Pendent Chain Functionalized Polyacetals That Display pH-Dependent Degradation: A Platform for the Development of Novel Polymer Therapeutics

Ryan Tomlinson,[†,‡] Marcus Klee,[†] Shane Garrett,[†] Jorge Heller,[§] Ruth Duncan,[*,‡] and Steve Brocchini[*,†]

Biomedical Polymers Group, Department of Pharmaceutics, The School of Pharmacy, University of London, 29-39 Brunswick Square, London WC1N 1AX, UK; Centre for Polymer Therapeutics, Welsh School of Pharmacy, University of Wales, King Edward VII Avenue, Cardiff, CF10 3XF UK; and A. P. Pharma, 123 Saginaw Drive, Redwood City, California 94063

Received May 21, 2001; Revised Manuscript Received September 30, 2001

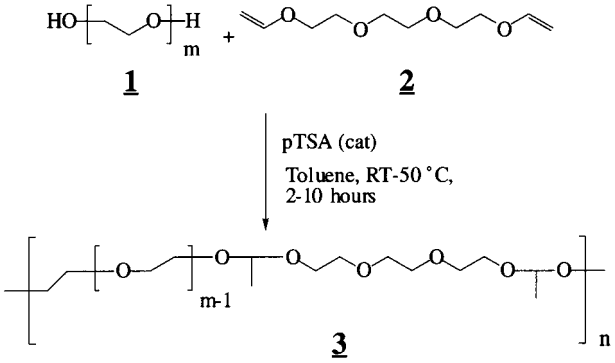
ABSTRACT: The synthetic polymers that are used to prepare polymer therapeutics reaching clinical use are predominantly nonbiodegradable, and this severely limits the molecular weight range that will give certainty of safe elimination. The aim of this study was to synthesize water-soluble, biocompatible, amino-functionalized polyacetals that would display pH-dependent degradation and, moreover, be suitable for drug conjugation. To test the feasibility of the synthetic procedure, polyacetals were first prepared by the reaction of a diol (e.g., poly(ethylene glycol) (PEG)) and a divinyl ether (e.g., tri(ethylene glycol) divinyl ether) using an acid catalyst. Using PEG_{3400}, these polyacetals had a M_w of 36 000−43 000 g/mol (M_w/M_n = 1.6−1.8) and displayed pH-dependent degradation. An enhanced rate of hydrolysis was seen at pH 5.5 (41% M_w loss in 25 h) compared to pH 7.4 (10% M_w loss in 73 h). The polymers and their degradation products were nontoxic toward B16F10 cells in vitro (IC_{50} > 5 mg/mL), and they were also nonhemolytic (rat red blood cells). Several approaches were examined to produce amino-functionalized polyacetals. It was found that modification of either the divinyl ether or PEG monomer was not the best strategy. However, terpolymerization, for example using the hydrolytically stable diol 9-fluorenylmethyloxycarbonyl (Fmoc)-serinol, PEG_{3400}, and tri(ethylene glycol) divinyl ether, did produce functionalized polyacetals of M_w 20 000−77 000 g/mol and M_w/M_n = 1.8−2.0. Varying the ratios of diol monomer gave a family of polymers containing different amounts of pendent group. One of these amino-polyacetals was used to prepare a polymer containing ^{125}I-labeled Bolton−Hunter reagent (74 μCi/mg), introduced to facilitate a preliminary biodistribution study after intravenous administration to rats. The polyacetals showed no preferential accumulation in the major organs (at 1 h; liver (4.2 % dose), lung (0.7%), and kidney (1.1%)), and the log blood clearance with time was linear over 24 h. These novel, biodegradable polyacetals have potential for further development as polymer therapeutics and more generally as a new family of biodegradable polymers.

1. Introduction

Conjugation of a water-soluble polymer to bioactive agents, e.g., drugs or proteins, has been successfully used as a strategy to develop novel polymer therapeutics (reviewed in refs 1−4). Although many polymers have been examined preclinically, almost all conjugates reaching clinical evaluation have used either PEG[3,4] or copolymers of *N*-(2-hydroxypropyl)methacrylamide (HPMA).[1,5−7] Both polymers are well tolerated in man, but they share the disadvantage that the main chain is not biodegradable in vivo, and this restricts use to a molecular weight <40 000 g/mol (depending on structure)[8,9] if renal elimination is to be ensured. Clinical use of higher molecular weight nonbiodegradable polymers (e.g., poly(vinylpyrrolidone)) can give rise to lysosomal storage disease syndrome.[10,11]

There is a pressing need to identify novel, water-soluble polymers that are biocompatible, that have sufficient functionality to allow carriage of a drug payload, and that will degrade following parenteral administration. Availability of such polymers would allow design of polymer therapeutics for repeated chronic administration and as treatments for illnesses other than life-threatening infectious diseases and cancer. It is recognized that use of higher molecular weight polymers would allow optimization of tumor targeting of anticancer conjugates.[12] One option is to use natural polymers. Some polysaccharides are degraded enzymatically by mammalian enzymes, e.g., dextran,[13,14] and thus can be administered over a wider molecular weight range. However, chemical modification

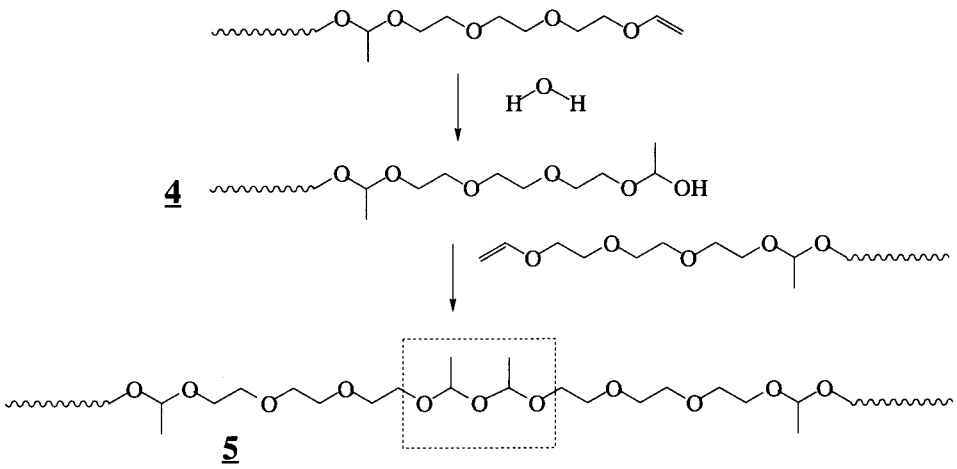
Scheme 1. Synthesis of Polyacetals 3



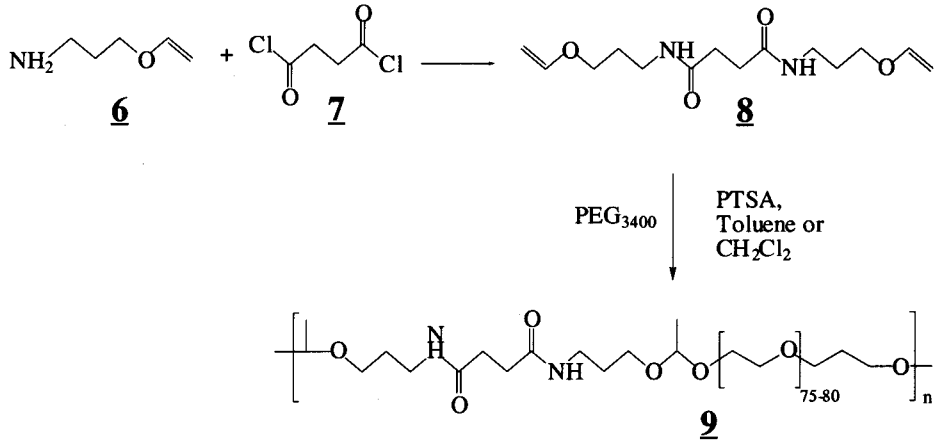
[†] University of London.
[‡] University of Wales.
[§] A. P. Pharma.
* Corresponding authors. R. Duncan: e-mail DUNCANR@cf.ac.uk; Tel +44 (0) 292 087 4180; Fax +44 (0) 292 087 4536. S. Brocchini: e-mail Steve.Brocchini@ulsop.ac.uk; Tel +44 (0) 207 753 5802; FAX +44 (0) 207 753 5942.

10.1021/ma0108867 CCC: $22.00
Published on Web 12/18/2001

Scheme 2. Potential Competition Reactions from Trace Water during Polymerization



Scheme 3. Synthesis of Model Divinyl Ether 8 Using the Commercially Available Amino Vinyl Ether 6



of polysaccharides can lead to toxicity[15] and also causes a loss in degradability of the polymer backbone.[13] Polyamino acids, particularly polyglutamates, have been explored as a potentially biodegradable platform for drug conjugation, and a polyglutamate−paclitaxel conjugate is currently in phase I clinical trial as an anticancer conjugate.[16] However, polysaccharides and poly(amino acid)s share the disadvantage that they can be immunogenic[17] so repeated administration must be treated with care.

The aim of this study was to design novel polymers that would display main-chain hydrolytic degradation and, in particular, that would degrade more rapidly at the acidic pH values encountered in endosomes and lysosomes[18] following endocytic internalization by cells. The acetal moiety was chosen as it undergoes faster hydrolysis under mildly acidic conditions, and polymers can be prepared under mild conditions from diols and divinyl ethers.[19] To determine the reproducibly of synthesis and the stability of the isolated and stored product, polyacetals **3** (Scheme 1) were first prepared by the reaction of PEG **1** and tri(ethylene glycol) divinyl ether **2** using an acid catalyst. The rate and pH dependence (pH 7.4−pH 5.5) of hydrolytic degradation of polyacetals **3** was determined using GPC, and biocompatibility was assessed by measuring cell cytotoxicity and hemolytic activity. To introduce the functionality required for conjugation of model compounds and drugs, amino-polyacetals **16** (Scheme 4) were synthesized. A conjugate of the amino-polyacetal **16** and ^{125}I-labeled Bolton and Hunter reagent was then used to assess the biodistribution after intravenous (iv) administration to rats.

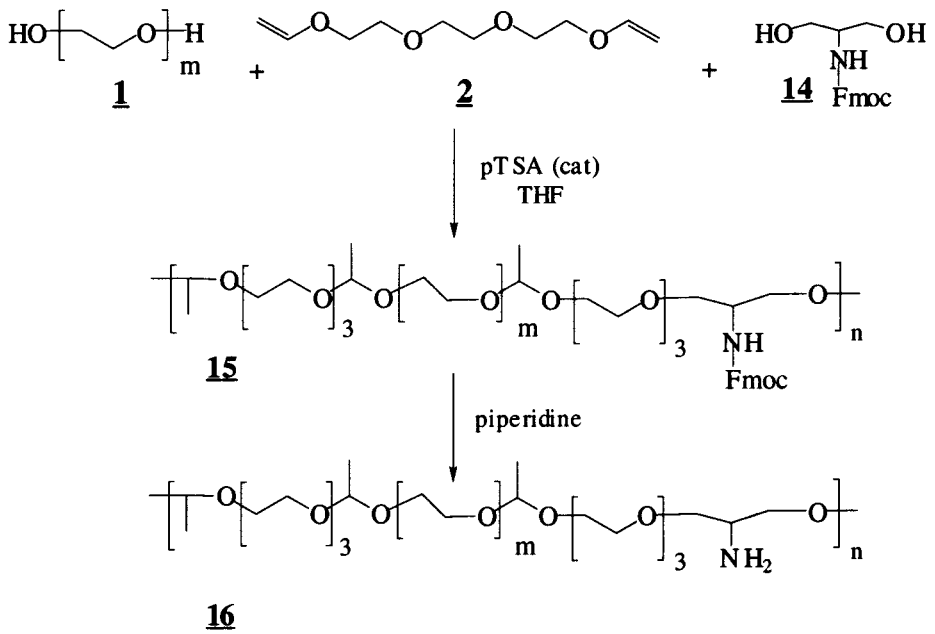
2. Experimental Section

2.1. Instruments. NMR analysis was performed using a Bruker AM250 or AM500 FT-NMR spectrometer. GPC analysis was performed using a JASCO HPLC pump and Gilson 133 refractive index detector and analyzed using Caliber software (Polymer Laboratories). THF or DMF with 0.1% LiCl was used as an organic solvent eluent (Styragel HR3 and HR4 columns in series), and for aqueous phase GPC PBS buffer was used (Waters Ultrahydrogel 1000 and 250 columns in series).

2.2. Materials. Tri(ethylene glycol) divinyl ether, PEG, and *p*-toluenesulfonic acid monohydrate (*p*-TSA) were used as supplied from Aldrich (Dorset, UK). Lancaster (Lancashire, UK) supplied the 2-amino-1,3-propanediol (serinol), and 9-fluorenylmethyl chloroformate (Fmoc-Cl), dichloromethane (DCM), tetrahydrofuran (THF), and toluene were purchased from BDH (Dorset, UK). Before use, THF was distilled from sodium benzophenone and DCM was distilled from CaH. Medical grade oxygen, nitrogen, and CO_2 (all 95% v/v) and liquid nitrogen were supplied by BOC (Surrey, UK). Isofluorane was from Abbott Labs (Kent, UK) and Amersham Pharmacia Biotech (Hertfordshire, UK) supplied the ^{125}I-labeled Bolton−Hunter reagent. All other reagents were of general laboratory grade and were purchased from Aldrich.

Bantin and Kingman Ltd. (Hull, UK) supplied the Wistar rats. B16F10 murine melanoma cells were kindly donated by Prof. I. Hart (St. Thomas's Hospital, London, UK). Tissue culture grade dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), trypan blue, and optical grade DMSO were from Sigma (Dorset, UK)

Scheme 4. Terpolymerization To Give Polyacetal 16 with Amine Functionalized Pendent Chains for Drug Conjugtion



Trypsin, fetal calf serum (FCS), and RPMI 1640 were from Gibco BRL Life Technologies (Paisley, UK).

2.3. Synthesis of Polyacetal 3. Representative Polymerization in Toluene. PEG (M_n = 3400 g/mol; 17.0 g, 5.0 mmol, 1.0 equiv), *p*-TSA (0.03 g, 0.15 mmol, 0.03 equiv), and toluene (60 mL) were added to a 100 mL round-bottom flask equipped with a stirrer and fitted with a thermometer, a Dean Stark trap, and a condenser. An azeotropic distillation of the stirred toluene solution (oil bath, T = 150 °C) under nitrogen was conducted for 2 h. The solution was then allowed to cool to ~50 °C, and tri(ethylene glycol) divinyl ether (1.073 g, 1.083 mL, 5.2 mmol, 1.04 equiv) was added by syringe. Within 1 min the reaction mixture became visibly more viscous, and after 15 min the viscosity appeared to be very high. Toluene (30.0 mL) was added to decrease the viscosity, and the clear colorless reaction mixture was stirred a further 2 h at ambient temperature. Aqueous $NaHCO_3$ (8.0% w/v, 2.0 mL) was added to the reaction mixture. It was then rapidly stirred for 15 min. The aqueous phase was allowed to settle, and the toluene phase was carefully decanted into stirred hexane (200 mL) to precipitate the polyacetal. After stirring in hexane for an additional 10 min, the polyacetal was collected and placed into a fresh solution of hexane and stirred for a further 10 min. The polyacetal was again collected and then dried in a vacuum at 50 °C for 4 h to give a white fluffy solid. The molecular weight was M_w = 42 800 g/mol, M_n = 26 700 g/mol, and M_w/M_n = 1.6 as determined by aqueous GPC calibrated with PEG standards. NMR (δ_H, 500 MHz, $CDCl_3$): 1.25−1.30 (3H, d, acetal CH_3), 4.75−4.78 (1H, q, acetal −CH−).

2.4. Synthesis of Fmoc-Protected Serinol 14. Serinol (1.0015 g, 11 mmol) was dissolved in a 10% solution of Na_2CO_3 in water (26.5 mL, 25 mmol). Dioxane (15 mL) was then added, and the mixture was stirred in an ice water bath. Fmoc-Cl (2.851 g, 11 mmol) was carefully added and allowed to react at ice bath temperature for 4 h and room temperature for 8 h. The mixture was diluted with water (100 mL), and the product was extracted from ethyl acetate (2 × 100 mL). The organic phase was dried ($MgSO_4$) and the solvent removed. The crude product was recrystallized from ethyl acetate−hexane to yield a white crystalline solid. Yield 90%. NMR (δ_H, 500 MHz, $CDCl_3$): 3.5 (4H, d, −C**H**$_2$−OH), 3.8 (1H, d,d, −C**H**N−), 4.3 (1H, m, Ar−C**H**−Ar), 4.7 (2H, d, O−C**H**$_2$−), 7.3−7.8 (8H, m, Fmoc Ar).

2.5. Synthesis of the Amino-Polyacetal 16. PEG_{3400} (5.0052 g, 1.5 mmol) and *p*-TSA (12 mg, 0.05 mmol) were accurately weighed into a 100 mL double-necked round-bottom flask equipped with a stirrer. The compounds were dried at 80 °C under high vacuum for 3 h to remove water and allowed to cool. The reaction vessel was purged with nitrogen, and a solution of the Fmoc diol **14** (0.4616 g, 1.5 mmol) in freshly distilled THF (10 mL) was added. A solution of tri(ethylene glycol) divinyl ether (0.601 mL, 3 mmol) in freshly distilled THF (10 mL) was then added by syringe to the reaction mixture, and the solution was stirred for 3 h. Additional THF was added if the reaction mixture became too viscous. Triethylamine (0.3 mL) was added to quench the reaction, and the mixture was poured into a stirred solution of hexane (100 mL) to precipitate a white solid. After 10 min the precipitate was filtered, stirred again in hexane, and isolated. Residual solvent was removed in a vacuum. Polyacetal **15** had a molecular weight of 25 000 g/mol (M_w); M_w/M_n = 2.0 as determined by aqueous GPC calibrated with PEG standards. NMR (δ_H, 500 MHz, $CDCl_3$): 1.23−1.30 (3H, m, acetal CH_3), 4.75−4.78 (1H, m, acetal −CH−), 7.27−7.73 (8H, m, Ar).

The Fmoc group was removed by adding polyacetal **15** (2.0509 g) to a stirred solution of 20% piperidine in acetonitrile (CH_3CN) at ambient temperature. TLC (ethyl acetate, UV) was used to monitor the reaction that was complete after 0.5 h. The mixture was washed with hexane, and the CH_3CN was removed in vacuo. The resulting oil was poured into stirred hexane to yield the desired polyacetal as a white solid **16** in quantitative yield. NMR (δ_H, 500 MHz, $CDCl_3$) indicated complete removal of the Fmoc protection by the absence of characteristic peaks at 7.3−7.8, 4.6, and 4.3. The molecular weight of polyacetal **16** by GPC using PEG standards was M_w 25 000 g/mol (M_w/M_n = 2.0), and this was the same as seen for the Fmoc-protected polyacetal **15**.

2.6. Radiolabeling of Amino-Polyacetal 16. The polymer was radiolabeled using the Bolton and Hunter method.[20] Briefly, amino-polyacetal **16** (50 mg) was dissolved in 500 μL of 0.1 N borate buffer pH 8.5. In a fume cupboard, 500 μCi (18.5 MBq) of ^{125}I-labeled (di-iodo) Bolton−Hunter reagent in benzene with 2% (v/v) DMF was carefully dried using a stream of nitrogen gas. The polyacetal solution was added to the reagent and allowed to react for 15 min with periodic agitation. A small aliquot was removed (10 μL) as an archive sample and to determine labeling efficiency. The remaining mixture (490 μL) was diluted with PBS, pH 7.4, (10 mL) and dialyzed at 4 °C in a Spectra Por dialysis membrane (molecular weight cutoff of 1000 Da) against PBS, pH 7.4 (5 L), to remove any free Bolton−Hunter reagent. The dialysate was changed twice

a day until no radioactivity could be detected, and then the radiolabeled polyacetal was transferred to a vial and stored at −18 °C. The labeled polymer (before and after dialysis) was subjected to paper electrophoresis in barbitone buffer pH 8.6 to ascertain the characteristics of the radioactive species in the samples.

2.7. Investigation of pH-Dependent Degradation. Polyacetal **3** derived from PEG_{3400} was used in the initial study. The polyacetal (3 mg/mL) was incubated at 37 °C in PBS (0.1 mol/L) at pH 7.4, 6.5, and 5.5 (the latter pHs adjusted by addition of NaH_2PO_4). Generally, the pH values remained constant, but it was sometimes necessary to adjust the pH of the solutions with NaH_2PO_4 or Na_2HPO_4 during the experiment. At various time points (up to 21 days) aliquots were removed, and the molecular weight was determined by GPC as described above. Polyacetal **16** degradation was also investigated using essentially the same method, but in this case the incubation pH was adjusted when necessary with dilute NaOH or HCl.

2.8. Hemolytic Activity of Polyacetals and Their Degradation Products. The method used was previously described.[21] To obtain rat red blood cells (RBC), blood was taken from an adult male Wistar rat (by cardiac puncture) immediately after death and placed in a lithium/heparinized tube (10.0 mL) on ice. PBS (2.0 mL prechilled) was added, and the diluted blood was centrifuged three times (1500 g, 10 min). Each time the supernatant was removed along with the heparin beads, and fresh PBS was added. A 2% (w/v) solution of RBC was then prepared in PBS. To determine hemolytic activity polyacetals or their degradation products (100 μL in PBS, 0−5 mg/mL)) were placed in 96 well micro-titer plates and RBCs (100 μL of the 2% (w/v) solution) was added before incubation for 24 h at 37 °C. Triton X-100 (1% solution) was used as a reference control to measure 100% hemolysis. Polyethylenimine (PEI) and dextran were used as positive and negative reference polymers, respectively. After 24 h samples were centrifuged (1500*g*, 10 min), and the supernatant (100 μL) was pipetted into a 96 well micro-titer plate and hemoglobin release measured (absorbance at 550 nm using a microtiter plate reader). The degree of lysis was expressed as a percentage of the Triton X-100 value.

2.9. Cytotoxicity of Polyacetals and Their Degradation Products. B16 F10 cells were grown in RPMI 1640 with 5.0 mM L-glutamine and 10% (v/v) FCS. Cells were seeded into 96 well microtiter plates at a density of 1×10^4 cells/well, and 24 h later the polymers were added to give a final concentration range of 0−5 mg/mL. Poly(L-lysine) and dextran were used as positive and negative reference controls, respectively. The cells were then incubated for 72 h prior to the assessment of their viability using the MTT assay.[22] Results were expressed as viability (%) relative to a control containing no polymer.

2.10. Evaluation of the Body Distribution of ^{125}I-Labeled Polyacetal 16. Adult male Wistar rats of known weight (200−250 g) were lightly anaesthetized using fluorothane, and the polymer (100 μL, $\sim 5 \times 10^5$ counts per minute (cpm)) was injected iv via the tail vein. Rats were killed at 2, 5, 30 min and 1, 2, 5, 24 h ($n = 3$), a blood sample was taken, and the following organs were removed and homogenized in water to a known volume: heart, liver, spleen, lungs, kidneys, thyroid, and bladder. Urine and feces samples were also collected. Samples of homogenate (1.0 mL) were assayed for radioactivity. The total amount of radioactivity per organ was expressed as a percentage of the injected dose administered.

3. Results and Discussion

Most of the biodegradable, water-soluble, synthetic polymers thus far proposed for drug conjugation have been prepared from amino acids or other metabolites (for example, refs 23−26). However, such polymers and also the PEG−peptide block polymers prepared for intracellular enzymatic hydrolysis[27] do not show pH-dependent degradation. One exception is a water-soluble polymer synthesized using a degradable element derived from *cis*-aconitic acid.[28] While this polymer undergoes faster degradation at acidic pH values, there still remain significant synthetic challenges for preparing well-defined structures using this approach. Polyacetals seem to offer an ideal opportunity to reproducibly prepare a novel family of biodegradable polymers with broad application.

3.1. Optimization of the Synthesis of Polyacetals. Polyacetals **3** were prepared by the reaction of a diol (e.g., PEG **1**) and a divinyl ether (e.g., tri(ethylene glycol) divinyl ether **2**) using an acid catalyst (Scheme 1). PEG was selected as the diol because it is generally recognized as safe (GRAS) by Drug Regulatory Authorities and is widely used in pharmaceutical and consumer products.[29] No small molecule byproduct is generated during this polymerization reaction. Alternative methods for preparing polyacetals include acetalization and transacetalization reactions that generate low molecular weight byproducts (e.g., water or alcohol). Complete removal of these byproducts often requires quite vigorous reaction conditions (e.g., prolonged heating at reduced pressure) for reproducible polymerization and to ensure the polyacetal does not degrade on storage.

Initial studies focused on the preparation and preliminary evaluation of polyacetal **3**. An azeotropic distillation of a toluene solution of PEG and *p*-TSA was cooled to 30−50 °C. Upon addition of divinyl ether **2**, an exothermic reaction typically followed with a rapid increase in viscosity over 15−45 min. After 2−12 h a small volume of concentrated aqueous Na_2CO_3 was added to the stirred reaction mixture to extract the *p*-TSA. Removal of all *p*-TSA was required to prevent degradation of the polymer during isolation and storage. The polymer was isolated by precipitation into hexane. Molecular weight characteristics depended on the molecular weight of the PEG used. The weight-average molecular weight (M_w) of the polyacetals **3** prepared using PEG_{400} were typically in the range 7000−9000 g/mol ($M_w/M_n = 2.0-2.9$), and with PEG_{3400} they were 36 000−43 000 g/mol ($M_w/M_n = 1.6-1.8$). H NMR spectra clearly showed the acetal quartet at 4.8 ppm and the acetal methyl doublet at 1.3 ppm with the correct relative integration (1:3 protons). Polyacetals **3** derived from PEG_{400} were colorless oils, and those derived from PEG_{3400} were white solids. All polymers prepared under these conditions were water-soluble, and there was no evidence of degradation (by GPC) when the polymer was stored in glass bottles at ambient conditions on a laboratory bench over a 6 month period.

One concern was the potential for competing reactions due to adventitious water that would consume vinyl ether functionality resulting in disruption of the strictly alternating structure of the polymer main chain and loss of control of molecular weight. For example, competing water may in principle form hemiacetal intermediates (e.g., **4**) after reaction with a vinyl ether moiety with subsequent formation of unstable intermediates such as **5** (Scheme 2). Optimally all water must be removed to ensure only PEG-derived hydroxy groups can participate in the polymerization. An azeotropic distillation ensured that the amount of remaining water (measured in a Dean Stark trap) in the PEG toluene solution was very low (∼0.05%).[30] Conducting the polymerization ≥5 g scale of PEG ensured that the reaction was reproducible, and it was possible to compensate for the known

Table 1. Molecular Weight Characteristics of Polyacetal 15 Prepared with Different Ratios of PEG_{3400} 1 and Fmoc Protected Serinol 14

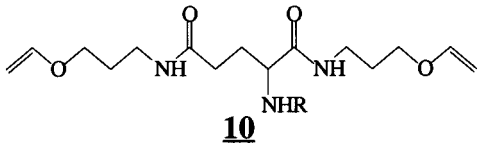
ratio **1**:**14**	M_w (g/mol)	M_n (g/mol)	M_w/M_n
1:1	24 900	12 700	1.96
1:1	19 800[a]	10 700	1.86
1:1	20 500[b,c]	11 600	1.77
4:1	77 500[a]	39 400	1.97
9:1	41 300[a]	22 600	1.83

[a] Triethylene glycol divinyl ether **2** distilled prior to use. [b] GPC conducted in THF (1.0 mL/min) using PEG standards. [c] Single μL amounts of divinyl ether **2** were added after the initial reaction period.

amount of trace water remaining by adding a corresponding amount of the divinyl ether **2**.

Another important factor in this reaction was the polydispersity of PEG, typically in the range 1.04−1.10 when PEG is obtained from commercial sources. Addition of a 1:1 stoichiometric ratio of PEG **1** and divinyl ether **2** was not possible without knowledge of the exact PEG hydroxyl equivalence. Although titration of the PEG hydroxyl content is possible,[31] it was found that additions of single (microliter) aliquots of divinyl ether during the polymerization reaction resulted in increased molecular weight.

3.2. Incorporation of Pendent Chain Functionality. To incorporate a functional pendent chain subsequently suitable for drug conjugation, it was first decided to prepare functionalized divinyl ethers. Derivatization of PEG was not attempted due to (1) the small but inherent polydispersity of PEG and (2) the difficulty to functionalize and purify completely PEG macromonomers needed for polymerization. Also, it was intended to have PEG diols as a "safe" degradation product, and this potentially may not have been possible with derivatized PEG macromonomers. To examine the polymerization of different divinyl ether monomers, reactions were conducted with the model divinyl ether **8** (Scheme 3). Because of the poor solubility of **8** in toluene, the polymerization was conducted in dichloromethane (DCM) to give polyacetal **9** ($M_w \sim 35\,000$ g/mol; $M_w/M_n = 1.9$) after first removing the water via the toluene azeotrope and then distilling off the toluene.



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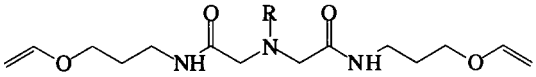

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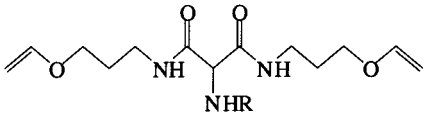

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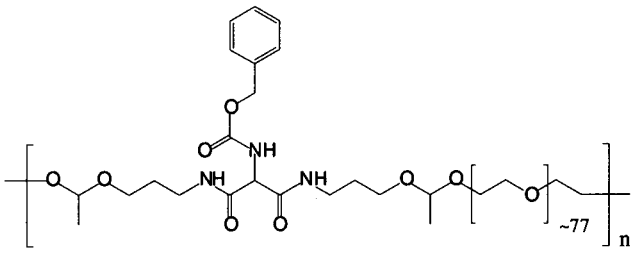
Several functionalized divinyl ethers were examined (e.g., **10**−**12**). To prevent competition during polymerization, it was necessary to protect the amine with a base labile group (e.g., R = Fmoc or Cbz). Reproducible preparation of Fmoc-protected divinyl ether monomers that were pure enough for polymerization proved troublesome. Although Cbz-protected monomers **10**−**12** were more easily purified and polymerized (e.g., **13**), deprotection by hydrogenation was incomplete, and as anticipated, residual palladium-activated charcoal could not completely be separated from the final product.



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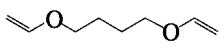
Since monomers **10**−**12** were poorly soluble in toluene, other solvents were examined for the polymerization. PEG and *p*-TSA catalyst were dried in the reaction vessel at 80 °C under high vacuum for 3 h and cooled to ambient temperature, and the divinyl ether monomer was added as a solution in either freshly distilled THF or DCM. In the case of the model polyacetalization using PEG_{3400} **1** and tri(ethylene glycol) divinyl ether **2** (Scheme 1) this procedure gave polyacetal **3** of M_w = 48 000 g/mol ($M_w/M_n = 1.9$). Use of dehydrating agents (e.g., $MgSO_4$) and molecular sieves during polymerization failed to increase polymer molecular weight.

It was concluded that modification of either the divinyl ether or PEG to introduce pendent chain conjugating functionality was not the best strategy, so a terpolymerization process was explored (Scheme 4). The comonomer diol **14** provided an amine pendent functionality in a hydrolytically stable diol that was easily prepared from serinol. One crystallization from chloroform provided **14** in purity sufficient for polymerization. Divinyl ether **2** could be distilled prior to use to ensure its purity. The molecular weight characteristics of the resultant polyacetals **15** (GPC, DMF eluent, PEG standards) are shown in Table 1. Varying the ratios of **1** to **14** during synthesis gave a family of polymers containing different amounts of pendent group as determined by comparing the H NMR integrals for the Fmoc and acetal methyl groups. For example, a ratio of 1:1 of PEG **1** (3400 g/mol) to **14** gave a polyacetal **15** with 4−5 pendent amine groups with a near equal incorporation of PEG. Using the polymer derived from a 1:1 ratio of diols (Table 1) with a starting M_w 24 900 g/mol, it was found that the Fmoc group could be completely removed to give the amine pendent functionalized polyacetal **16**. The deprotection reaction was carried out in 20% piperidine in acetonitrile, and the polymer was isolated by precipitation as a white solid. GPC analysis (using either DMF or PBS at pH 7.4 as the eluent) indictated there was no significant change in the molecular weight characteristics ($M_w = 25\,000$ g/mol; $M_w/M_n = 2.0$) caused by removal of the Fmoc group.



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The commercially available divinyl ether **17** was also examined and used as supplied. The molecular weight characteristics of water-soluble polyacetals prepared by polymerization of this monomer with PEG alone or by

Table 2. Molecular Weight Characteristics of Polyacetals Prepared with the Butanediol Derived Divinyl Ether 17

diol	M_w (g/mol)	M_n (g/mol)	M_w/M_n
PEG_{400}	21 400[a]	8 500	2.54
PEG_{400}:**14** (4:1)	22 400[a]	8 800	2.54
PEG_{3400}	98 100[b]	55 200	1.78
PEG_{3400}:**14** (1:1)	90 000	34 000	2.63

[a] Single μL amounts of divinyl ether **17** were added after the initial reaction period. [b] GPC conducted in DMF (1.0 mL/min) using PEG standards.

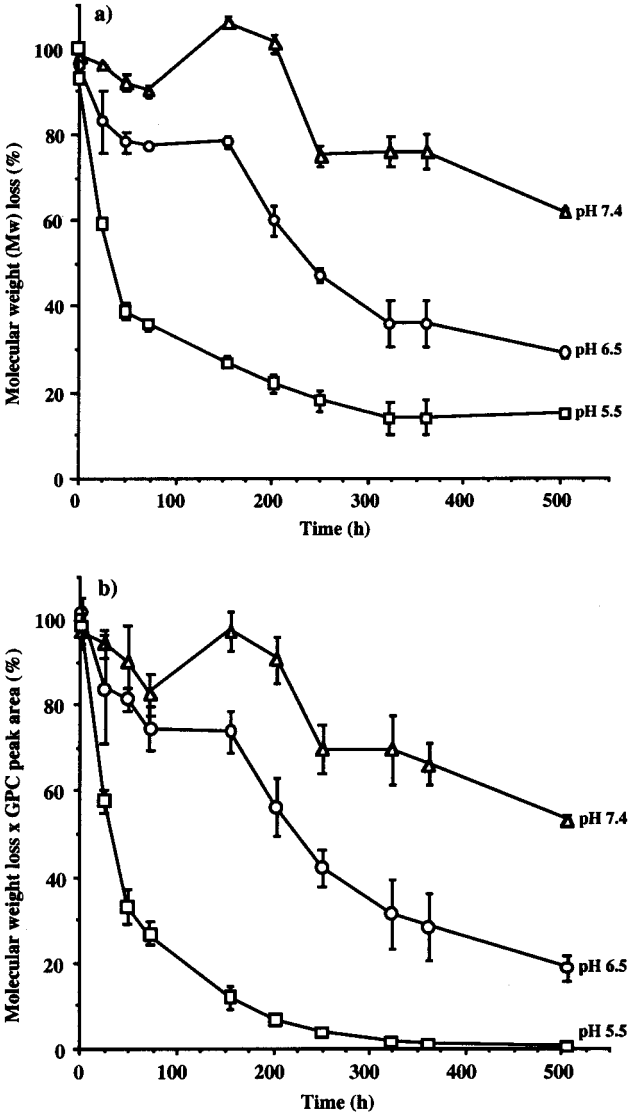


Figure 1. **(a)** pH-dependent degradation profile of polyacetal **3** obtained at the physiologically relevant pH values of 7.4 (blood), 6.5 (endosome), and 5.5 (lysosome). **(b)** Degradation profile of polyacetal **3** showing the relative amount of the polyacetal remaining at each time point. Mean values ± SD ($n = 3$) are shown.

terpolymerization with PEG and **14** are shown in Table 2 (GPC, THF eluent, PEG standards). Divinyl ether **17** was previously used to prepare polyacetals,[19] and since this monomer is more hydrophobic than **2**, it was hoped there would be less trace water to compete during polymerization. The polyacetals derived from this monomer tended to display higher molecular weights, which at present is a factor of 2−4 times, compared to polyacetals derived from the more hydrophilic divinyl ether **2**. The use of monomer **17** provides a wider range of polyacetals for study and indicates that different

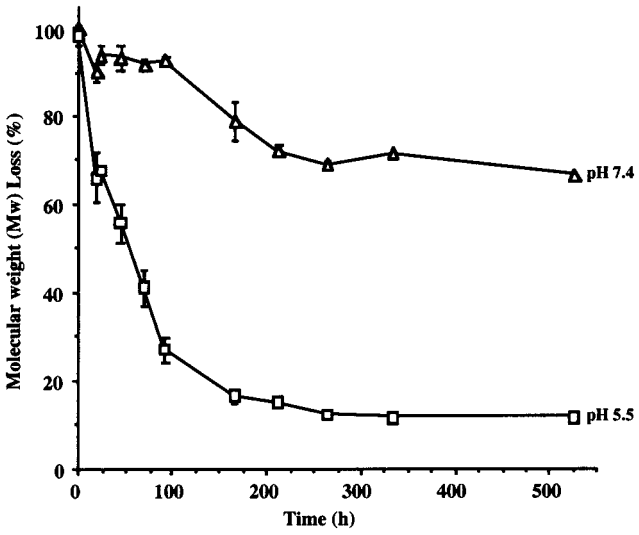
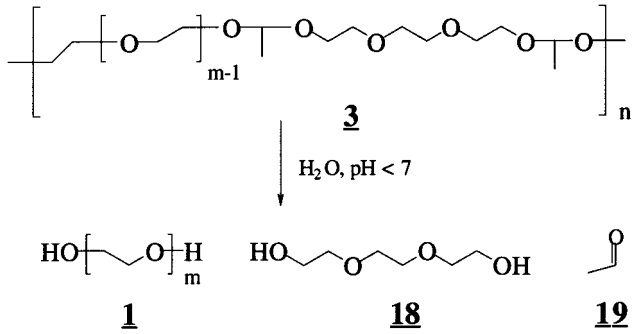


Figure 2. Degradation profile of polyacetal **16** with a starting weight-average molecular weight (M_w) of 25 000 g/mol ($M_w/M_n = 2.0$). Mean values ± SD ($n = 3$) are shown.

Scheme 5. Degradation Products That Are Expected from the Hydrolytic Degradation of Polyacetal 3



commercially available divinyl ethers may yield polymers with different molecular weight characteristics.

3.3. Degradation of Polyacetals. Following synthesis, it was important to determine the rate of degradation of the polyacetals at pH conditions that might be encountered in a biological environment. Polyacetals **3** derived from either PEG_{400} or PEG_{3400} completely degraded within 10−15 min to the monomeric PEG when stirred at ambient temperature in aqueous 0.1 M HCl (pH 1) (results not shown). A more controlled degradation study was required to provide an indication of the degradation profile that might be encountered following cellular uptake. For example, pH 7.4 was chosen to mimic the bloodstream and pH 6.5 and 5.5 to mimic the intracellular compartments, endosomes and lysosomes, respectively. Using polyacetal **3** derived from PEG_{3400} ($M_w \sim 42\,000$ g/mol; $M_w/M_n = 1.6$), it was clearly shown that the rate of degradation seen over 3 weeks was faster at pH 5.5 > pH 6.5 > pH 7.4 (Figure 1a). Considering the molecular weight of the PEG unit (3400 g/mol) data shown in Figure 1a suggests that all the acetal moieties have been hydrolyzed at pH 5.5 after 10−12 days.

Generally, degradation profiles for biomedical polymers are obtained under constant sink conditions, and the results correlated with mass loss of the polymer. Since the polyacetals are water-soluble, the relative peak areas used in the GPC derived molecular weight calculations provided a suitable alternative to indicate the relative amount of the polyacetal which remained

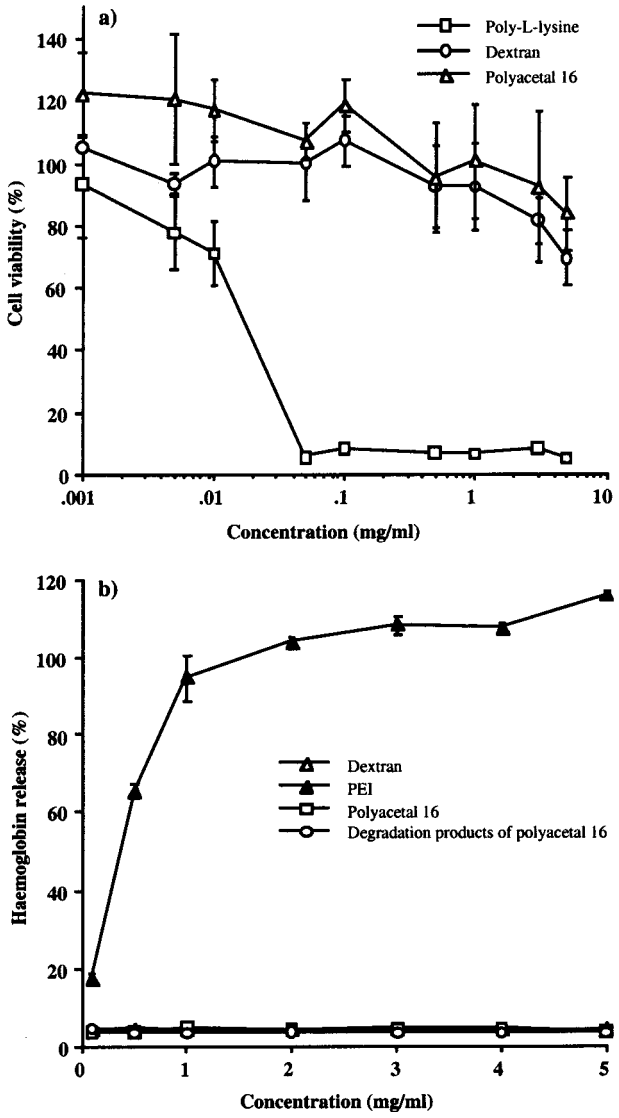
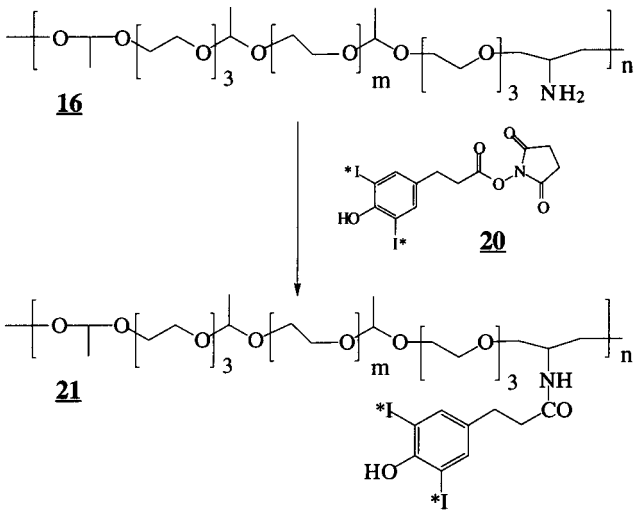


Figure 3. (a) Cytotoxicity assay of amino-polyacetal **16** (B16F10 cell culture) over 72 h. The positive control was polylysine and the negative control was dextran. (b) Red blood cell (RBC) lysis assay of amino-polyacetal **16** and its degradation products over 24 h. The negative control was dextran, and the positive control was PEI. Mean values ± SE (n = 6) are shown.

at each time point. As the polymer degraded there was a notable loss of polyacetal molecular weight with an increase in the presence of PEG_{3400}. As more PEG_{3400} was generated, there was a decrease in the peak area for the part of the GPC trace used to calculate the polymer molecular weight at each time point (Figure 1b). Amino-polyacetal **16** (M_w = 25 000 g/mol; M_w/M_n = 2.0) also displayed an enhanced rate of degradation at pH 5.5 (Figure 2). The molecular weight reduction after 24 h was ~40%, and nearly all the acetal groups appeared to have undergone hydrolysis within 5−6 days which was comparable to the nonfunctionalized polyacetal **3** (Figure 1).

Two observations from these degradation studies are important for the design of polymer therapeutics. As there was a significant amount of degradation at pH 5.5 during the first 2 days (~50 h), this confirms that polyacetal degradation would be fast intralysosomally following pinocytic internalization into the cell. Although polyacetal degradation proceeded more slowly at pH 7.4, the polymer displayed notable degradation

Scheme 6. Conjugation of ^{125}I-Labeled Bolton−Hunter Reagent 20 to Amine Functionalized Polyacetal 16



over a 3 week period. This indicates that these polyacetals may potentially be suitable for parenteral administration at molecular weights greater than the renal threshold. This is an important feature for the design of long-circulating polymer drug conjugates (advantageous for various applications including improved tumor targeting) without concern that a storage disease syndrome might emerge on repeated administration.

3.4. Biocompatibility and Preliminary Body Distribution Studies. It is critical that the novel polymers designed for parenteral use are biocompatible (with respect to their proposed route and frequency of administration). The polymer metabolites must also be nontoxic. Hydrolytic degradation of polyacetals yields an equivalent of an aldehyde for each acetal group that is hydrolyzed. For example, degradation of polyacetal **3** gives the starting PEG **1**, triethylene glycol **18**, and acetaldehyde **19** (Scheme 5). Neither polyacetal **3** (results not shown) nor polyacetal **16** (concentrations up to 5 mg/mL) was cytotoxic toward B16F10 cells (Figure 3a), and polyacetal **3** degradation products were also nontoxic in this assay. Additionally, these polyacetals and their degradation products were nonhemolytic over 24 h (Figure 3b), indicating suitability for iv injection.

It is important that polymeric platforms used for drug targeting do not rapidly localize in liver and lung. This severely limits their usefulness for targeting to other disease sites such as cancer. To monitor the in vivo body distribution of polyacetal **16**, the polymer was radiolabeled with ^{125}I-labeled Bolton−Hunter reagent **20** to give compound **21** (Scheme 6).

The labeling efficiency of the product was 73.6%, and on purification only 1.8% free ^{125}I-iodide remained. The radiolabeled polymer had a specific activity of ~74 μCi/mg and was stable for ≥3 weeks. Following iv administration ^{125}I-labeled polyacetal **16** displayed a linear log blood clearance profile over 24 h (Figure 4a), and elimination was accompanied by appearance of radioactivity in the urine. Urine radioactivity increased to 67.2% of the administered dose after 24 h. In real terms, polyacetal clearance is biphasic with an initial rapid rate of elimination. This can be attributed to the polydispersity of the sample (M_w/M_n = 2.0), the lower M_w fraction being cleared more quickly than the higher M_w which may be approaching the renal threshold for that

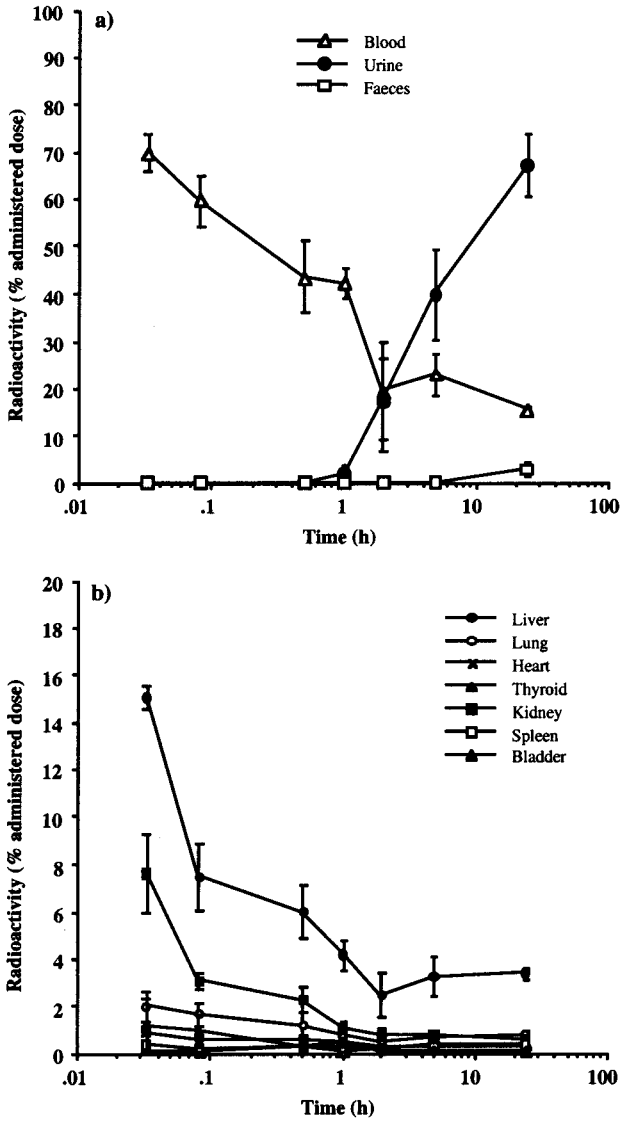


Figure 4. (a) Distribution of radioactivity in the blood, urine, and feces following iv injection. (b) Distribution of radioactivity in the major organs following iv injection. Mean values ± SE ($n = 3$) are shown.

polymer.[8] No specific organ, including the liver, showed significant accumulation of radioactivity (Figure 4b). These observations suggest that this novel family of biodegradable polyacetals have the potential for further development, either as long-circulating platforms for drug delivery (in this case a higher molecular weight product would be required) or as targetable drug carriers (introduction of targeting moieties would be required).

4. Conclusions

Water-soluble, hydrolytically labile polyacetals with pendent amine functionality for drug conjugation applications were synthesized by a terpolymerization process. These polyacetals clearly display pH-dependent degradation (with faster rates of hydrolysis at acidic pH), and their lack of toxicity in the preliminary biocompatibility studies suggests that they have potential for development as new biodegradable polymers for parenteral use. As the polyacetal is not inherently hepatotropic, there is potential to use the polymer for development in a variety of polymer therapeutic applications.

Acknowledgment. We thank A. P. Pharma for financial support and Rhondalea McDonald for technical assistance.

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MA0108867