Synthesis and Characterization of New Biomedical Polymers: Serine- and Threonine-Containing Polyphosphazenes and Poly(L-lactic acid) Grafted Copolymers

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ABSTRACT: Polyphosphazenes that contain serine and threonine side groups have been synthesized by the macromolecular substitution technique, and their structure and properties were determined. Serine and threonine have two different sites for covalent linkage to the polyphosphazene backbone. For one series of polymers, serine ethyl ester and threonine ethyl ester units were linked to the polyphosphazene skeleton via the *N*-terminus. The free hydroxyl groups on each side group were then used to graft poly(L-lactide). Graft densities of 1:0.5, 1:2, and 1:4 grafts per repeat unit were controlled through the stoichiometric ratios of sodium naphthalenide to polyphosphazene side groups during the synthetic procedures. For the second series of macromolecules, the hydroxyl function of serine or threonine was used as the site for covalent attachment to the polyphosphazene backbone, a process that required protection of both the *N*- and *C*-termini. Secondary reactions to remove the protective groups then yielded side units with free amino and carboxylic acid functions. These polymers (with serine or threonine groups linked via the hydroxyl function) are water-soluble over a broad pH range but have the ability to form ionic cross-links in the presence of calcium ions.

Introduction

Numerous polymers have received attention as biomaterials.[1,2] Of these, macromolecules that contain amino acid units are potentially useful in many different medical applications such as bioerodible sutures,[3,4] tissue engineering scaffolds,[5−7] and drug delivery matrices.[8−10] Amino acid based polymers generally possess good mammalian cell compatibility and yield benign hydrolysis products. However, polymers composed of amino acids alone have limited thermal stability and are insoluble in many solvents, which makes these materials difficult to fabricate and utilize.

Polyphosphazenes are hybrid macromolecules with a backbone of alternating phosphorus and nitrogen atoms and with two organic side groups linked to each phosphorus atom. Most of the known polyphosphazenes have been synthesized by replacement of the chlorine atoms in poly(dichlorophosphazene) by organic groups. The molecular structure of the organic side groups has a strong influence on the physical and chemical properties of these polymers.[11] Alkoxides, aryloxides, and primary amines are the most common reagents used for chlorine replacement with poly(dichlorophosphazene),[12−14] and certain physical properties, such as hydrolytic sensitivity and chemical reactvity, can be controlled by the nature of the side groups and by their substitution pattern.[15−17] Some amino-substituted polyphosphazenes are susceptible to hydrolysis.[18,19] Polymers with alkoxy or aryloxy side groups are generally more stable in aqueous media. This property allows secondary reactions to be carried out on the alkoxy or aryloxy side groups, including processes that require acidic or basic reaction conditions. However, amines are generally more reactive toward poly(dichlorophosphazene) than are alcohols or phenols, although conversion of an alcohol or phenol to its metal alkoxide or aryloxide markedly increases the reactivity. The relatively low reactivity of a hydroxyl group in a multifunctional nucleophile minimizes the need for protection/deprotection chemistry during the introduction of complex side groups. This allows considerable choice of side groups for linkage to the polymer backbone.

A few polyphosphazenes that contain simple amino acid esters side chains have been investigated for their bioerodible medical applications.[20−22] For example, earlier work has shown that the hydrolysis rates of N-functional amino acid ester substituted polyphosphazenes decrease as the steric bulk of the substituent on the α-carbon of the amino acid residue increases. This creates options for controlling the bioerosion of the final material.[18,20,23] Polyphosphazenes that bear nonpolar amino acid esters have good mammalian cell compatibility as well as near-neutral hydrolysis products. The synthesis of these polymers is relatively straightforward because they have only a single reactive site (the amino group) for coupling to the polyphosphazene backbone. Tyrosine-based reactants offer more possibilities because tyrosine has two different points of covalent attachment to the polyphosphazene backbone (the free amino terminus and the phenolic hydroxyl group at the α-carbon). Thus, two types of tyrosine-based polyphosphazenes are known: those that bear tyrosine groups linked through the amino functionality and those linked through the hydroxyl site. The amino-linked tyrosine-based polymers are hydrolytically sensitive. The analogue linked through the hydroxyl function are soluble in aqueous acidic and basic pH media, are hydrolytically stable, and can form hydrogels in the presence of multivalent cations, such as Ca^{2+} or Al^{3+}.[24] This development suggested that the use of other multifunctional side groups could confer additional useful properties to this class of macromolecules.

More complex amino acids, such as serine and threonine esters, also have two different functional sites as potential points of linkage to the polyphosphazene. Use of these reactants has

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Table 1. Weight and Molar Equivalents Used for the Synthesis of Polymers 4a-c and 5a-c

polymer	poly (dichlorophosphazene) (mg, mmol)	sodium (mg, mmol)	naphthalene (mg, mmol)	L-lactide (mg, mmol)
4a	500,1.62	149,6.47	829,6.47	4660,32.3
4b	500,1.62	18.6,0.808	104,0.808	583,4.04
4c	500,1.62	9.29,0.404	51.8,0.404	466,3.23
5a	500,1.48	136,5.93	760,5.93	4270,29.6
5b	500,1.48	17.0,0.741	95.0,0.741	534,3.71
5c	500,1.48	8.52,0.371	4.75,0.371	427,2.96

not been examined previously as side groups on polyphosphazenes because of the danger of cross-linking during macromolecular substitution. However, the presence of up to three different functional sites on these amino acids should allow access to macromolecules with different properties by the use of different functional sites for coupling to the polyphosphazene skeleton. We report here the first synthesis of serine and threonine based polyphosphazenes and describe the subsequent secondary reactions to yield novel hybrid macromolecules.

Experimental Section

Reagents and Equipment. All polymer synthesis reactions were carried out under a dry argon atmosphere using standard Schlenk line techniques. Tetrahydrofuran and triethylamine (EMD) were dried using solvent purification columns.[25] Serine ethyl ester hydrochloride (Bachem), threonine (Bachem), Boc-serine methyl ester (Aldrich), and Boc-threonine methyl ester (Aldrich) were used as received. Threonine ethyl ester hydrochloride was synthesized following procedures reported in the literature.[26] Poly(dichlorophosphazene) was prepared by the thermal ring-opening polymerization of recrystallized and sublimed hexachlorocyclotriphosphazene (Fushimi Chemical Co., Japan) in evacuated Pyrex tubes at 250 °C. ^{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. The elution times were compared to polystyrene standards. Infrared spectroscopy samples were analyzed using a Digilab FTS 7000 spectrometer with 32 scans per sample.

Synthesis of Polyphosphazenes with N-Linked Serine Ethyl Ester or Threonine Ethyl Ester (Polymers 2 and 3). Both of the syntheses leading to polymers **2** and **3** were carried out in a similar fashion. The synthesis of polymer **2** is given as an example. Poly(dichlorophosphazene) (2.00 g, 17.3 mmol) was dissolved in dry THF (200 mL). Serine ethyl ester hydrochloride (8.20 g, 48.3 mmol) was suspended in dry THF (150 mL), and triethylamine (14.4 mL, 104 mmol) was added. The mixture was stirred and refluxed for 24 h, then filtered, and added to the polymer solution. The polymer solution was stirred at room temperature for 24 h and then refluxed for an additional 24 h. The solution was filtered and then concentrated to dryness. The polymer was redissolved in methanol and dialyzed against methanol for 3 days, followed by precipitation from THF into hexanes. The final product was isolated as a brittle, yellow material (yield = 85−87%). The molecular structures of polymers **2** and **3** were analyzed with ^{1}H and ^{31}P NMR techniques.

Synthesis of Poly(L-lactide) Graft Copolymers 4a,b,c and 5a,b,c. The syntheses of polymers **4** and **5** were carried out in a similar manner, with the reaction amounts described in Table 1. The synthesis of polymer **4a** is given here as an example. All reactions took place in an argon atmosphere glovebox. Polymer **2** (500 mg, 1.62 mmol) was dissolved in dry THF (5 mL). Sodium (149 mg, 6.47 mmol) and naphthalene (829 mg, 6.47 mmol) were suspended in dry THF (2 mL). A sodium/naphthalide suspension (1 mL) was added to the solution of polymer **2** in THF, and the mixture was stirred at room temperature for 20 min. L-Lactide (4.66 g, 32.3 mmol) was then dissolved in dry THF (5 mL), and then this solution was added to the solution of polymer **2**. The reaction mixture was stirred at room temperature overnight, followed by termination with acetic acid. The product was precipitated from THF into cold methanol (three times). The final polymer was isolated as a brittle, white material (yield = 50−70%). The molecular structures of polymers **4** and **5** were analyzed with ^{1}H and ^{31}P NMR techniques, together with diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy.

Synthesis of Polymers 6a and 7a. The synthesis of polymers **6a** and **7a** followed similar procedures. The preparation of polymer **6a** is described. Poly(dichlorophosphazene) (2.20 g, 19.0 mmol) was dissolved in dry THF (150 mL). Boc-serine methyl ester (10.0 g, 45.6 mmol) dissolved in dry THF was added to a suspension of sodium hydride (60% suspension in mineral oil, 1.83 g, 45.6 mmol) in dry THF (150 mL). After the mixture was stirred overnight at room temperature, the amino acid ester solution was added to the polymer solution. This reaction mixture was refluxed for 48 h, then concentrated to dryness, and redissolved in methanol. Purification involved dialysis of the product against methanol for 3 days followed by removal of solvent. The final polymer structure was determined by ^{1}H and ^{31}P NMR techniques (yield = 86−90%).

Synthesis of Polymers 6b and 7b. The synthesis of polymer **6b** is described. Polymer **6a** (300 mg, 0.623 mmol) was suspended in THF (10 mL). Potassium *tert*-butoxide (979 mg, 8.72 mmol) was dissolved in THF (20 mL) and cooled to 0 °C. Water (84.1 mg, 4.67 mmol) was added to the potassium *tert*-butoxide solution, and the medium was mixed at 0 °C. This solution was added to the polymer suspension, and the reaction mixture was stirred at room temperature for 36 h. Aqueous hydrochloric acid (1 M) was added to the polymer solution, and the product was then concentrated to dryness. A brown solid was isolated. This product was dissolved in a 50% solution of trifluoroacetic acid and dichloromethane and stirred at room temperature overnight. The solvent was removed, and the white product was recovered (yield = 90%).

Results and Discussion

Synthesis Principles. In this work the amino and hydroxyl groups of serine and threonine were employed in different synthesis pathways for coupling to the polyphosphazene skeleton. In one set of experiments, the free amino terminus of the serine and threonine ethyl esters was used for linkage to the polyphosphazene backbone. After the coupling, structural characterization of the resultant polymers indicated that the hydroxyl groups in the side units had not participated in chlorine replacement even in the presence of triethylamine as a hydrochloride acceptor. Because the hydroxyl function remained free after linkage of the amino acid ester to the backbone, this site could be used for the formation of a poly(L-lactide) graft structure. The graft densities were controlled through the stoichiometry of the reactants.

For the second set of polymers, the hydroxyl terminus of the amino acid ester was used for replacement of the chlorine atoms in poly(dichlorophosphazene). This required that both the *N*- and carboxylic acid-termini of serine or threonine were protected with *tert*-butoxycarbonyl and a methyl ester unit, respectively. After linkage to the polyphosphazene skeleton, the subsequent deprotection of the amino and carboxylate units generated water-soluble polymers irrespective of the pH of the aqueous media. The reaction conditions used for deprotection of the amimo and carboxylate sites did not alter the chain lengths of the products. The oxygen-linked serine- or threonine-substituted polyphosphazenes with free carboxylic acid functional groups became ionically cross-linked in the presence of multivalent cations such

Scheme 1. Synthesis of Polyphosphazenes 2 and 3 with Serine or Threonine Ethyl Ester Side Groups Attached via the *N*-Terminus of the Amino Acid Ester

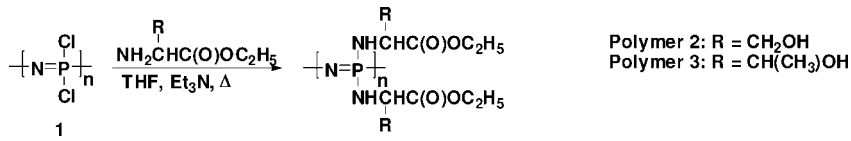


Table 2. Molecular Characterization and Thermal Transitions of Polymers 2 and 3[a]

polymer	^{31}P NMR (ppm)	^{1}H NMR (ppm)	molecular weight[a] (g/mol)	T_g (°C)
2	−0.9	1.1 (6H), 3.2 (2H), 4.0 (4H), 4.2 (4H)	265 000	25.4
3	−1.3	1.2 (6H), 1.4 (6H), 3.1 (2H), 4.1 (4H), 4.2 (2H)	244 000	24.7

[a] The GPC molecular weights were calculated as M_w.

as calcium ions. Each of these amino acid side substituted polyphosphazenes is potentially useful as a biomedical material.

Synthesis of Polyphosphazenes with N-Linked Serine Ethyl Ester or Threonine Ethyl Ester (Polymers 2 and 3). The *N*-terminus of serine ethyl ester and threonine ethyl ester was utilized to replace all the chlorine atoms of poly(dichlorophosphazene) (**1**), as shown in Scheme 1. Molecular structure characterization was conducted by ^{31}P and ^{1}H NMR spectroscopy. These data are shown in Table 2. The hydroxyl function of the amino acid ester did not react with poly(dichlorophosphazene) under these conditions. The linkage of the amino groups to the polyphosphazene backbone was confirmed by ^{31}P NMR spectra based on a single, broad shift at ∼0 ppm, which was evidence for a fully amino-substituted polyphosphazene with no P−Cl bonds remaining. The resultant products were isolated as yellow, brittle solids that were soluble in tetrahydrofuran, chloroform, and methanol.

Molecular weights and glass transition temperatures (T_g) were determined by GPC and DSC techniques (Table 2). The T_g of polymers **2** and **3** were 25.4 and 24.7 °C, respectively, with no crystalline transitions detected. The lack of crystallinity suggests that the substituent at the α-carbon of the amino acid ester creates enough free volume to prevent ordered packing of the side groups and indirectly allows extensive segmental motion of the polyphosphazene backbone.

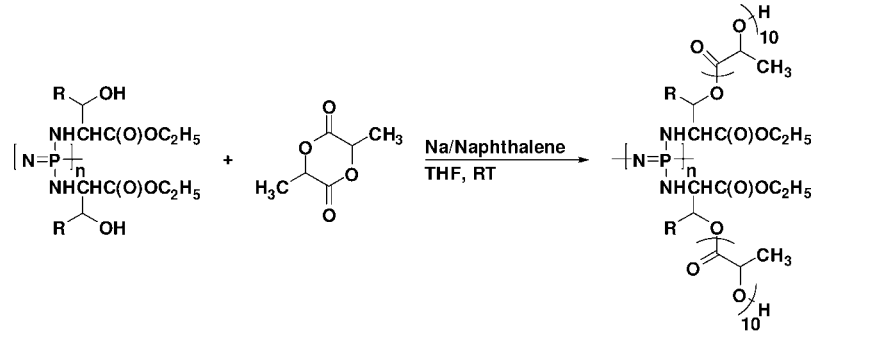
Synthesis of Poly[bis(serine ethyl ester)phosphazene]-*graft*-poly(L-lactic acid) and Poly[bis(threonine ethyl ester)-phosphazene]-*graft*-poly(L-lactic acid) (Polymers 4 and 5). Following the synthesis of polymers **2** and **3**, secondary reactions were conducted using the free hydroxyl functions at the α-carbon atom of the serine or threonine residues. These hydroxyl groups were reactive sites for the formation of polyester grafts linked covalently to the side group structure. The growth of poly(L-lactic acid) from the alcohol terminus was accomplished by an anionic ring-opening polymerization of the L-lactide initiated by sodium naphthalide. This synthesis is described in Scheme 2, with the molar amounts given in Table 1. The lengths of the poly(L-lactic acid) side chains were controlled by the reactant stoichiometry to within 10 repeating units.

Specifically, the graft density of poly(L-lactic acid) (PLA) depended on the concentration of the sodium/naphthalenide complex used to initiate the lactide polymerization. Six different macromolecules were synthesized, starting from polymers **2** and **3**, with average graft densities of 0.25 0.5, and 2 grafts per phosphazene repeat unit. Proton NMR and IR techniques confirmed that the PLA grafts were present and were covalently linked to the polyphosphazene backbone. The loss of the O−H vibrational stretching modes at ∼3200 cm^{-1} in the infrared spectra suggested that the hydroxyl groups had been converted to ester linkages by connection to PLA. The P−N vibrations were detected at 1214 cm^{-1}. Proton NMR spectra confirmed the presence of the $-CH_3$ protons from PLA at 1.6 ppm. DSC analysis of polymers **4a** and **5a** revealed a thermal transition at 40 °C with a crystalline melting transition at 50 °C. However, the ^{31}P NMR spectra were inconclusive because no phosphorus signal was detected. This was probably caused by the poor solubility of the polymer in the solvent and the ∼5% detection limit for ^{31}P nuclei. However, when deprotected polymers **4b**, **4c**, **5b**, and **5c** were synthesized, the ^{31}P NMR spectra showed a strong signal from the phosphorus nuclei. As the graft density decreased, the crystalline melting transition disappeared. This suggests that the grafts were spaced far enough apart to avoid packing into crystalline domains. The graft lengths were also short enough to minimize the formation of microcrystallinities.

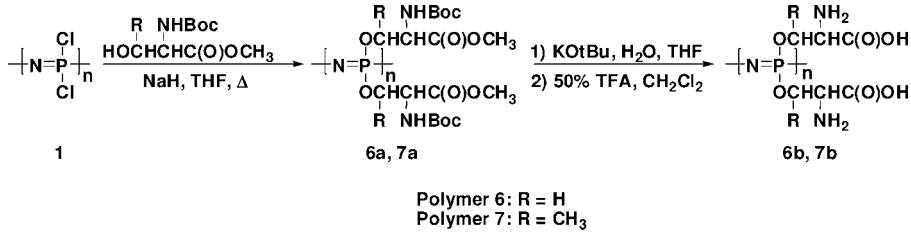
Synthesis of Polyphosphazenes with O-Linked Boc-serine Methyl Ester and Boc-threonine Methyl Ester (Polymers 6a and 7a). Boc-protection of the *N*-terminus of the amino acid allowed the hydroxyl function of serine or threonine to be converted to the sodium alkoxides and used for chlorine replacement with poly(dichlorophosphazene). Without amine protection, and in the presence of sodium hydride, there would be a competitive replacement of chlorine atoms along the polyphosphazene backbone by both nucleophilic sites and a likelihood of cross-link formation. A single-substituent polyphosphazene (i.e., only one type of side group along the chain) was synthesized with the boc-serine and boc-threonine methyl esters, as outlined in the first step in Scheme 3. The methyl ester protecting groups were used because of commercial availability. The ester protection was subsequently removed after the amino acid was linked to the polyphosphazene backbone. Structural characterization of polymers **6a** and **7a** was conducted with the use of ^{1}H and ^{31}P NMR techniques. A shift at −8.2 ppm in the ^{31}P NMR spectra confirmed the linkage of the protected amino acid through an oxygen linkage to the backbone. The absence of a peak at 0 ppm further confirmed the proposed substitution pattern. The proton NMR spectra showed all the expected peaks for the boc-protected amino acid esters, especially the presence of the boc-protection group at 1.4 ppm and the methyl ester at 3.7 ppm. The molecular weight of polymer **6a** was M_w = 822 kg/mol and for polymer **7a** was M_w = 869 kg/mol. This corresponds to ∼1710 repeat units for each polymer.

Deprotection of Polymers 6a and 7a. The amino and carboxylic acid termini of the side groups in polymers **6a** and **7a** were deprotected sequentially by a base-catalyzed cleavage of the methyl ester units, followed by acid catalyzed removal of the boc-protection group, as outlined in the second step of Scheme 3. The structures of polymers **6b** and **7b** were confirmed by proton NMR techniques, particularly the disappearance of two signals—from the *tert*-butyl protons of the boc-protection group at 1.4 ppm and the methyl ester protons at 3.7 ppm. The deprotection conditions did not affect the main chain length of polymers **6b** and **7b** since the molecular weights were measured to be M_w = 820 kg/mol and M_w = 866 kg/mol, respectively. Polymers **6b** and **7b** were soluble in water, irrespective of the

Scheme 2. Synthesis of Polymers 4a−c and 5a−c That Contain Poly(L-lactide) Grafts Grown from the Alcohol Function of Serine or Threonine Ethyl Ester



Scheme 3. Synthesis of Polymers 6 and 7 with Serine and Threonine Covalently Linked to the Polyphosphazene Backbone via the Hydroxyl Function of the Amino Acid



pH. There was no detectable hydrolysis of polymers **6b** and **7b** after dissolution in aqueous media. This is a characteristic of polyphosphazenes with P−OR side groups.

Ionic Cross-Linking of Polymers 6b and 7b. Polymers **6b** and **7b** were dissolved in water and treated with an aqueous solution of calcium chloride. The solutions became viscous and formed a gel after ~30 min. The gel slowly swelled with the addition of water. Aqueous sodium chloride was then added and the gel redissolved. The calcium ions act as ionic cross-linking agents that are bound to the carboxylate groups of different polymer chains. The sodium ions displace the calcium ions and cause the hydrogel to dissolve. This procedure is explained in Figure 1.

Synthetic Conclusions and Biomedical Potential

This is the first reported synthesis of polyphosphazenes that contain serine or threonine side groups. The multiple functionality of serine and threonine allows different properties to be generated according to the site of linkage to the polymer skeleton. When the carboxylic acid units remain blocked by ester formation, the unprotected hydroxyl functionality can be utilized for secondary reactions such as the formation of poly(L-lactide) grafts from hydroxyl units. The hydroxyl function was also utilized for direct linkage of the amino acid to the backbone through oxygen atoms when both the amino terminus and the carboxcylic acid groups are protected. In this way boc-serine methyl ester and boc-threonine methyl ester were linked to the polyphosphazene backbone. The *N*- and *C*-termini were subsequently deprotected to yield polymers that were ionic in character. The alkoxy linkage of the side groups to the backbone yielded polymers that were both soluble in aqueous media and hydrolytically stable. The anionic charge on the carboxylate function on the amino acid was used for ionic cross-linking with divalent ions to form hydrogels. The gels redissolved following the addition of monovalent ions such as sodium. This behavior could potentially be used for drug delivery applications in which drug molecules trapped in the hydrogel matrix are released following exposure to monovalent cations or for the control of the activity of living cells trapped in the hydrogel matrix.

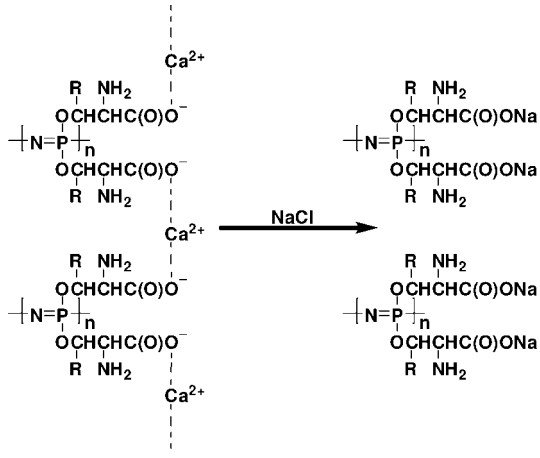


Figure 1. Calcium cross-linked serine and threonine containing polyphosphazenes.

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