Macromolecules
ARTICLE

Macromolecules **2010**, *43*, 5205–5210 **5205**
DOI: 10.1021/ma1007013

Synthesis and Characterization of Methionine- and Cysteine-Substituted Phosphazenes

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Received April 1, 2010; Revised Manuscript Received May 13, 2010

ABSTRACT: The preparation of phosphazenes that possess reversible cross-linking groups to control mechanical stability and hydrolysis has been accomplished using cysteine and methionine amino acid side groups. Small molecule models and linear polymeric phosphazenes that contain methionine ethyl ester and cysteine ethyl disulfide ethyl ester side groups were synthesized. Protection of the free thiol groups was carried out to circumvent unwanted cross-linking of the phosphazenes through the cysteine ethyl ester N- and S-termini. Cyclic trimeric cysteine ethyl disulfide ethyl ester model compounds were deprotected by S−S bond cleavage using β-mercaptoethanol, dithiothreitol (DTT), and zinc in aqueous hydrochloric acid. For the high polymeric derivatives, the extent of S−S bond cleavage varied depending on the deprotection method used. With the exception of the Zn/HCl method, the resultant deprotected polymers were soluble in common organic solvents and underwent minimal chain cleavage during the reaction sequence. The protected or deprotected high polymers are candidates for reversible cross-linking in drug delivery systems and for cross-link stabilization of tissue engineering scaffolds.

Introduction

Thiol-containing polymers are potentially useful biomaterials due to their ability to reversibly form disulfide cross-links. Cysteine and methionine units, together with their polymeric derivatives, have been used for the cross-linking of polymer chains,[1−3] biological assays,[4,5] mucoadhesion,[6] nitric oxide generation,[7−10] metal ion detection,[11] and in soft and hard tissue engineering.[12,13] However, most of these polymers yield physiologically questionable hydrolysis products and often lack controllable cross-linking that could be useful to improve mechanical properties in tissue engineering applications. Moreover, most of the properties of these polymers rely on the surface modification of a bulk material, and these useful properties are lost when the material undergoes surface hydrolysis. For these reasons, new biomaterials are needed to avoid the loss of useful characteristics during surface erosion and also to provide controllable cross-linking to improve mechanical strength. The formation of benign hydrolysis products is an additional requirement.

Polyphosphazenes are hybrid organic−inorganic polymer systems composed of a backbone that contains alternating phosphorus and nitrogen atoms with two organic side groups covalently linked to each phosphorus atom. The macromolecular substitution technique, which involves replacement of chlorine atoms in poly(dichlorophosphazene) by organic groups, is a preferred synthesis method for many of these polymers.[14] This method allows facile changes to be made in the polymer side groups to produce materials that are useful for hydrophobicity[15] and hydrolytic sensitivity.[16−20] Most polyphosphazenes with nitrogen-linked amino acid ester side groups are hydrolytically sensitive, and hydrolysis yields phosphates, ammonia, alcohol, and the corresponding amino acid.[17,23] Such properties are valuable for the development of tissue regeneration matrices and for controlled drug delivery applications. However, the development of new macromolecular substitution processes often requires exploratory chemistry carried out on small molecule analogues—in this case on cyclic trimeric phosphazenes.[14]

In this study we have synthesized several novel polyphosphazenes with sulfur-containing amino acid ester side groups. These include methionine ethyl ester-based polymers and various cysteine ethyl ester derivatives in which the S−H units were first protected, before linkage of the side groups to the phosphazene skeleton, and then deprotected to form the free thiol derivatives.[21−25]

Experimental Section

Reagents and Equipment. All synthesis reactions were carried out under a dry argon atmosphere using standard Schlenk line techniques except when aqueous solutions were used. Tetrahydrofuran and triethylamine (EMD) were dried using solvent purification columns.[26] Dichloromethane (EMD), acetic acid (EM Science), hexanes (EMD), ethyl acetate (EMD, diethyl disulfide[25] (Alfa Aesar), β-mercaptoethanol (EMD), dithiothreitol (ChemSampCo), 30% hydrogen peroxide (VWR), cysteine ethyl ester hydrochloride (Alfa Aesar), methionine ethyl ester hydrochloride (Bachem), and 230−400 mesh silica gel (EMD) were used as received. Poly(dichlorophosphazene) was prepared via the thermal ring-opening polymerization of recrystallized and sublimed hexachlorocyclotriphosphazene (Fushimi Pharmaceutical Co., Japan) in evacuated Pyrex tubes at 250 °C.[27] ^{31}P and ^{1}H NMR spectra were obtained with a Bruker 360 WM instrument operated at 145 and 360 MHz, respectively. Glass transition temperatures were measured with a TA Instruments Q10 differential scanning calorimetry apparatus with a heating rate of 10 °C/min and a sample size of ca. 10 mg. Gel permeation chromatograms were obtained using a Hewlett-Packard HP 1100 gel permeation chromatograph equipped with two Phenomenex Phenogel linear 10 columns and a Hewlett-Packard 1047A refractive index detector. The samples were eluted at 1.0 mL/min with a 10 mM solution of tetra-*n*-butylammonium nitrate in THF, and the elution times were calibrated with polystyrene standards. Mass

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Published on Web 05/27/2010

spectrometric analysis data were collected using the turbospray ionization technique on an Applied Biosystems API 150EX LC/MS mass spectrometer.

Reaction Intermediates. *Protection of Cysteine Ethyl Ester Hydrochloride with Ethyl Ethanethiolsulfinate.* Cysteine ethyl ester hydrochloride salt (10.00 g, 53.86 mmol) and an appropriate amount of the above mixture containing 0.5 mol equiv of ethyl ethanethiolsulfinate[25] (3.723 g, 26.93 mmol) were stirred in 100 mL of absolute ethanol at 25 °C for 24 h. The solvent was removed via evaporation to leave a white solid as the crude product. The crude product was recrystallized by dissolution in a minimal amount of dichloromethane, followed by an addition of 5 times the volume of diethyl ether with respect to the amount of dichloromethane. After 1 day, crystals were obtained, filtered, and dried under reduced pressure. The yield was 72%. ^{1}H NMR ($CDCl_3$), ppm: δ 1.26 (m, 6H, OCH_2CH_3 and SCH_2CH_3), 2.70 (q, 2H, SCH_2CH_3), 3.40 (d, 2H, SCH_2CH), 4.26 (q, 2H, OCH_2CH_3), 4.51 (t, 1H, SCH_2CH), 3.40 (d, 2H, SCH_2CH), 8.79 (s-broad, 3H, NH_3Cl).

Small Molecule Model Compounds. *Synthesis of Hexa(methionine ethyl ester)cyclotriphosphazene.*. Hexachlorocyclotriphosphazene (2.00 g, 5.75 mmol) was dissolved in dry THF (10 mL). Methionine ethyl ester hydrochloride (14.8 g, 69.0 mmol) and triethylamine (24.0 mL, 173 mmol) were allowed to react in 150 mL of THF. This solution was refluxed for 24 h, filtered, and added to the hexachlorocyclotriphosphazene solution. The resultant solution was stirred at 25 °C for 96 h followed by removal of all the solvent. The precipitate was dissolved in dichloromethane, and extractions against deionized water were completed. The dichloromethane layer was dried over anhydrous magnesium sulfate, filtered, and dried under vacuum for 1 week. The yield was 67% based on hexachlorocyclotriphosphazene. ^{31}P NMR ($CDCl_3$), ppm: δ +16.28 (3P, s). ^{1}H NMR ($CDCl_3$), ppm: δ 1.30 (3H, t, CH_3), 2.11 (5H, (t)beta-CH_2, (s)beta-CH_3), 2.67 (2H, m, beta-CH_2), 3.67 (1H, t, CH), 4.21 (2H, q, CH_2). MS ($ESCI^+$) m/z = 1191 ($[M+H]^+$) m/z was calculated for $C_{42}H_{84}N_9P_3O_{12}S_6$.

Synthesis of Hexa(cysteine ethyl disulfide ethyl ester)cyclotriphosphazene.. Hexachlorocyclotriphosphazene (5.00 g, 14.4 mmol) was dissolved in dry THF (10 mL). Cysteine ethyl disulfide ethyl ester hydrochloride (24.1 g, 115 mmol) and triethylamine (40.1 mL, 288 mmol) were allowed to react in 150 mL of THF. This solution was refluxed for 24 h, filtered, and added to the hexachlorocyclotriphosphazene solution. The resultant solution was stirred at 25 °C for 96 h followed by removal of all solvent. The residue was dissolved in dichloromethane, and extractions against deionized water were carried out. The dichloromethane layer was dried over anhydrous magnesium sulfate, filtered, and dried under vacuum for 1 week. The yield was 82% based on hexachlorocyclotriphosphazene. ^{31}P NMR ($CDCl_3$), ppm: δ +15.38 (3P, *s*). ^{1}H NMR ($CDCl_3$), ppm: δ 1.26 (3H, t, CH_3), 1.26 (3H, t, CH_3 from ethyl disulfide), 2.67 (2H, q, CH_2 from ethyl disulfide), 3.19 (2H, q, CH_2), 3.57 (1H, t, CH), 4.19 (2H, q, CH_2). MS ($ESCI^+$) m/z = 1384 ($[M + H]^+$) m/z was calculated for $C_{42}H_{84}N_9P_3O_{12}S_{12}$.

Model Compound Deprotection Reactions. *Deprotection of Hexa(cysteine ethyl disulfide ethyl ester)cyclotriphosphazene with β-Mercaptoethanol.* Hexa(cysteine ethyl disulfide ethyl ester)cyclotriphosphazene (1.00 g, 0.722 mmol) was dissolved in dichloromethane (100 mL). β-Mercaptoethanol (0.564 g, 7.22 mmol) was added to the trimer solution and allowed to react for 24 h at 25 °C. The trimer solution was dried and redissolved in methanol. Column chromatography was completed using silica gel and a mixture of ethyl acetate:hexanes (80:20) as the mobile phase. Multiple degrees of deprotection were achieved, and the products were dried under reduced pressure to yield 0.335 g of hexa-deprotected product for a 45% yield. ^{31}P NMR ($CDCl_3$), ppm: δ +15.38 (3P, s). ^{1}H NMR ($CDCl_3$), ppm: δ 1.26 (3H, t, OCH_2CH_3), 1.26 (3H, t, CH_3 from ethyl disulfide), 2.67 (2H, q, CH_2 from ethyl disulfide), 3.21 (2H, d, CH_2S), 3.59 (1H, t, CH), 4.18 (2H, q, OCH_2CH_3). m/z = 1023, 1083, and 1143 ($[M + H]^+$) m/z was calculated for $C_{30}H_{60}N_9P_3O_{12}S_6$, $C_{32}H_{64}N_9P_3O_{12}S_7$, and $C_{34}H_{68}N_9P_3O_{12}S_8$, respectively.

Deprotection of Hexa(cysteine ethyl disulfide ethyl ester)cyclotriphosphazene with Dithiothreitol (DTT). Hexa(cysteine ethyl disulfide ethyl ester)cyclotriphosphazene (1.00 g, 0.722 mmol) was dissolved in dichloromethane (100 mL). Dithiothreitol (DTT) (0.891 g, 5.78 mmol) was added to the trimer solution and allowed to react for 24 h at 25 °C. The trimer solution was dried and redissolved in methanol. Column chromatography was completed using silica gel and a mixture of ethyl acetate: hexanes (80:20) as the mobile phase. The product was dried under reduced pressure to yield 0.635 g of product for an 86% yield. ^{31}P NMR ($CDCl_3$), ppm: δ +15.38 (3P, s). ^{1}H NMR ($CDCl_3$), ppm: δ 1.27 (3H, t, OCH_2CH_3), 3.21 (2H, d, CH_2S), 3.59 (1H, t, CH), 4.18 (2H, q, OCH_2CH_3). m/z = 1023 ($[M + H]^+$) m/z was calculated for $C_{30}H_{60}N_9P_3O_{12}S_6$.

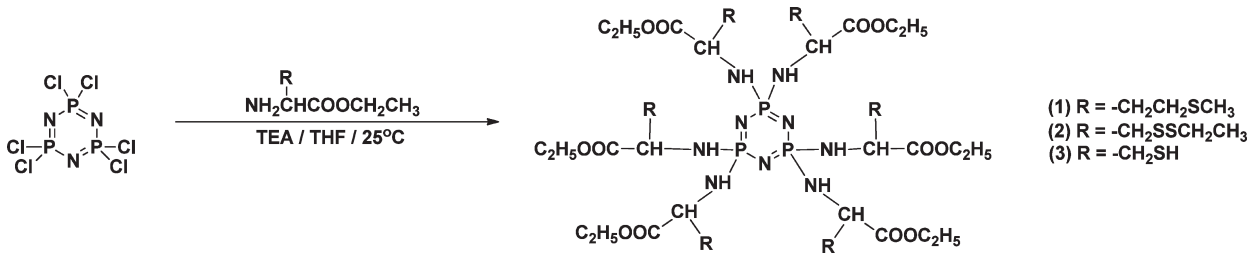
Deprotection of Hexa(cysteine ethyl disulfide ethyl ester)cyclotriphosphazene with Zinc/HCl. Hexa(cysteine ethyl disulfide ethyl ester)cyclotriphosphazene (1.00 g, 0.722 mmol) was dissolved in dichloromethane (100 mL). Zinc powder (0.472 g, 7.22 mmol) and 12 M hydrochloric acid (260 uL, 21.7 mmol) were added to the trimer solution and allowed to react for 24 h at 25 °C. The trimer solution was dried and redissolved in methanol. Column chromatography was completed using silica gel and a mixture of ethyl acetate:hexanes (80:20) as the mobile phase. The product was dried under reduced pressure to yield 0.5193 g of yellow oil for a 73% yield. ^{31}P NMR ($CDCl_3$), ppm: δ + 15.38 (3P, s). ^{1}H NMR ($CDCl_3$), ppm: δ 1.27 (3H, t, OCH_2CH_3), 3.21 (2H, d, CH_2S), 3.59 (1H, t, CH), 4.18 (2H, q, OCH_2CH_3). m/z = 1041 ($[M + NH_4]^+$) m/z was calculated for $C_{30}H_{60}N_9P_3O_{12}S_6$.

Polymer Syntheses. *Poly[bis(methionine ethyl ester)phosphazene]*. Poly(dichlorophosphazene) (2.00 g, 17.3 mmol) was dissolved in dry THF (200 mL). Methionine ethyl ester hydrochloride (10.33 g, 48.3 mmol) and triethylamine (14.5 mL, 104 mmol) were allowed to react in THF (200 mL). This solution was refluxed for 24 h, filtered, and added to the polymer solution. The polymer solution was stirred at room temperature for 24 h and was refluxed for another 48 h. The solution was concentrated and precipitated from THF into hexanes five times. The polymer was soluble in THF and chloroform. The yield was 81% based on poly(dichlorophosphazene). ^{31}P NMR (*d*-THF), ppm: δ −0.2255. ^{1}H NMR (*d*-THF), ppm: δ 1.30 (3H, CH_3), 2.11 (5H, beta-CH_2, beta-CH_3), 2.67 (2H, beta-CH_2), 3.67 (1H, CH), 4.21 (2H, CH_2).

Poly[bis(cysteine ethyl ester)phosphazene]. Poly(dichlorophosphazene) (2.00 g, 17.3 mmol) was dissolved in dry THF (200 mL). Cysteine ethyl ester (12.8 g, 69.0 mmol) and triethylamine (19.3 mL, 138 mmol) were allowed to react in 200 mL of THF. This solution was refluxed for 24 h, filtered, and added to the polymer solution. The solution was then refluxed for 48 h, at which point the polymer precipitated from the THF solution. The polymer was concentrated and dialyzed versus methanol for 3 days. This polymer was soluble in acetic acid and trifluoroacetic acid. The yield was 76% based on poly(dichlorophosphazene). ^{31}P NMR ($CDCl_3$), ppm: δ −0.7220 (P−NHR_2), 0.00 (P−OH), −7.666 (P−SR). ^{1}H NMR ($CDCl_3$), ppm: δ 1.25 (3H, OCH_2CH_3), 3.24 (2H, CH_2S), 3.71 (1H, CH), 4.15 (2H, OCH_2CH_3)

Poly[bis(cysteine ethyl disulfide ethyl ester)phosphazene]. Poly(dichlorophosphazene) (2.00 g, 17.3 mmol) was dissolved in dry THF (200 mL). Cysteine ethyl disulfide ethyl ester (7.95 g, 38.0 mmol) and triethylamine (12.1 mL, 86.5 mmol) were allowed to react in 150 mL of THF. This solution was refluxed for 24 h, filtered, and added to the polymer solution. The solution was then refluxed for 28 h, and the product was precipitated into hexanes. The polymer was redissolved in THF and purified by dialysis versus methanol for 3 days. This polymer was soluble in chloroform, tetrahydrofuran, acetic acid, and trifluoroacetic acid. The yield was 66% based on poly(dichlorophosphazene). ^{31}P NMR ($CDCl_3$), ppm: δ −0.7220. ^{1}H NMR ($CDCl_3$), ppm: δ 1.26 (3H, CH_3), 1.26 (3H, CH_3 from ethyl disulfide), 2.67 (2H, CH_2 from ethyl disulfide), 3.19 (2H, CH_2), 3.57 (1H, CH), 4.19 (2H, CH_2).

Scheme 1. Synthesis of (1) Hexa(methionine ethyl ester)cyclotriphosphazene], (2) Hexa(cysteine ethyl disulfide ethyl ester)cyclotriphosphazene, and (3) Hexa(cysteine ethyl ester)cyclotriphosphazene



Polymer Deprotection Reactions. *Attempted Deprotection of Poly[bis(cysteine ethyl disulfide ethyl ester)phosphazene] with 2.0 mol equiv of Dithiothreitol (DTT) per Repeat Unit.* Poly[bis-(cysteine ethyl disulfide ethyl ester)phosphazene] (2.00 g, 4.33 mmol) was dissolved in dichloromethane (150 mL). Dithiothreitol (1.34 g, 8.66 mmol) was added to the polymer solution and allowed to react for 24 h at 40 °C, It was then concentrated and purified by dialysis versus acetone for 24 h. The solvent was removed by rotary evaporation and was dried under reduced pressure. Only 4.4% of the disulfide bonds were deprotected to the free thiol form with an 88% yield. ^{31}P NMR ($CDCl_3$), ppm: δ −0.7220. ^{1}H NMR ($CDCl_3$), ppm: δ 1.27 (3H, OCH_2CH_3) and (3H, SCH_2CH_3), 2.68 (2H, SCH_2CH_3), 3.19 (2H, CH_2SH), 3.21 (2H, CH_2SS), 3.59 (1H, CH), 4.18 (2H, OCH_2CH_3).

Deprotection of Poly[bis(cysteine ethyl disulfide ethyl ester)phosphazene] with 20.0 mol equiv of Dithiothreitol (DTT) per Repeat Unit. Poly[bis(cysteine ethyl disulfide ethyl ester)phosphazene] (2.00 g, 4.33 mmol) was dissolved in dichloromethane (150 mL). Dithiothreitol (13.36 g, 86.6 mmol) was added to the polymer solution, and the reaction was allowed to proceed for 24 h at 40 °C. The polymer solution was concentrated and purified by dialysis versus acetone for 24 h. The solvent was then removed from the resultant solution, and the residue was dried under reduced pressure. It was calculated that 38% of the disulfide bonds had been deprotected to the free thiol form with an 81% yield. ^{31}P NMR ($CDCl_3$), ppm: δ −0.7220. ^{1}H NMR ($CDCl_3$), ppm: δ 1.26 (3H, OCH_2CH_3) and (3H, SCH_2CH_3), 2.68 (2H, SCH_2CH_3), 3.19 (2H, CH_2SH), 3.21 (2H, CH_2SS), 3.58 (1H, CH), 4.17 (2H, OCH_2CH_3).

Deprotection of Poly[bis(cysteine ethyl disulfide ethyl ester)phosphazene] with 1.0 mol equiv of β-Mercaptoethanol per Repeat Unit. Poly[bis(cysteine ethyl disulfide ethyl ester)phosphazene] (2.00 g, 4.33 mmol) was dissolved in dichloromethane (100 mL). β-Mercaptoethanol (0.71 g, 9.10 mmol per polymer repeat unit) was added to the polymer solution and allowed to react for 48 h at 40 °C. This solution was concentrated and purified by dialysis versus methanol for 3 days. The polymer was isolated by solvent evaporation and was dried under reduced pressure. NMR evidence indicates that only 16% of the disulfide bonds had been deprotected to the free thiol with an 85% yield. ^{31}P NMR ($CDCl_3$), ppm: δ −0.7220. ^{1}H NMR ($CDCl_3$), ppm: δ 1.27 (3H, OCH_2CH_3) and (3H, SCH_2CH_3), 2.68 (2H, SCH_2CH_3), 3.19 (2H, CH_2SH), 3.21 (2H, CH_2SS), 3.59 (1H, CH), 4.18 (2H, OCH_2CH_3).

Deprotection of Poly[bis(cysteine ethyl disulfide ethyl ester)phosphazene] with 5.0 mol equiv of β-Mercaptoethanol per Repeat Unit. Poly[bis(cysteine ethyl disulfide ethyl ester)phosphazene] (2.00 g, 4.33 mmol) was dissolved in a mixture of ethanol (100 mL) and dichloromethane (50 mL). β-Mercaptoethanol (1.69 g, 21.7 mmol per polymer repeat unit) was added to the polymer solution and allowed to react for 48 h at 40 °C. The polymer solution was concentrated and purified by dialysis versus methanol for 3 days. The product was isolated and dried under reduced pressure. Approximately 52% of the disulfide bonds had been deprotected to the free thiol form with an 83% yield. ^{31}P NMR ($CDCl_3$), ppm: δ −0.7220. ^{1}H NMR ($CDCl_3$), ppm: δ 1.26 (3H, OCH_2CH_3) and (3H, SCH_2CH_3), 2.68 (2H, SCH_2CH_3), 3.19 (2H, CH_2SH), 3.21 (2H, CH_2SS), 3.58 (1H, CH), 4.17 (2H, OCH_2CH_3).

Deprotection of Poly[bis(cysteine ethyl disulfide ethyl ester)phosphazene] with Zinc/HCl. Poly[bis(cysteine ethyl disulfide ethyl ester)phosphazene] (0.5 g, 1.08 mmol) was dissolved in tetrahydrofuran (35 mL). Zinc (0.708 g, 10.8 mmol) and 12 M hydrochloric acid (388 uL, 32.4 mmol) were added to the polymer solution, and the mixture was refluxed for 16 h. The solution was concentrated and purified by dialysis versus methanol for 3 days. The polymer was then isolated by solvent evaporation and dried under reduced pressure. ^{31}P NMR spectra suggested that the polymer had decomposed during deprotection.

Results and Discussion

Synthesis of Model Cyclic Trimers 1 and 2. Organic-type reactions carried out on polymer side groups are nearly always more challenging than their counterparts carried out on small molecules. For polyphosphazenes, this tendency can be exaggerated by the sensitivity of P−N bonds to undergo acid cleavage when hydrophilic aliphatic amino-linked side groups are present. Moreover, macromolecular substitution reactions, and especially side group protection and deprotection processes, can become complex unless they are optimized first using small molecule model compounds. In this work small molecule model phosphazene cyclic trimer reactions were carried out by the treatment of hexachlorocyclotriphosphazene with methionine ethyl ester and cysteine ethyl disulfide ethyl ester. An excess of each side group reagent, in the presence of triethylamine as the hydrochloride acceptor, was added to hexachlorocyclotriphosphazene in THF at room temperature as shown in Scheme 1. Complete chlorine replacement occurred to give hexa(methionine ethyl ester)cyclotriphosphazene] (**1**) and hexa(cysteine ethyl disulfide ethyl ester)cyclotriphosphazene (**2**), which yielded ^{31}P NMR signals at +15.8 and +15.3 ppm, respectively. The resulting cyclic trimers were yellow-orange oils that were soluble in common organic solvents. The yields of these compounds were in the range of 67−85% based on hexachlorocyclotriphosphazene. Hexa(cysteine ethyl disulfide ethyl ester)cyclotriphosphazene was further used as a model for cleavage of the disulfide protecting group.

Model Deprotections with Cyclic Trimer 2. The protective disulfide bonds in hexa(cysteine ethyl disulfide ethyl ester)-cyclotriphosphazene (**2**) were cleaved using β-mercaptoethanol, dithiothreitol (DTT), or with Zn^0 in aqueous HCl solution to form **3**. The use of β-mercaptoethanol to deprotect **2** went through several stages of deprotection to give hexa-, penta-, and tetra-cleavage of the disulfide bonds per trimer molecule, identified from the ^{1}H NMR chemical shifts. Mass spectrometry was also used to determine the extent of deprotection, and peaks at 1023, 1083, and 1143 confirmed the hexa-, penta-, and tetra-deprotection, respectively.

The use of dithiothreitol (DTT) as a reducing agent for disulfide bonds was also explored with compound **2**. DTT

Scheme 2. Synthesis of Poly[bis(methionine ethyl ester)phosphazene] (4)

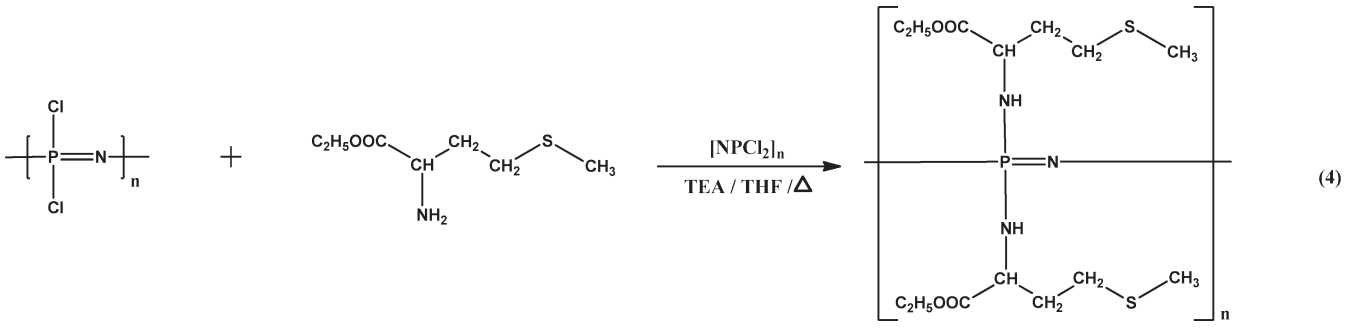


Table 1. Structural and Physical Properties of Polymers 4−6

polymer	^{31}P NMR (ppm)	^{1}H NMR (ppm)	T_g (°C)	M_w[a]	PDI
4	−0.2255	1.30 (3H, CH_3), 2.11 (5H, beta-CH_2, beta-CH_3), 2.67 (2H, beta-CH_2), 3.67 (1H, CH), 4.21 (2H, CH_2)	9	128 000	1.79
5[b]	−0.7220 (P−NHR_2), 0.00 (P−OH), −7.666 (P−SR)	1.25 (3H, CH_3), 3.24 (2H, CH_2), 3.71 (1H, CH), 4.15 (2H, CH_2)	17	N/A	N/A
6	−0.7220	1.26 (3H, CH_3), 1.26 (3H, CH_3 from ethyl disulfide), 2.67 (2H, CH_2 from ethyl disulfide), 3.19 (2H, CH_2), 3.57 (1H, CH), 4.19 (2H, CH_2)	22	160 000	1.81

[a] The GPC-derived molecular weight approximate to M_w. [b] ^{1}H NMR spectra taken in deuterated acetic acid, which causes acidic hydrolysis.

reduced all the disulfide bonds in **2** to the free thiol. The identity of this product was indicated by the absence of peaks from the thioethyl groups in the ^{1}H NMR spectra and was confirmed by mass spectrometry at m/z = 1123. IR verification was inconclusive due to the weak peaks that are associated with C−S and S−S stretches.[28] In addition, the S−H stretch is broadened out due to hydrogen bonding with the α nitrogen and is not detected in the IR spectra. The full deprotection of **2** is consistent with the stronger reduction ability of DTT for disulfide bonds when compared to β-mercaptoethanol.

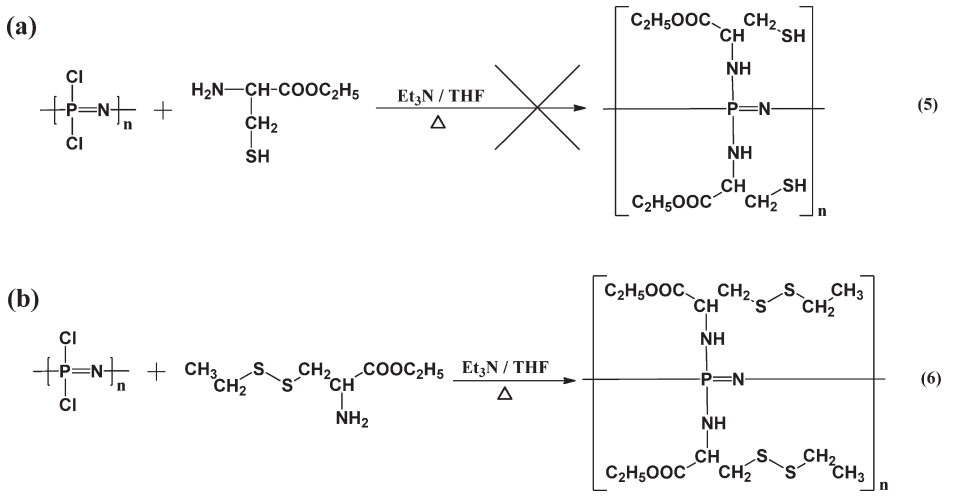
The third method for model deprotection of **2** was the use of zinc powder in an aqueous hydrochloric acid solution. Complete deprotection was accomplished to form **3**. Again, ^{1}H NMR spectroscopy was used to confirm the loss of the thioethyl groups together with the mass spectrometry peak of m/z = 1141 (NH_4^+). A ^{31}P NMR peak at +15.38 ppm was also detected, which is evidence that acidic hydrolysis of the phosphazene ring did not occur during the deprotection.

Synthesis of Polymers 4−6. Methionine is an essential amino acid that contains a thioether group and is thus a monofunctional reagent. Therefore, unlike the cysteine ethyl ester derivative, the methionine sulfur site requires no protection before reaction with poly(dichlorophosphazene). As shown in Scheme 2, methionine ethyl ester reacts cleanly with poly(dichlorophosphazene) in the presence of triethylamine to produce polymer **4**. The complete replacement of chlorine atoms in poly(dichlorophosphazene) by amino acid esters is accompanied by the complete loss of ^{31}P NMR peaks at −16 ppm $(NPCl_2)_n$ and at −7 ppm $(NPCl_1NHR_1)_n$. The physical and structural properties of **4** are shown in Table 1. The ethyl ester of methionine was used because of its structural similarity to cysteine and its utility as a standard for comparison with the thiol functionality in cysteine.

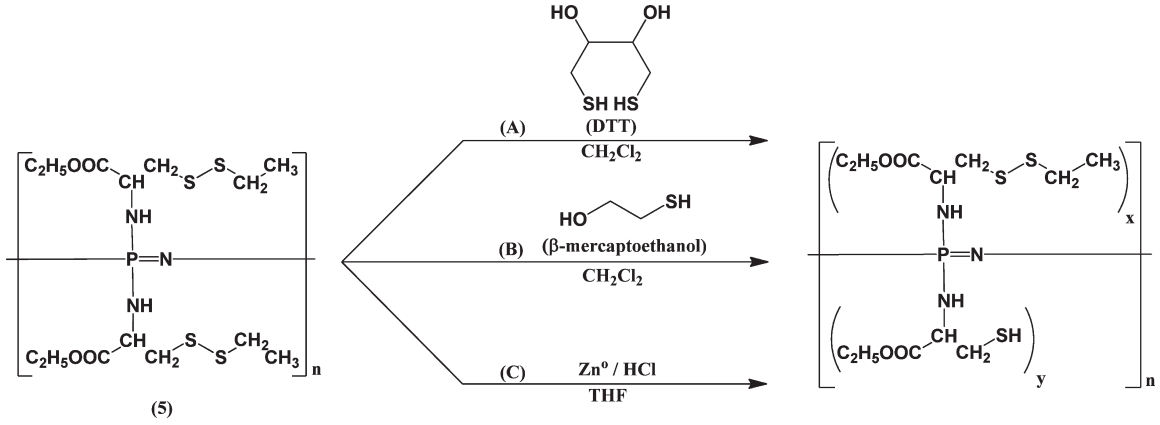
The synthesis of high polymeric phosphazenes with cysteine side groups and their bifunctional character proved to be a greater challenge. The simplest route to these macromolecules was impractical. Thus, cysteine ethyl ester was allowed to react with poly(dichlorophosphazene) in an attempt to form polymer **5** directly. However, the initial ^{31}P NMR spectra indicated that both the thiol and amino functional units replaced chlorine atoms in the polymer. At an early stage in the reaction, the substitution ratio between the thiol and amine reaction sites was equal. However, over time the signal associated with the phosphorus-thiol (P−S) linkage (at +26 ppm) disappeared, leaving only the phosphorus amine (P−N) signal (−1 ppm). This multiple substitution phenomenon was also detected with the small molecule cyclic trimers. The polymer remained soluble in THF at this stage, which was an indication that the free thiol groups had not undergone oxidative dimerization with their counterparts on other chains. However, purification by either dialysis or precipitation in nonsolvents yielded a product that was thereafter insoluble in common organic solvents. It is solubility in acetic acid or trifluoroacetic acid was accompanied by hydrolytic decomposition. Hydrolysis in strong acids is typical of amino acid-substituted polyphosphazenes.[19,20,29] Three possible processes might explain the insolubility in organic media. First, not all of the P−S bonds may have been displaced by P−NHR units. This could yield a cross-linked polymer by the coupling of cysteine units on one chain via the N-terminus of cysteine to another polymer chain via the S-terminus, a process that would be facilitated by the proximity of functional groups in the solid state. Another possibility is that the thiol functionalities are oxidized to disulfide bonds during precipitation or during dialysis. Finally, a combination of both cross-linking during synthesis and oxidation during purification could yield an insoluble product. Small molecule model deprotection reactions of trimer **2** under atmospheric conditions suggested that oxidation to form disulfide bonds is slow but possible. Therefore, it appears that formation of covalent cross-links is the main reason for the insolubility. From these results it was concluded that protection of the thiol group before reaction of the amino acid ester with poly(dichlorophosphazene) is a necessary step to produce un-cross-linked polymers.

The protection of cysteine ethyl ester before linkage to the polyphosphazene was via the deliberate use of disulfide bonds to prevent the formation of cross-links. Thus, ethanethiolsulfinate was synthesized by the reaction of diethyl disulfide with a hydrogen peroxide/acetic acid mixture. The purified product was then allowed to react with cysteine ethyl ester to form the disulfide-protected thiol while leaving an unprotected amino terminus for linkage to the polyphosphazene backbone. Following this step, the cysteine ethyl

Scheme 3. Attempted Synthesis of (a) Poly[bis(cysteine ethyl ester)phosphazene] (5) and Synthesis of (b) Poly[bis(cysteine ethyl disulfide ethyl ester)phosphazene] (6)



Scheme 4. Deprotection of Poly[bis(cysteine ethyl disulfide ethyl ester)phosphazene] Using (A) Dithiothreitol, (B) β-Mercaptoethanol, and (C) Zinc/Hydrochloric Acid



disulfide ethyl ester was allowed to react with poly(dichlorophosphazene) to produce polymer **6** as shown in Scheme 3. The physical and structural properties of polymers **4**, **5**, and **6** are shown in Table 1. The molecular weights for **4** and **6** are typical for poly(amino acid ester phosphazenes) due to hydrolysis induced by hydrogen chloride that is generated during the side group replacement of chlorine in poly(dichlorophosphazene).[17−20,28] In addition, initial hydrolysis of **4** and **6** revealed a sharp ^{31}P peak at 0.0 ppm (phosphate) which is an indicator of sensitivity to hydrolysis that is typical for amino acid linkages to polyphosphazenes.[17,20,28] A complete kinetic study and cellular study is to be completed to determine the rates of hydrolysis and effects on cellular adhesion.

Deprotection of Polymer 6. Polymer **6** was deprotected via three common sulfur−sulfur bond cleavage methods.[25] The first was the use of dithiothreitol (DTT), which is a strong reducing agent for disulfide linkages. DTT was used in varying molar equivalents to produce the free thiol functionality shown in Scheme 4A. Solutions of DTT (2.0 and 20.0 mol equiv) per repeat unit of poly[bis(cysteine ethyl disulfide ethyl ester)phosphazene] were treated in this way and resulted respectively in deprotection of only 4.4% and 38% of the disulfide bonds to form the free thiol. The percent deprotection for all polymers was determined by a comparison of protons between 2.68 (2H, SCH_2CH$_3$) and 4.17 (2H, OCH_2CH$_3$) in the ^{1}H NMR spectrum. The values for x and y in Scheme 4 are determined by the percent deprotection, where a 38% deprotection in disulfide bonds using DTT would result in an x of 0.76 and a y of 1.24. Higher molar equivalences than 20.0 M DTT to poly[bis(cysteine ethyl disulfide ethyl ester)phosphazene] did not bring about any appreciable increase in the extent of deprotection of the disulfide linkages. However, these partially deprotected macromolecules remained soluble in organic solvents.

The second method used the weaker disulfide reducing agent, β-mercaptoethanol (Scheme 4B). Use of 1.0 M and 5.0 mol equiv of β-mercaptoethanol per polymer repeat unit resulted respectively in 16% and 52% disulfide cleavage to form the free thiol, as determined by ^{1}H NMR methods. As with DTT, molar equivalences of β-mercaptoethanol higher than 5.0 M per polymer repeat unit did not increase the number of cleaved disulfide bonds. The T_g of polymer **6** with 52% disulfide deprotection was lowered slightly from 22 to 18 °C with no appreciable change in the molecular weight. Again, these partially deprotected derivatives remained soluble in organic solvents.

The DTT route to deprotection of disulfide linkages should have yielded the highest percentage of deprotection due to its high reactivity toward disulfide bonds. However, the β-mercaptoethanol route gave a higher percentage of cleaved disulfide linkages to form the thiol functionality, possibly because of steric hindrance around the disulfide groups in the polymer. Although DTT is a strong reducing agent for disulfide bonds, it requires appreciable free volume

at the reaction site to cyclize DTT. Cyclization of DTT must occur for reduction of the cysteine disulfide to be successful. The steric hindrance of the side groups presumably inhibits this process. Unlike DTT, β-mercaptoethanol has a lower free volume requirement and can deprotect a larger fraction of the sterically hindered disulfide linkages such as those found in polymer **6**. It is also important to note that both the DTT and β-mercaptoethanol deprotection routes gave polymers that were initially soluble in common organic solvents such as chloroform and tetrahydrofuran. Although not all of the S−H groups are deprotected by this route, it is clear that this polymer has the capacity to cleave disulfide bonds that are useful for reversible cross-links and modification of its properties through reactions at the regenerated −SH groups.

The use of zinc powder in the presence of hydrochloric acid was a third method used to cleave disulfide bonds and produce thiol functionality (Scheme 4C). This approach was attempted to avoid the steric hindrance problems encountered in the previous two methods. Although 100% of the disulfide bonds were reduced to the free thiol by this method, as indicated by ^{1}H NMR spectroscopy, the polymer backbone underwent concurrent acid cleavage, clearly detected by ^{31}P NMR spectroscopy.

Conclusions

In this work, the first cyclic trimeric phosphazenes and high polymeric phosphazenes that contain methionine ethyl ester and cysteine ethyl disulfide ethyl ester have been synthesized. The small molecule hexa(cysteine ethyl disulfide ethyl ester)cyclotriphosphazene was deprotected using β-mercaptoethanol, dithiothreitol (DTT), or zinc in aqueous hydrochloric acid. Deprotection by β-mercaptoethanol resulted in hexa-, penta-, and tetra-disulfide cleavage, while deprotection by DTT and zinc in a hydrochloric acid solution resulted in complete cleavage of the disulfide bonds.

At the polymeric level, the deprotection of poly[bis(cysteine ethyl disulfide ethyl ester)phosphazene] by dithiothreitol or β-mercaptoethanol yielded polymers that were un-cross-linked and soluble in common organic solvents. Poly[bis(cysteine ethyl disulfide ethyl ester)phosphazene] deprotected by dithiothreitol (DTT) achieved a maximum of 38% disulfide cleavage, while deprotection by β-mercaptoethanol cleaved a maximum of 52% disulfide bonds. Nevertheless, even the polymers with free thiol and some protected side groups may prove to be useful in tissue engineering applications since hydrolysis of these species may still yield biocompatible products while retaining the disulfide cross-linking properties needed for materials strengthening. It is also recognized that high concentrations of these side groups may prove detrimental when the polymers are used as tissue engineering scaffolds. Therefore, limited amounts of these side groups can be used together with more compatible amino acid esters while achieving similar affects.

Acknowledgment. We thank the Fushimi Pharmaceutical Co., Japan, for the partial support of this work.

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