers). The conjugate sizes are mostly defined by the polymer chain(s) present in the conjugate; the model protein globule is small as compared to the hydrophilic polymer coil and does not contribute much to the hydrodynamic diameter. Therefore, this approximation closely corresponds to the total molecular weight of the polymer chain(s) present in the conjugate molecule.

A Cary 300Bio UV–vis spectrophotometer equipped with a Peltier-thermostated multicell block was used for spectroscopic measurements and enzyme kinetics studies. Radioactivity measurements were carried out using a Wallac Wizard 1480 gamma counter (Perkin-Elmer). Gamma scintigraphy was performed using an Ohio Nuclear gamma camera with a medium energy collimator.

Polymer Synthesis

PHF is a semisynthetic acyclic polyacetal prepared via complete lateral cleavage of dextran B-512 with periodate. Dextran B512, a product of *Leuconostoc mesenteroides* strain B-512, is a nearly linear (1 $\rightarrow$ 6)-poly- α -D-glucose with ca. 5% (1 $\rightarrow$ 3; β) branching, of which 95% are only one or two residues long.¹² Periodate oxidation of (1 $\rightarrow$ 6)-polyglycoside in controlled conditions starts with breaking up either the C2–C3 or C3–C4 bond, resulting in the formation of dialdehydes IIa and IIb.¹³ The slower oxidation stage, cleavage of C3, leads to dialdehyde III (Scheme 1).

Borohydride reduction of aldehyde groups of dialdehydes IIa, IIb, and III gives polyals with pendant hydroxymethyl groups IV and (from IIa and IIb) vicinal glycol groups VI. Control of the oxidizer/substrate stoichiometry and reaction conditions enables generation of polymers with a desirable number of vicinal diol groups, which can be subsequently used as selective reactive sites for further polymer modification and conjugation. Both PHF- and PHF-diols with vicinal diol content ranging from 2% to 20% (mol) were prepared and used in this study.

Poly-1-hydroxymethylethylene Hydroxymethyl-formal, IV (PHF)

PHF was prepared via exhaustive lateral cleavage of carbohydrate rings by periodate oxidation. Dextran (in most syntheses, $M_n = 188$ kDa (15.15 g, 93.4 μ mol by glucopyranoside) was dissolved in 300 mL of deionized water. Dextran solution was treated with 47.95 g (224.2 mmol) of sodium metaperiodate dissolved in 350 mL of deionized water at 0–5 °C in a light-protected glass reactor for 3 h. The precipitated sodium iodate was removed by filtering the reaction mixture (1 μ m glass filter). The pH of the filtrate was adjusted to 8.0 with 5 N NaOH, and the resultant solution was treated with sodium borohydride (7.4 g, 200 mmol, dissolved in 100 mL of deionized water) for 2 h. Then, the pH was adjusted to approximately 6.5 with 1 N HCl. The obtained macromolecular product was desalted and concentrated on a CH2PR flow dialysis system (Amicon, Beverly, MA) equipped with a hollow fiber cartridge, cutoff 30 kDa, by passing approximately 6 volumes of deionized water through the polymer solution. Alternatively, the product was purified on a Sephadex G-25 preparative column using deionized water as an eluent. PHF was recovered from aqueous solutions by lyophilization. Average polymer yields ranged from 70% to 80%. SEC analysis of a typical PHF prepared from dextran 188 kDa showed peak molecular weight at 130 kDa, $M_n = 92$ kDa, and polydispersity index $PDI = M_w/M_n = 2.5$. The structures of all obtained polymers, as examined by ^{13}C and ^1H NMR, were consistent with the expected acyclic polyacetal structure.^{10,28}

PHF-glycol

PHF-glycol was prepared by controlled dextran cleavage that was stopped at stage II, with subsequent reduction of intermediates (IIa, IIb). The starting glucopyranoside/periodate molar ratio was 1:1.90. The presence of PHF-diol structure VI in the resultant polymers was determined by ^1H NMR spectroscopy in DMSO- d_6 /D $_2$ O (95:5 v/v), which showed that the C1-H signal at δ 4.62 (t, $J = 5.2$ Hz) is specific for IV and the signal of the C1 acetal hydrogen at δ 4.49 (d, $J = 5.2$ Hz) is characteristic for structure V.²⁴ At the same time, no C4-H signals at δ 3.10–3.20 (m) were registered, indicating the absence of C3–C4 diols (reduced IIb). The amount of PHF-diol structures (V), as determined by NMR, was approximately 2%. SEC analysis has shown no substantial difference between the molecular weight distributions of the starting dextran and the resultant PHF-glycol. The product was isolated as described above.

PHF-Succinate (PHF-SA)

PHF (100 mg), succinic anhydride (7.5 mg, 0.075 mmol), and DMAP (1.2 mg, 0.01 mmol) were dissolved in 5 mL of anhydrous pyridine. After 18 h of agitation at 40 °C, pyridine was

removed in vacuum. The residue was suspended in deionized water, and the pH was adjusted to 7.0 by addition of 1 N NaOH. The succinylated PHF was purified on a Sephadex G-25 column with deionized water as an eluent and recovered from aqueous solution via lyophilization. The succinic acid (carboxyl) content, as determined by potentiometric titration, was 11.3% (mol/mol of monomer). The ^1H NMR spectrum of the polymer (D_2O) contained signals characteristic for methylene protons of succinic acid ester at δ 2.62 (t) and δ 2.46 (t).

Aminoxy (O-Hydroxylamino) Reagents

Two types of aminoxy reagents were developed and tested, each containing a protected aminoxy group and either a maleimide group for thiol modification (*N*-(5-(2,5-dioxo-2,5-dihydro-pyrrol-1-yl)-5-oxo-hexenyloxy)-acetimidic acid ethyl ester, VII), or

the *N*-hydroxysuccinimide ester group for amino group modification (*N*-(3-(3-(2,5-dioxo-pyrrol-1-yl)-propionylamino)-2-hydroxy-propoxy)-acetimidic acid ethyl ester, VIII). The

aminoxy group in either reagent can be deprotected in situ in mild acidic conditions and used for selective condensations with aldehyde and ketone groups resulting in oxime bonds.

Reagent VII was prepared in three steps. First, ethyl-(*N*-hydroxyacetimidate) was reacted with 1.1 equiv of epichlorohydrin in acetone in the presence of 2 equiv of NaOH (refluxing acetone, continuous addition of NaOH over 5 h). The reaction mixture was filtered to remove NaCl, acetone was evaporated under reduced pressure, and the resultant epoxide IX was isolated by vacuum distillation. Then, IX

was reacted with a 10-fold excess of ammonia in methanol (48 h, ambient temperature). Methanol was removed under reduced pressure, then the resultant amine X was isolated by vacuum distillation. Finally, X was reacted with 3-male-imidopropionic acid in the presence

of DCC in butanol. The product (VII) was purified chromatographically (chloroform/ SiO₂), and the solvent was removed under vacuum. The product was obtained as a colorless oil which crystallized after one month of storage at –8 °C. The structure was verified by NMR.

Reagent VIII was prepared in two steps. First, ethyl-(*N*-hydroxyacetimidate) was reacted with 6-bromohexanoic acid in methanol, in the presence of NaOH (slow addition), at 40 °C overnight. The product (XI) was extracted with ether and

purified chromatographically (chloroform/SiO₂). Then, acid XI was reacted with *N*-hydroxysuccinimide in the presence of DCC in methylenechloride (ambient temperature, overnight). The reaction mixture was filtered, extracted with ether, washed with water, and dried over anhydrous MgSO₄. The product (VIII) was isolated chromatographically (chloroform/SiO₂), dried in vacuum, and obtained as a colorless oil that crystallized after ca. 2 months of storage at –8 °C. The structure was verified by NMR.

Protein Conjugates

Protein conjugates with PHF-SA (CII, C-VIII, Table 1) were prepared via EDC-mediated coupling. PHF-diol conjugates were prepared via diol conversion into aldehyde with subsequent reductive amination¹⁴ (C-I, Table 1) or, alternatively, nonreductive amination utilizing aminoxy (*O*-hydroxylamino) containing bifunctional reagents developed in our laboratory (C-III–C-VII, C-IX, Table 1). Nonreductive coupling of the aminoxy coupling reagents, or their unprotected analogues, with carbonyl containing compounds was conducted at pH 3–4 to preserve the *N*-oxysuccinimido- and maleimido- functions for the subsequent stage of protein coupling. Representative procedures illustrating the process of PHF conjugation are given below for trypsin.

PHF–Trypsin Conjugates, C-I (Reductive Amination)

The solution of PHF-diol with $M_n = 150$ kDa (200 mg) and diol content of 10% (mol/mol of monomer) was dissolved in 2 mL of deionized water and combined on ice with 30.1 mg (0.14 mmol) of NaIO₄ in 0.25 mL of deionized water. After a 1 h incubation, the activated polymer was combined with 31.8 mg of trypsin in 6.0 mL of 0.1 M phosphate buffer, pH 5.5, and 18 mg (0.29 mmol) sodium cyanoborohydride, incubated on ice for 1 h, then at 8 °C for 18 h. The low pH was used to reduce the reactivity of amines and thus suppress cross-linking. The macromolecular product was desalted by gel filtration on a Sephadex G25 column equilibrated with deionized water and separated from unreacted trypsin on a Superose-6 column. Trypsin conversion to conjugate (conjugation efficacy) was 61% (HPSEC BioSil-125, detected by UV at 280 nm).

PHF-SA-Trypsin Conjugates, C-II

PHF-SA solution with $M_n = 176$ kDa, 100 mg in 2.0 mL of deionized water, was combined with 3.0 mL of 5.0 mg/mL trypsin solution in 0.1 M phosphate buffer, pH 7.4. Then, EDC (20 mg) was added to the reaction mixture in 500 μ L of cold (0–5° C) deionized water. The

conjugation efficacy after 3 h of incubation, according to HPLC (UV at 280 nm), was 97%. The reaction mixture was separated from low molecular weight components and concentrated to approximately 10 mg/mL on a PM-30 ultrafiltration membrane in 0.05 M PBS, pH 7.0. The conjugate was separated from unbound trypsin on a Superose-6 column using 0.5 M PBS, pH 7.0, as a running buffer. The resultant conjugate was aliquoted and stored frozen at -40°C . SEC analysis of this conjugate gave $M_n = 245$ kDa, PI = 1.8, and peak polymer MW = 260 kDa. The amount of trypsin per conjugate, as estimated by HPLC and measured photometrically at 280 nm, was 10.7% w/w.

PHF Conjugation with Aminoxy Cross-Linkers

The reaction conditions for conjugation between aminoxy-containing cross-linkers (VII and VIII) and aldehyde-containing polymers were optimized using a model reaction between glycolic aldehyde and VII. The course of the reaction was monitored by the increase of optical density at 230 nm. It was found that in aqueous media at pH 3.0–4.5 a stoichiometric mixture of 0.01 M aldehyde and 0.01 M VII quantitatively converts to oxime within 5 min. The degree of PHF-diol modification with oxime linker is easily controlled by varying the PHF-diol/oxidizer ratio, i.e., by controlling the number of aldehyde groups in PHF, which are subsequently quantitatively conjugated with an aminoxy reagent.

PHF–AO(NHS)–Trypsin Conjugates, C-III

The solution of PHF-diol with $M_n = 150$ kDa (200 mg) and diol content of 2% (mol/mol of monomer) was dissolved in 2 mL of deionized water and combined on ice with 15 mg (0.07 mmol) of NaIO_4 in 0.125 mL of deionized water. After a 1 h incubation, the product was desalted by gel filtration on Sephadex G-25, using deionized water as an eluent. The obtained solution was diluted with 3.0 mL of ethyl alcohol and combined with 92.6 mg of VIII in 2 mL of ethanol. The pH was adjusted to 3.0 with 1 M NaHSO_4 , and the mixture was agitated for 2 h on ice. The pH was adjusted to 7, and the product was desalted on Sephadex G-25. The obtained product was combined with 32 mg of trypsin dissolved in phosphate buffer (pH 8.6), and the mixture was incubated on ice for 3 h. HPSEC (UV at 280 nm) analysis of the reaction mixture showed 98% trypsin conversion to conjugate. The reaction mixture was desalted and concentrated to approximately 10 mg/mL on a PM-30 ultrafiltration membrane in 0.05 M PBS, pH 7.0. The conjugate was separated from unbound trypsin on a Superose-6 column (Pharmacia) with 0.5 M PBS, pH 7.0, as a running buffer. The resultant conjugate was aliquoted and stored frozen at -40°C .

PHF–AO(MI)–Trypsin Conjugates, C-VII

PHF-aldehyde was prepared as described above from PHF-diol, $M_n = 150$ kDa. The PHF-aldehyde from 100 mg of PHF-diol in 5.0 mL of deionized water was combined with 46 mg of VII. The pH was adjusted to 3.0 with 1 M NaHSO_4 , and the mixture was agitated for 2 h on ice. Then, the pH was adjusted to 6.5, and the oxime-modified polymer (PHF–AO(MI)) was desalted on Sephadex G-25. Trypsin was treated with sodium borohydride (molar ratio $\text{NaBH}_4/\text{trypsin} = 1.4:1$, phosphate buffer, pH 8.6, 30 min) to convert ca. 30% of the thiol bridges into mercapto groups available for the reaction with maleimide. The obtained PHF–AO(MI) product was combined with 16 mg of reduced trypsin dissolved in phosphate

buffer, pH 8.6, and incubated for 2 h on ice and for 18 h at 8 °C. HPSEC (UV at 280 nm) analysis of the reaction mixture showed 75% trypsin association with the polymer. The reaction mixture was desalted and concentrated to approximately 10 mg/mL on a PM-30 ultrafiltration membrane in 0.05 M PBS, pH 7.0. The conjugate was separated from the unbound trypsin on a Superose-6 column with 0.5 M PBS, pH 7.0, as a running buffer, desalted on Sephadex G-25, and lyophilized. SEC analysis of this conjugate (Biosil SEC-400) showed substantial presence of a high molecular weight fraction eluted with the void volume, peak MW ~500 kDa.

Chymotrypsin conjugates (C-VIII, C-IX, Table 1) were prepared and isolated analogously.

Trypsin Conjugate Modification with DTPA and ^{111}In Labeling

For animal studies, protein conjugates C-II and C-III were modified with DTPA (through protein amino groups) and labeled with ^{111}In . EDC-mediated coupling was carried out in aqueous Na_2gDTPA solution, pH 7.5, at a DTPA/EDC/amine molar ratio of 500:50:1 (amine content in trypsin calculated by lysine). These conditions were previously developed for polyamine modification without cross-linking,¹⁵ based on earlier publications.^{16,17} The resultant DTPA-labeled conjugates were purified by gel chromatography on Sephadex G-25. The DTPA to protein molar ratio, as determined by Cu(II) photometric assay at 775 nm, was approximately 1:4. Unmodified proteins were labeled analogously.

Labeling with ^{111}In was carried out by transchelation from [^{111}In]citrate. The labeling solution was prepared by mixing carrier-free [^{111}In]indium chloride in 0.05 M HCl with a 20-fold volume of 0.5 M sodium citrate, pH = 5.6. The resultant [^{111}In]indium citrate solution was added to unbuffered solutions of DTPA-modified conjugates and proteins at 0.2-1 mCi of ^{111}In per 1 mg of dry substance. The labeled conjugates were separated by gel chromatography on Sephadex G-25, with simultaneous media replacement to sterile isotonic saline for injections. Labeling efficacy after transchelation, as estimated by HPLC equipped with a gamma detector, exceeded 90%. Radiochemical purity after desalting was >99%.

Blood Clearance and Biodistribution Study

Animal experiments were performed in accordance with institutional guidelines. Adult male Sprague–Dawley CD rats (weight in the range of 150–200 g, Charles River Laboratories, Wilmington, MA), $n = 6$ per group, were injected with labeled protein conjugates and proteins via the tail vein, at 1 mg/kg by protein in 150 μL injections containing $10 \pm 3 \mu\text{Ci}$ of ^{111}In per animal (the exact activity was documented for each injection).

Blood samples were taken through a tail vein contralateral to the injection point 5, 10, 20, 30 min and 1, 2, 4, 8, and 24 h postinjection. For each animal, the label content in blood was expressed as a percent of the initial blood activity. The latter was calculated taking blood volume (7% of the body weight) as the initial distribution volume. Then, average numbers for each time point and standard deviations were calculated for each group.

Animals were euthanized at 24 h postinjection, and harvested organs (heart, lungs, liver, spleen, kidneys, adrenal glands, stomach, GI, testes, muscle, bone, brain and tail) were analyzed on a gamma counter. Animals with label extra-vasation in the tail tissue were

excluded from the experiment. The label accumulation was expressed as a percentage of the injected dose per gram of tissue.

Enzyme Activity

Esterase activity of the model enzymes was measured with model substrates BAEE (trypsin) and ATEE (R-chymotrypsin).^{18,19} The activity of trypsin was measured at 25 °C in 62.5 mM phosphate, pH = 7.6, photometrically via registering the kinetics of absorbance at 253 nm. The activity of α -chymotrypsin was studied by registering the kinetics of ATEE absorbance at 237 nm. The measurements were carried out at 25 °C in 62.5 mM Tris/HCl buffer containing 15 mM CaCl₂, pH = 7.8. The kinetic parameters of native and conjugated enzymes were determined from the initial reaction rates at various substrate concentrations, 5.0×10^{-5} to 5.0×10^{-4} M for BAEE and 5.0×10^{-5} to 1.0×10^{-4} M for ATEE, and enzyme concentrations of 5.0×10^{-8} M and 1.0×10^{-7} M for trypsin and α -chymotrypsin, respectively. The kinetic parameters $V_{\max}$ and K_m and the catalytic constant (k_{cat}) were obtained by fitting the initial velocity and substrate concentration data directly to the Michaelis–Menten equation using nonlinear regression.

Trypsin peptidase activity was also measured using bovine serum albumine (BSA) as a substrate, to estimate the steric hindrance introduced by the polyal chains with respect to a macromolecular substrate. The reaction was carried out in 0.1 M phosphate buffer, at 25 °C, pH = 7.6, at BSA and trypsin concentrations of 5 mg/mL and 0.2 mg/mL, respectively. The amount of intact BSA in the solution was determined by size exclusion HPLC (Biosil SEC-125 for trypsin and Biosil SEC-400 for PHF–trypsin conjugates experiments, UV detection at 280 nm) at 0, 10, 30, and 60 min and plotted against time. The effective rate of BSA digestion was calculated on the basis of the initial slope of the digestion curve.

Hydrolytic Stability of the Conjugates

Hydrolytic degradation of PHF and PHF–trypsin conjugates was studied at 37 °C in PBS at pH 7.4 and 5.5. The pH of the media remained constant over the course of the experiment. HPSEC analysis (Biosil SEC-400) of the reaction mixture aliquots taken at 24, 72, and 144 h was carried out to determine the molecular weight distributions and composition of the degradation products. The unbound and PHF-associated trypsin fractions were determined by integrating the absorbance at 280 nm for the respective peaks.

Results

PHF Conjugation with Proteins

Two model small proteins, trypsin and α -chymotrypsin, were used in this study as models for development and characterization of protein–polyal conjugates. Protein conjugates with PHF were prepared using three different conjugation approaches.

The first approach was based on acylation of the primary alcohol functionality present in PHF with succinic anhydride. Protein conjugation was subsequently conducted via carbodiimide-mediated coupling of the carboxy-modified polymer with the model protein (the method is targeted to coupling through, predominantly, lysine moieties). Under the

conditions used, approximately one-quarter to one-half of the 14 lysine moieties present in the protein molecule were expected to be reactive.

This approach resulted in up to 95–98% protein conversion to conjugates (see Figure 1) with 90–95% preservation of protein activity (see Tables 1–3).

Another conjugation technique used in this study was based on activation of pendant glycol groups introduced into the PHF structure via reduction of the intermediate oxidation products IIa/IIb (Scheme 1.) Diols were converted into active aldehyde groups immediately prior to conjugation. In one example, PHF-diol polyal protein conjugate was prepared by the conventional method of reductive amination (C-I, Table 1). Protein conversion was relatively low (~60%), which is in part due to the low pH in the reaction media that was used to suppress cross-linking.

Alternatively, conjugates C-II–C-VII and C-IX were prepared utilizing the aminooxy group containing bifunctional reagents VII and VIII developed in our laboratory for conjugation of carbonyl-containing compounds with molecules containing amino and sulfhydryl groups, respectively (Scheme 2). The method was developed as a replacement for the widely used hydrazone-based reagents for nonreductive coupling with aldehydes. Nonreductive amination with aminooxy reagents requires milder conditions, e.g., pH = 3–5 and as high as 6 if necessary, and results in a more stable oxime bond (hydrazone conjugates show significant instability even at pH 7–7.5²⁰).

Aminooxy-derivatized precursors (O-substituted hydroxylamines) are widely used in medicinal chemistry for fast, one-step coupling with carbonyl groups (ketones, aldehydes) with the formation of oxime bonds. Aminooxy-derivatized reagents were also used in bioconjugate chemistry for coupling of oxidized glycosylated proteins with amino-²¹ and sulf-hydryl-group-containing ligands.²² Our bifunctional reagents contained a protected aminooxy group and either a thiolreactive maleimide group (VII) or an amino-reactive *N*-hydroxysuccinimide ester group (VIII). Coupling of these aminooxy reagents with aldehydes (nonreductive amination) was carried out in aqueous media at pH 3 for 2 h. When unprotected analogues of VII and VIII or their hydrochloride salts were used, the pH of the reaction mixture during the coupling was maintained in the range of 3.5–4.5. In these mild acidic conditions, the *N*-oxysuccinimido and maleimido functions were preserved for the subsequent stage of protein coupling. In PHF-diols, glycol content as low as 2% mol (or approximately 20 diol moieties per PHF molecule of MW = 150 kDa) was sufficient for quantitative coupling of proteins at a protein to PHF ratio of 1:1 mol/mol.

Conjugates with protein to PHF ratios from 1:1 to 4:1 (mol/mol) were successfully prepared utilizing the above strategies. In most cases, the desirable degree of modification was achieved with high yields (85–95%). Conjugates prepared in one step using aminooxy-NHS coupling reagent VIII and via EDC-mediated coupling to PHF-SA gave the highest conjugate yields (up to 95–98% for 1:1 conjugates) by protein. Both aminooxy reagents have shown a high degree of flexibility with respect to the conjugation sequence and conditions. The sufficient stability of aminooxy, NHS, and maleimido coupling groups in various activation conditions enabled us to change the reaction order at will and, where

required, isolate and purify the intermediate aminooxy-functionalized proteins and NHS or MI-functionalized PHF. As indicated by HPSEC, under the mildly acidic conditions used in the polyal/aminooxy coupling reaction the polyal backbone remained stable, and the protein adducts were obtained with nearly theoretical yields.

The observed effective molecular weights of the conjugates, in many cases 1.5 to 2 times higher than that of PHF, were apparently due to the partial cross-linking of polyal chains via protein molecules. This process, however, had no noticeable effects on enzymatic activity of the conjugates. Comparative evaluation of enzymatic activity was performed using conjugates with polymer/protein molar ratios of approximately 1:1 (conjugates 2, 3, and 8, Table 1). When tested with synthetic substrates, no significant changes in the Michaelis–Menten parameters (K_M , k_{kat}) and no pH optimum shifts were observed as compared to native enzymes (Table 2). Conjugates retained from 85% to 95% of the native enzyme activity. With BSA as a substrate (not shown), a more significant 50–70% decrease in enzyme activity was observed, most likely due to the expected steric hindrance, which is in agreement with earlier literature data on trypsin conjugated with uncharged polymers.²³

pH-Dependent Hydrolytic Degradation of PHF–Protein Conjugates

All PHF- and PHF-diol-based conjugates exhibited a pH-dependent profile of hydrolytic degradation, being essentially stable under neutral and mildly basic conditions (pH = 7.0–10.5).

Hydrolytic degradation of PHF and two different PHF–trypsin conjugates, PHF-SA–trypsin (protein content 10% w/w, M_n = 250 kDa) and PHF-AO–trypsin (protein content 25% w/w, M_n 350 kDa), was studied at pH 7.4 and 5.5. No substantial changes in the polymer molecular weight distribution nor noticeable accumulation of unbound trypsin were observed at 37 °C pH = 7.4 over a 144 hour period. On the contrary, incubation at 37 °C, pH = 5.5 resulted in a steady decrease in the effective molecular weight and in protein release for both conjugates (see Table 3 and Figures 2 and 3). The HPLC data indicated that, while the absolute rate of the PHF peak reduction was higher in the oxime-linked conjugates than in succinamide ester-linked conjugates (Table 3), the relative rate of accumulation of trypsin and smaller UV-absorbing fragments for oxime-linked conjugates was higher (Figure 3). The latter is possibly due to either a lower rate of autolysis in the SA-linked conjugates, or differences in the extinction coefficients of polymer degradation products, or both.

Biokinetics and Biodistribution of [¹¹¹In]DTPA-Labeled Model PHF–Protein Conjugates

Biokinetics of PHF-SA–trypsin, PHF-AO–trypsin (protein content 10% and 25%, and M_n 250 kDa and 350 kDa, respectively) and unmodified trypsin (24 kDa) were studied to determine the effect of PHF modification on protein biokinetics and biodistribution. The data demonstrated significant improvement in the protein circulation, as compared to the unmodified protein (Figure 4). At 24 h, the AUC was 6.2-fold and 8.9-fold higher for the PHF-SA–trypsin and PHF-AO–trypsin than for the unmodified protein, respectively.

The unmodified trypsin was $68 \pm 7\%$ cleared from blood within 10 min (initial blood half-life 6 ± 1 min), followed by a longer clearance stage that is best described as 20% clearance with

a 1.3 h half-life and 10% with a 7.7 h half-life. When the relatively small protein size and the character of final biodistribution are considered (Figure 5), the first (main) phase is consistent with renal clearance and extravasation. The secondary (multiexponential) phase can be related to trypsin redistribution back from the interstitial compartment to blood and/or circulation of trypsin adducts with protease inhibitors of plasma.

PHF–trypsin conjugates showed a biphasic blood clearance pattern. Only $13\pm5\%$ and $8\pm6\%$ of activity were cleared from blood during the first 10 min for PHF-SA–trypsin and PHF–AO–trypsin, respectively. Approximately 60% and 53% were cleared with 24 and 30 min half-lives, while the rest remained in the circulation with a half-life of 15 and 18 h, respectively. The first phase here was consistent with extravasation and, possibly, partial renal clearance of the low molecular weight fractions of the conjugates. The second phase was consistent with slower terminal clearance (hepatic uptake and accumulation in tissues). The data are also consistent with the previously observed clearance rates of small molecule PHF derivatives.¹¹ The long circulation indicates preservation of the conjugate integrity in plasma within the time frame of the experiment.

The final biodistribution (Figure 5) showed a 5-fold reduction in the label accumulation in kidneys ($p < 0.01$) for either conjugate, as compared to that of unmodified trypsin. High renal uptake is characteristic for small proteins and many peptides; thus, the data suggest that small protein modification with PHF can be an effective method for suppressing the renal uptake. Adrenals were another organ where label uptake was significantly lower (3-fold, $p < 0.01$). The data also shows a significant reduction in hepatic accumulation for at least one conjugate (trypsin–AO–PHF, $p < 0.05$). However, since a significant part of the protein was still circulating in blood at the time of the study (24 h), the final hepatic uptake would not be necessarily significantly lower although undoubtedly much slower. No increase in the splenic accumulation was observed as a result of conjugation. No statistically significant changes were observed in other organs except skeletal muscles (2-fold higher, $p < 0.05$). Note that animals were not perfused, so the higher label content in the lung and heart (Figure 5) is consistent with the conjugate presence in the blood pool of these organs.

Discussion

This study was intended to obtain and characterize model protein conjugates of PHF, a lead material from the group of hydrophilic polyals developed in our laboratory.

Along with the conventional conjugation techniques employing acylation-based linking, novel bifunctional re-agents forming oxime bonds were used. Linkages formed by such reagents have the same pH sensitivity profile as the PHF backbone itself. PHF is a highly hydrophilic, essentially nontoxic semisynthetic polymer (no toxicity in mice at 4 g/kg iv²⁴) stable in physiological conditions but undergoing nonenzymatic hydrolysis at lysosomal pH. All previously tested PHF-containing preparations with MW exceeding 70 kDa exhibited long circulation in vivo, with no significant uptake in RES. The blood half-life of unmodified 500 kDa PHF was found to be over 24 h.^{10,28}

At a 1:1 polymer/protein ratio, two conjugation techniques gave the highest coupling yields (95–98% by protein): the one-step method utilizing aminooxy–NHS coupling reagent VIII and polymer succinylation followed by EDC-mediated acylation. Conjugate formation was accompanied with a 1.5 to 2.0-fold increase in the number-average molecular weight (M_n). This can be an indication of cross-linking as the result of protein coupling with more than one polymer chain, which can be expected considering the reaction conditions. The conjugation process caused no noticeable effect on the enzymatic activity of the conjugated proteins; from 85% to 95% of the original activity (as measured with synthetic substrates) was preserved in the conjugates. Other conjugation methods that were tested, such as reductive amination and coupling with the aminooxy–maleimido coupling reagent VII, showed lower conjugation yields with respect to the model protein used in this study (trypsin) but might provide efficient coupling routes for other proteins, especially where thiol groups are not hindered and where cross-linking is not a major concern.

The high site specificity of the aminooxy reagents, mild coupling conditions (pH range 3–6), and high reaction rate (50% conjugation in less than 10 min for unprotected oximes at pH 5.5) all show that this group of compounds has a significant potential for protein conjugate synthesis. As expected, the oxime-linked conjugate showed a sufficiently high degradation rate in acidic conditions that are characteristic for the intracellular vesicular environments into which polymers are transferred upon uptake by cells. Thus, the reversible, pH-sensitive character of the oxime linkage can be useful when a pH dependent (e.g., caveolar, endosomal, or lysosomal) drug release is desired.

The animal data demonstrated the feasibility of PHF for designing long-circulating protein conjugates. Basic biokinetics data were obtained for two model PHF-trypsin conjugates with M_n of ~250 kDa and ~350 kDa (SEC estimated particle size of approximately 14 and 15 nm and protein load of 10% and 25%, respectively). A 5 ± 1 -fold increase in the initial blood half-life and a 5-fold reduced renal uptake was observed for PHF-trypsin conjugates, as compared to that of the unmodified trypsin, with a compensatory increase in skeletal muscles and no organ specific accumulation. The amounts of trypsin conjugates remaining in circulation at 24 h postinjection were 9.5–12-fold higher than those of unmodified trypsin.

The data, notably obtained with unoptimized and unfractionated conjugates, are comparable with the prolongation of protein circulation and reduction of renal uptake reported for other small protein conjugates, e.g., PEG conjugates.^{25,26} In our other studies,²⁷ PHF conjugates showed a significantly lower renal vacuolization potential than analogously structured PEG conjugates. Thus, the available data in all aspects suggest that PHF (and, likely, other semisynthetic and fully synthetic hydrophilic polyals) have a significant potential as a platform for protein modification. PHF itself can be considered a viable and potentially superior biodegradable replacement for poly(ethylene glycol), especially in applications requiring chronic and/or high dose administration. Therefore, methods developed in this study are currently being extended to the synthesis of clinically relevant protein conjugates. The dependence of conjugate properties on the modification degree, protein load, linker structure, and length is a subject of ongoing research. Terminal (one-point) activation of PHF is expected to open the way to un-cross-linked conjugates which may have further improved pharmacokinetics.

Conclusion

Bioconjugates comprising hydrophilic, fully degradable polyacetal modules and model enzymes were successfully prepared with high yields and preservation of activity, utilizing conjugation strategies based on acylation as well as reductive and nonreductive amination. Aminooxy reagents, which have been developed to address the problem of preparation of bioconjugates fully biodegradable upon cell uptake, enabled efficient protein modification and resulted in long-circulating protein conjugates. The data suggests several potential applications for both hydrophilic polyacetals and aminooxy reagents in protein modification, in particular where chronic or high-dose administration is required.

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Scheme 1.

Preparation of Acyclic Hydrophilic Acetals from Dextran B-512

Figure 1.
The dynamics of trypsin conjugation with PHF-succinate (size exclusion HPLC, UV detection).

Scheme 2.
Conjugation of Proteins with PHF Using Aminoxy–NHS and Aminoxy–Maleimide Linkers

Figure 2. Degradation of the PHF-AO-trypsin conjugate at 37 °C, pH 5.5 (size exclusion HPLC, UV detection). The free enzyme peak may include smaller UV-absorbing fragments.

Figure 3. Kinetics of accumulation of trypsin and low molecular weight fractions as percent of the total UV-absorbing material in the process of conjugate hydrolysis. Conditions: 37 °C, pH = 5.5 (size exclusion HPLC peak integration).

Figure 4. Blood radioactivity following intravenous injection of ^{111}In -labeled trypsin and trypsin conjugates in rat. Triangles, trypsin; squares, PHF-SA-trypsin; circles, PHF-AO-trypsin. Lines: best fits, three-exponential for trypsin and biexponential for the conjugates (see text).

Figure 5. Biodistribution of ^{111}In -labeled trypsin and trypsin conjugates 24 h after intravenous injection in rat.

Table 1

Composition and Yield (by Protein) of PHF-Protein Conjugates

No.	carrier	linker	enzyme	enzyme molecules per PHF chain	protein modification %
C-I	PHF-diol	direct amination	trypsin	1	61
C-II	PHF	SA	trypsin	1	97
C-III	PHF-diol	AO-NHS (VIII)	trypsin	1	98
C-IV	PHF-diol	AO-NHS (VIII)	trypsin	2	85
C-V	PHF-diol	AO-NHS (VIII)	trypsin	4	72
C-VI	PHF-AO-NHS ^a	AO-NHS (VIII)	trypsin	1	87
C-VII	PHF-diol	AO-MI (VII)	trypsin	1	75
C-VIII	PHF	SA	chymotrypsin	1	95
C-IX	PHF-diol	AO-NHS(VII)	chymotrypsin	1	93

^a PHF-glycol with a 2,3-diol content of 2% was modified with VIII. Isolated and lyophilized PHF-AO-NHS was used sequentially for preparation of the protein conjugate in one step without additional activation.

Table 2

Kinetic Parameters of Trypsin-PHF Conjugates

enzyme/conjugate	$K_m \text{ M} \times 10^5$	$k_{\text{cat}} \text{ s}^{-1}$	$k_{\text{cat}}/K_m \text{ M}^{-1} \text{ s}^{-1}$
trypsin	1.79 ± 0.28	16.0 ± 0.1	8.9×10^5
PHF-AO-trypsin (C-II) ^a	1.40 ± 0.15	16.0 ± 0.2	11.4×10^5
PHF-SA-trypsin (C-VI)	1.41 ± 0.35	16.5 ± 0.7	11.8×10^5
α -chymotrypsin	126 ± 26	135 ± 36	1.07×10^5
PHF-SA-chymotrypsin (C-VIII)	115 ± 19	128 ± 17	1.11×10^5

^aNumbers in parentheses refer to the conjugate numbers in Table 1.

Table 3

Degradation of PHF and PHF-Trypsin Conjugates in PBS at pH 5.5

time h	M_n	peak MW	polydispersity index $PDI = M_w/M_n$	residual content of PHF % to initial	trypsin release %
PHF					
0	92000	130000	2.6	100.0	n/a
72	86000	99000	2.0	98.5	n/a
144	66000	97000	3.6	89.2	n/a
PHF-AO-Trypsin (1:2)					
0	344000	429000	2.5	100.0	n/a
72	270000	363000	2.8	96.1	5.4
144	174000	255000	4.6	89.8	10.5
PHF-SA-Trypsin (1:1)					
0	246000	260000	1.8	100.0	n/a
72	177000	209000	2.0	99.8	4.7
144	153000	176000	3.1	98.6	5.4