Macromolecules

ARTICLE

pubs.acs.org/Macromolecules

Polyphosphazenes Containing Vitamin Substituents: Synthesis, Characterization, and Hydrolytic Sensitivity

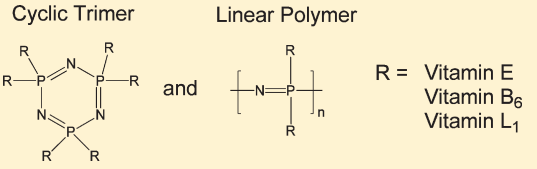
Nicole L. Morozowich,[†] Arlin L. Weikel,[†] Jessica L. Nichol,[†] Chen Chen,[†] Lakshmi S. Nair,[‡] Cato T. Laurencin,[‡,§] and Harry R. Allcock*[,†]

[†]Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802, United States

[‡]Department of Orthopaedic Surgery, University of Connecticut, Farmington, Connecticut 06030, United States

[§]Department of Chemical, Materials and Biomolecular Engineering, University of Connecticut, Storrs, Connecticut 06269, United States

ABSTRACT: Novel polyphosphazenes containing various vitamin substituents were synthesized and characterized, and their sensitivity to hydrolysis and pH behavior was investigated. Vitamins L_1, E, and B_6 were used because of their biocompatibility, their importance in a variety of biological functions, and their potential to increase the mechanical properties of the resulting polymers, thus making these materials promising candidates for hard tissue engineering scaffolds. Chlorine replacement reactions were carried out initially with the small molecule, hexachlorocyclotriphosphazene, as a model for high polymeric poly(dichlorophosphazene). Because of the steric hindrance generated by vitamin E as a substituent, co-substituted polymers were synthesized with either glycine ethyl ester or sodium ethoxide as the second substituent. Similarly, vitamin B_6 was co-substituted with glycine ethyl ester or phenylalanine ethyl ester to favor biodegradability. To prevent cross-linking via multifunctional reagents, the hydroxyl groups in vitamin B_6 were protected and subsequently deprotected under acidic conditions after side group linkage to the polymer backbone. The glass transition temperatures of the polymers ranged from −24.0 to 44.0 °C. Hydrolysis of the polymers in deionized water at 37 °C was used as an initial estimate of their hydrolytic sensitivity. Different solid polymers underwent 10−100% weight loss in 6 weeks with the generation of a broad pH range of ∼2.5−9. The weight loss during preliminary hydrolysis experiments was attributed to cleavage of the polymer backbone and/or the polymers becoming soluble in the aqueous media during hydrolytic reactions.



INTRODUCTION

The development of new biomedical materials is a major challenge in synthetic polymer chemistry. The challenge involves a balancing of synthesis methodology with the need for specific physiochemical and mechanical properties. Synthetic polymers are attractive candidates for use in a wide variety of biomedical applications, such as drug delivery,[1] adhesives,[2] antimicrobial coatings,[3] and tissue engineering.[4,5] The polymer properties required for tissue engineering vary significantly depending on the type of tissue to be regenerated. However, all candidate polymers need to be biocompatible, promote cellular growth and differentiation, degrade into nontoxic products which can be excreted or metabolized, provide sufficient mechanical stability, and have high porosity regardless of tissue type.[6] Moreover, polymers used as scaffolding materials for hard tissue engineering require a high degree of mechanical stability to support bone growth.[7] In earlier work a range of polymers has been investigated for this application such as poly(lactic acid) and poly(lactic-*co*-glycolic acid). However, the primary concern with these polymers is the acidic byproduct produced during hydrolysis that can cause inflammation and tissue necrosis at and around the implant site.[8] Another concern with these materials and with other synthetic polymers is their inability to stimulate specific cellular responses.[9] Naturally derived polymers, such as collagen and chitosan, favor cellular adhesion, proliferation, and differentiation, although these macromolecules do not provide the mechanical strength necessary for hard tissue engineering.[10] Therefore, a need exists to develop novel polymers that have high mechanical stability while incorporating biomolecules that can stimulate cellular responses.

Polyphosphazenes are highly tunable polymers that can be synthesized for use in a variety of biomedical applications, such as drug delivery[11] or tissue engineering.[12] Polyphosphazenes are hybrid organic−inorganic systems with alternating phosphorus and nitrogen atoms in the skeleton and with two (usually organic) side units covalently bonded to each phosphorus atom. An important reaction intermediate for these syntheses is poly(dichlorophosphazene), $(NPCl_2)_n$, the chlorine atoms of which can be replaced by organic units via macromolecular substitution with various nucleophiles to generate biostable or bioerodible polymers.[13] Previous research in our program has focused on the synthesis of amino acid ester-substituted polyphosphazenes because of their hydrolytic sensitivity.[14−16] Hydrolysis of these polymers produces a buffering medium that consists of phosphates and ammonia, together with the corresponding amino acid and alcohol.[17] These polymers have also been co-substituted with more sterically hindered nucleophiles, such as *o*-cresol, *m*-cresol, and *p*-phenylphenol, to increase the glass transition

Received: December 1, 2010
Revised: January 17, 2011
Published: February 14, 2011

temperature and improve the mechanical properties while maintaining hydrolysis sensitivity.[18,19] However, the phenols employed as cosubstituents in earlier work are potentially toxic and need to be replaced by substituents that are biologically compatible but equally sterically hindered.[20] Biocompatible substituents, such as known biomolecules, should provide the steric hindrance required to maintain high mechanical stability and be conducive to cellular growth.

Vitamins are important in a variety of biological functions.[21] For example, B vitamins are essential for growth, development, and maintaining healthy cells,[22] vitamin E is a natural antioxidant which protects cells from damage caused by free radicals and improves the calcium content/mechanical properties of bone tissue,[23−25] and vitamins D and A are important for bone growth.[21] An example of the effect of incorporating vitamins into the polymeric scaffold was shown by the incorporation of vitamin E into a poly(3-hydroxybutyrate)/bioglass composite scaffold.[26] A significant increase in the activity of osteoblasts (bone forming cells) occurred while decreasing the bone resorption activity of osteoclasts (bone removing cells). A disadvantage of incorporating vitamins into the polymeric scaffold as a nonbonded additive is that they may diffuse from the polymer matrix and not provide a significant long-term effect.[27] Thus, in this work we chose vitamins that are suitable for covalent linkage to the polyphosphazene skeleton via macromolecular substitution reactions. Covalently bound vitamins would then provide a platform to guide incoming cells, while releasing the vitamin substituents during the scaffold degradation. Here we describe the synthesis of both small molecule model phosphazene analogues containing vitamin E (DL-α-tocopherol), L_1 (ethyl 2-aminobenzoate), and B_6 (pyridoxine) and their corresponding polymers. Other related polymers that contain vitamin E and B_6 were co-substituted with amino acid ester units, glycine and phenylalanine ethyl ester, to overcome the steric restrictions that accompany the use of vitamin E and the biostability of vitamin B_6. A preliminary hydrolysis study in deionized water at physiological temperature (37 °C) was carried out to monitor the weight loss and pH change over a 6 week period.

EXPERIMENTAL SECTION

Reagents and Equipment. All synthesis reactions were carried out using standard Schlenk line techniques and a dry argon atmosphere. The glassware was dried overnight in an oven at 125 °C before use. Tetrahydrofuran and triethylamine (EMD) were dried using solvent purification columns with the final water content monitored by Karl Fisher titration.[28] Dichloromethane (EMD), diethyl ether (EMD), methanol (EMD), hexanes (EMD), pyridoxine hydrochloride (TCI), trifluoroacetic acid (EM Science), glycine ethyl ester hydrochloride (Chem Impex), phenylalanine ethyl ester hydrochloride (Chem Impex), sodium hydride (60% in mineral oil, Sigma-Aldrich), Sephadex G-25 (VWR), and Sephadex LH-20 (VWR) were used as received. Ethyl 2-aminobenzoate (Sigma) and DL-α-tocopherol (TCI) were dried under vacuum at 60 °C for 96 h before use. DL-α-Tocopherol was further dried using activated molecular sieves (4 A, powder, Alfa Aesar). Spectra/Por molecular porous cellulose dialysis membranes with molecular weight cutoff of 12 000−14 000 were used for purification of the polymers. Poly(dichlorophosphazene) was prepared by the thermal ring-opening polymerization of recrystallized and sublimed hexachlorocyclotriphosphazene (Fushimi Chemical Co., Kyoto, Japan) in evacuated Pyrex tubes at 250 °C.[29] ^{31}P and ^{1}H NMR spectra were obtained with a Bruker 360 WM instrument operated at 145 and 360 MHz, respectively. ^{31}P shifts are reported in ppm relative to 85% H_3PO_4 at 0 ppm. 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 chromatography was performed using a Hewlett-Packard 1047A refractive index detector and two Phenomenex Phenogel linear 10 columns. 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 calibrated with polystyrene standards. Mass spectrometric analysis data were collected using a turbospray ionization technique on an Applied Biosystems API 150EX LC/MS mass spectrometer. pH values were measured using a VWR Symphony SB70P pH meter.

Synthesis of 3,4′-*O*-Isopropylidene. This compound was synthesized using a published procedure with a modification to the starting material preparation and purification.[30] To a suspension of pyridoxine hydrochloride (5.00 g, 24.3 mmol) and 2,2-dimethoxypropane (50.1 mL, 408 mmol) in 75 mL of acetone was added *p*-toluenesulfonic acid monohydrate (18.5 g, 97.2 mmol). This mixture was stirred for 20 h under an inert atmosphere. The dark brown solution was neutralized with aqueous sodium bicarbonate, concentrated under reduced pressure, and extracted with dichloromethane several times. The organic layers were combined and dried with magnesium sulfate. The product was recrystallized from dichloromethane/ether. The yield was 80%. ^{1}H NMR (360 MHz, $CDCl_3$); δ 7.86 (s, 1H, NC*H*C), 4.93 (s, 2H, CHCCH_2OH), 4.55 (s, 2H, CCH_2OC), 2.36 (s, 3, CCH_3), 1.54 (s, 6H, CH(CH_3)$_2$). MS (ESI$^+$, 50% MeOH/50% DCM): m/z 196 ([M + H]).

Synthesis of Cyclic Trimer Models (2−5). *Synthesis of 1,3,5-Tri(DL-α-tocopherol)-1,3,5-tri(ethylglycinato)cyclotriphosphazene (**2**).* Hexachlorocyclotriphosphazene (1.00 g, 2.88 mmol) was dissolved in dry THF (10 mL). DL-α-Tocopherol (8.67 g, 20.1 mmol) was dissolved in THF (100 mL), and this solution was added to a suspension of sodium hydride (0.792 g, 20.0 mmol) in THF (35 mL). After the formation of the sodium salt, the solution was added to the hexachlorocyclotriphosphazene solution dropwise over a period of 1 h, and the mixture was stirred for 24 h at room temperature and then for 72 h at reflux. A solution of glycine ethyl ester hydrochloride (1.61 g, 11.5 mmol) and triethylamine (3.00 mL, 23.0 mmol) in THF (50 mL) was refluxed for 24 h, filtered, and added to the refluxing trimer solution. The resultant reaction mixture was refluxed for 168 h and was monitored by mass spectrometry for the appearance of **2** at 1729 m/z. The product was then subsequently dried under reduced pressure. The oily residue was soluble in dichloromethane, and the excess starting material was removed by flash chromatography. The oily residue was dried under vacuum for 1 week. Physical and structural characterization data are presented in Table 1.

*Synthesis of 1,3,5-Tri(DL-α-tocopherol)-1,3,5-tri(ethoxy)cyclotriphosphazene (**3**).* Hexachlorocyclotriphosphazene (1.00 g, 2.88 mmol) was dissolved in dry THF (10 mL). DL-α-Tocopherol (4.95 g, 11.5 mmol) was dissolved in THF (50 mL), and this was added to a suspension of sodium hydride (0.449 g, 11.2 mmol) in THF (50 mL). After the formation of the sodium salt, the solution was added to the hexachlorocyclotriphosphazene solution dropwise over a period of 1 h, and the reaction mixture was stirred for 24 h at room temperature then for 72 h under reflux. Ethanol (16.8 mL, 287.7 mmol) was distilled from calcium hydride, added directly to sodium (0.661 g, 28.8 mmol), and allowed to react for 72 h at room temperature. When all the sodium had been consumed, the sodium salt was added to the trimer solution and the mixture was refluxed for 72 h. The reaction was monitored by MS for the appearance of **3** at 1558 m/z. It was subsequently dried under reduced pressure. The oily residue was soluble in dichloromethane/methanol, and the excess starting material was removed by size exclusion column chromatography using Sephadex GH-20. The oily residue was dried under vacuum for 1 week. Physical and structural characterization data are presented in Table 1.

*Synthesis of Hexa(ethyl-2-aminobenzoate)cyclotriphosphazene (**4**).* Hexachlorocyclotriphosphazene (2.00 g, 5.75 mmol) was dissolved in dry THF (20 mL). Ethyl 2-aminobenzoate (11.65 g, 69.0 mmol)

Table 1. Characterization Data for Small-Molecule Model Reactions

trimer	^{31}P (ppm)	^{1}H (ppm)	*m/z*
2	18.5[a]	4.20 (2H, q, gly), 3.42 (2H, t, gly), 2.60 (2H, t, toco), 2.17 (9H, t, toco), 1.78 (2H, m, toco), 1.54 (3H, m, toco), 1.39 (4H, m, toco), 1.26 (13H, m, toco), 1.15 (4H,m, toco and 3H, gly), 0.85 (12H, m, toco)	1729[c]
3	14.3[a]	4.02 (2H, q, ethoxy), 2.60 (2H, t, toco), 2.17 (9H, t, toco), 1.78 (2H, m, toco), 1.54 (3H, m, toco), 1.39 (4H, m, toco), 1.26 (13H, m, toco and 3H, d, ethoxy), 1.15 (4H,m, toco), 0.85 (12H, m, toco)	1558[c]
4	3.61[a]	7.6 (1H, d, NHCC*H*CH), 7.0 (1H, t, CCHC*H*CH), 6.4 (2H, t, CC*H*CHC*H*CH), 4.10 (2H, t, CH_2CH_3), 1.1 (3H, d, CH_2CH_3)	1120.6[c]
5a	17.67[a]	7.91 (s, 1H, NC*H*C), 4.78 (s, 2H, CHCCH_2OH), 4.73 (s, 2H, CCH_2OC) 2.40 (s, 3, CCH_3), 1.45 (s, 6H, CH(CH_3)$_2$)	1383[c]
5b	17.01[b]	7.91 (s, 1H, NC*H*C), 5.12 (s, 2H, CHCCH_2OH), 4.80 (s, 2H, CCH_2OC), 2.58 (s, 3, CCH_3)	1144[d]

[a] NMR spectra was measured in $CDCl_3$. [b] NMR spectra were in D_2O. [c] Mass spectra for solution in 50% MeOH/50% DCM. [d] Mass spectra for solution in 100% MeOH.

and triethylamine (19.2 mL, 138.0 mmol) were then added to the hexachlorocyclophosphazene solution. The mixture was stirred for 4 h at room temperature, then dimethylaminopyridine (0.703 g, 5.75 mmol) was added, and the solution was refluxed for 96 h followed by removal of all the solvent. The oily residue was dissolved in dichloromethane, and the excess starting material was removed by flash chromatography. The dichloromethane solution was extracted with deionized water and subsequently dried over magnesium sulfate, filtered, and dried under vacuum for 1 week. A red/orange oil was obtained with 62% yield based on the amount of hexachlorocyclotriphosphazene. Physical and structural characterization data are presented in Table 1.

*Synthesis of Hexa(3,4′-O-isopropylidene)cyclotriphosphazene (**5a**).* 3,4′-*O*-Isopropylidene (4.49 g, 23.0 mmol) was dissolved in dry THF (400 mL) and added to a suspension of sodium hydride (0.909 g, 22.7 mmol) in THF (50 mL). The resultant solution was stirred at room temperature for 24 h, at which point the sodium salt precipitated from solution. Hexachlorocyclotriphosphazene (1.00 g, 28.8 mmol) dissolved in THF (10 mL) was added dropwise to the salt solution over a period of 2 h, during which the sodium salt dissolved. The resulting solution was then refluxed for 24 h followed by removal of the solvent under reduced pressure. The oily residue was dissolved in dichloromethane, extracted with deionized water, and subsequently dried over magnesium sulfate, filtered, and dried under vacuum for 1 week. An orange solid was obtained. The yield was 75.4% based on the amount of hexachlorocyclotriphophazene. Physical and structural characterization data are shown in Table 1.

*Deprotection of Hexa(3,4′-O-isopropylidene)cyclotriphosphazene (**5a**) To Form Hexa(pyridoxine)cyclotriphosphazene (**5b**).* Hexa(3,4′-*O*-isopropylidene)cyclotriphosphazene (**5**) (0.50 g, 0.360 mmol) was dissolved in methanol (2 mL). To this was added 80% trifluoroacetic acid (2 mL, 53.0 mmol) in methanol, and the mixture was stirred at 50 °C. After 48 h the solvent was removed under reduced pressure, and the oily residue was redissolved in dichloromethane and extracted with deionized water. The water layer was concentrated and purified by size exclusion column chromatography using Sephadex G-25. The product was dried under vacuum for 1 week to give a yellow oil in 40% yield. Physical and structural characterization data are given in Table 1.

Polymer Syntheses (7−12). The syntheses followed a similar pattern, and the differences are emphasized in the following descriptions.

*Synthesis of Poly[((DL-α-tocopherol)$_{0.66}$(ethylglycinato)$_{1.34}$)phosphazene] (**7**).* Poly(dichlorophosphazene) (2.00 g, 17.3 mmol) was dissolved in dry THF (200 mL). DL-α-Tocopherol (8.18 g, 18.9 mmol) was dissolved in dry THF (75 mL) and was added to a suspension of sodium hydride (0.687 g, 17.3 mmol) in THF (50 mL). The reaction was allowed to proceed for 24 h, and the mixture was then added dropwise to the polymer solution. After 24 h at room temperature and 72 h refluxing, a suspension of glycine ethyl ester hydrochloride (4.82 g, 34.5 mmol) and triethylamine (12.0 mL, 86.3 mmol) in THF (50 mL) was refluxed for 24 h and added to the polymer solution by filter addition. The resultant solution was refluxed for 48 h, then dimethylaminopyridine (2.10 g, 17.3 mmol) was added, and the solution was refluxed for an additional 72 h, at which point ^{31}P NMR spectra were consistent with complete chlorine replacement. The reaction mixture was cooled to room temperature, filtered, concentrated, and dialyzed versus THF/methanol (75/25) for 3 days. A brown solid was obtained in a 73% yield. Physical and structural characterization data are shown in Table 3.

Table 2. Deprotection Conditions for Polymers 11a and 12a

polymer	amount of acid	temp (°C)	time (h)	% removed
11a	4.27 g; 74.2 mmol	25	50	95
12a	3.22 g; 69.9 mmol	25	50	95

*Synthesis of Poly[((DL-α-tocopherol)$_{0.94}$(ethoxy)$_{1.06}$)phosphazene] (**8**).* Poly(dichlorophosphazene) (2.00 g, 17.3 mmol) was dissolved in dry THF (200 mL). DL-α-Tocopherol (8.18 g, 18.9 mmol) was dissolved in dry THF (75 mL) and was added to a suspension of sodium hydride (0.687 g, 17.3 mmol) in THF (50 mL). After 24 h the reactant solution was added dropwise to the polymer solution. After refluxing for 48 h, sodium ethoxide (2.64 g, 38.8 mmol) was added, and the solution was refluxed for an additional 168 h. The reaction mixture was concentrated, precipitated into methanol (3 times), and dried under vacuum for 5 days. A light brown solid was obtained, and the yield was 85.9%. Physical and structural characterization data are given in Table 3.

*Synthesis of Poly[bis(ethyl-2-aminobenzoate)phosphazene] (**9**).* Poly(dichlorophosphazene) (3.00 g, 25.8 mmol) in dry THF (300 mL) was treated with ethyl 2-aminobenzoate (13.39 g, 90.6 mmol) and triethylamine (39.7 mL, 285 mmol). The reaction mixture was refluxed for 120 h and was subsequently cooled to room temperature, filtered, and dialyzed versus dichloromethane/methanol (50/50) for 3 days. The resultant polymer was dried under vacuum for 5 days to give an off-white solid in a 43% yield. Physical and structural characterization data are presented in Table 3.

*Synthesis of Poly[bis(3,4′-O-isopropylidene)phosphazene] (**10a**).* Poly(dichlorophosphazene) (1.00 g, 8.63 mmol) was dissolved in dry THF (100 mL). 3,4′-*O*-Isopropylidene (5.95 g, 28.4 mmol) in dry THF (25 mL) was added to a suspension of sodium hydride (1.10 g, 27.6 mmol) in THF (100 mL). This solution was allowed to react for 24 h at room temperature, at which point the sodium salt precipitated from solution. The polymer solution was then added dropwise to the suspension over a period of an hour. This mixture was refluxed for 72 h, concentrated, and dialyzed versus methanol for 3 days. After dialysis, the polymer solution was concentrated and washed with triethylamine for 3 h. The resultant solution was dried, dissolved in dichloromethane, and extracted with water to remove the triethylamine hydrochloride salts. A brown solid was obtained in 64% yield. Physical and structural characterization data are listed in Table 3.

Table 3. Characterization Data for Polymers 7−12

polymer	^{1}H NMR (ppm)	^{31}P NMR(ppm)	M_w (g/mol)	PDI	T_g (°C)
7	3.89 (2H), 3.55 (2H), 2.52 (2H), 2.09 (2H), 1.97 (9H), 1.49 (3H), 1.40 (4H), 1.25 (13H), 1.08 (7H), 0.82 (12H)	−1.27, 5.30[a]	178 956	1.80	−24.3
8	3.78 (2H), 2.43 (2H), 2.10 (2H), 1.95 (9H), 1.47 (3H), 1.33 (4H), 1.22 (13H), 1.14 (7H), 0.87 (12H)	−10.46, −14.3, −19.93[a]	705 170	1.48	−19.9
9	8.14 (1H), 7.87 (1H), 7.46 (1H), 6.75 (1H), 4.40 (2H), 1.38 (3H)	−13.1 (broad)[a]	131 410	2.43	26.0
10a	7.72 (1H), 4.60 (4H), 2.16 (3H), 1.23 (6H)	−3.47 (broad)[b]	306 825	1.63	30.8
11a	7.87 (1H), 4.75 (4H), 4.15 (2H), 3.75 (2H), 2.30 (3H), 1.25 (9H)	1.01, −4.31[b]	266 002	1.45	44.0, 30.7
12a	7.87 (1H), 7.12 (5H), 4.75 (4H), 3.91 (2H), 3.36 (1H), 2.84 (2H), 2.29 (3H), 1.35 (6H), 0.86 (3H)	−3.01 (broad)[a]	478 150; 86 839	1.89	35.4
10b	8.26 (1H), 5.10 (4H), 2.54 (3H)	−3.60 (broad)[c]	N/A	N/A	38.0
11b	8.16 (1H), 5.02 (4H), 4.00 (2H), 3.73 (2H), 2.52 (3H)	0.98, −4.64[c]	N/A	N/A	34.3
12b	7.91 (1H), 7.20 (5H), 4.96 (4H), 4.05 (2H), 3.36 (1H), 2.84 (2H), 2.35 (3H), 1.05 (3H)	−2.01 (broad), −4.40 (broad)[d]	N/A	N/A	40.0

[a] NMR spectra was measured in $CDCl_3$. [b] NMR spectra was obtained in MeOD. [c] NMR spectra were in d_4-acetic acid. [d] NMR spectra was measured in d_6-DMSO.

*Synthesis of Poly[((3,4′-O-isopropylidene)$_{1.0}$(ethylglycinato)$_{1.0}$)-phosphazene] (**11a**).* Polymers **11a** and **12a** were synthesized using similar procedures. Polymer **11a** is given as a representative example. Poly(dichlorophosphazene) (1.00 g, 8.63 mmol) was dissolved in dry THF (100 mL). 3,4′-*O*-Isopropylidene (2.34 g, 11.2 mol) in dry THF (25 mL) was added to a suspension of sodium hydride (0.414 g, 10.3 mmol) in THF (100 mL). This mixture was allowed to react for 24 h at room temperature, at which point the sodium salt precipitated from solution. The polymer solution was then added dropwise to the salt solution over a period of 3 h to obtain a partially substituted polymer. In a separate vessel, glycine ethyl ester hydrochloride (2.17 g, 15.5 mmol) was suspended in THF (50 mL) and triethylamine (5.53 mL, 39.7 mmol). This suspension was refluxed for 24 h, filtered, and added dropwise to the polymer solution over a period of an hour. The reaction mixture was stirred at room temperature for 24 h and was subsequently filtered, concentrated, and dialyzed versus methanol for 3 days. The resulting polymer was dried under vacuum for 5 days to give a brown solid in a 62.7% yield. Physical and structural characterization data are shown in Table 3.

Polymer Deprotection Reactions (10−12). The deprotection of polymers **10a**−**12a** followed similar procedures. A representative example of polymer **10a** is described below; deprotection conditions for polymers **11a** and **12a** are shown in Table 2. Poly[bis(3,4′-O-isopropylidene)phosphazene] (**10a**) (0.50 g, 1.08 mmol) was dissolved in 80% trifluoroacetic acid in methanol (6.24 g, 108 mmol). This solution was stirred at 50 °C for 48 h. It was then diluted with methanol and dialyzed versus methanol for 3 days. The solvent was removed under reduced pressure, and the residue was dried under vacuum for 24 h. A brown solid was obtained with a 63% yield. 95% of the acetal protecting group was removed. Physical and structural characterization data are presented in Table 3.

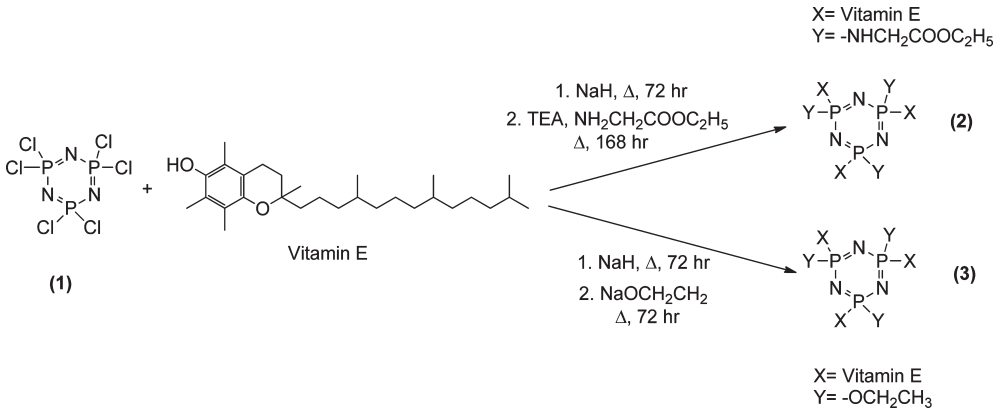
Hydrolysis Study of Polymers 7−12 and Detection of Hydrolysis Products. Polymers **7**−**9** were dissolved in tetrahydrofuran, and polymers **10a**−**12a** were dissolved in methanol (100 mg/1 mL). These were solution-cast into square films (4 × 4 cm). Polymers **10b**−**12b** were cast directly from the concentrated methanol dialysis solution. The films were divided into 18 samples ∼10 mg each and were placed in 5 mL of deionized water with a pH of 6.60. The samples were secured in a shaker bath maintained at 37 °C for 6 weeks. After each week, three samples were removed for each polymer. The aqueous media were decanted, and the pH was measured while the solid samples were dried under vacuum and then weighed. In addition, the hydrolysis medium was analyzed using ninhydrin and silver nitrate tests to detect the presence of ammonia and/or amino acids and phosphates. ^{1}H and ^{31}P spectroscopies were utilized to verify these results.

RESULTS AND DISCUSSION

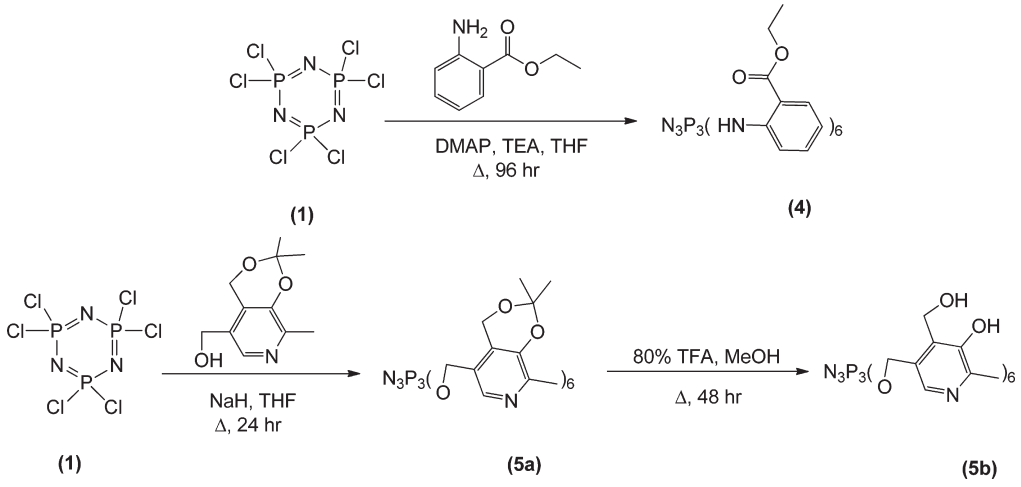
Synthesis of Model Compounds 2−5. In general, halogen replacement reactions using $(NPCl_2)_n$ become more challenging as the structural complexity of the nucleophile is increased. The influences of steric hindrance and multiple reactive functionalities are of special concern. The synthesis and characterization of small molecule phosphazenes is a necessary prelude to the synthesis of new phosphazene high polymers due to the synthetic challenges of macromolecular substitution reactions. Thus, small molecule model reactions were first attempted with the cyclic phosphazene analogue, hexachlorocyclotriphosphazene (**1**). All the vitamins used were substituted onto **1** in varying amounts. The physical and structural data are given in Table 1. Preliminary attempts were made to synthesize fully substituted vitamin-containing cyclophosphazenes. Thus, an excess of the side group nucleophile was allowed to react with **1** in THF, as shown in Schemes 1 and 2. The reactions were monitored by ^{31}P NMR spectroscopy and mass spectrometry techniques. It was not possible under these conditions to obtain full halogen replacement with vitamin E reactions (**2** and **3**). Thus, after the reaction had been allowed to proceed for several days, a second nucleophile was added to complete the substitution. Because of the bulky structure of vitamin E, small nucleophiles such as sodium ethoxide and glycine ethyl ester were used as cosubstituents. Complete chlorine replacement occurred in each case. The products contained equal numbers of the two side groups arranged in a nongeminal fashion as shown in Scheme 1. Other products with smaller amounts of vitamin E were also evident from these model reactions. However, a maximum of one chlorine atom per $NPCl_2$ unit could be replaced by vitamin E. This suggests that similar limits might prevail with the linear high polymer.

For nucleophiles **4** and **5** (vitamins L_1 and B_6) as substituents, all the chlorine atoms in $(NPCl_2)_3$ could be replaced. Reagent **4** gave only partial substitution after several days in refluxing THF at 70 °C, but the addition of dimethylaminopyridine (DMAP) induced full substitution after refluxing for 4 days.[31] The synthesis of **5** first required protection of two of the free

Scheme 1. Synthesis of [(Vitamin E)$_3$(glycine ethyl ester)$_3$cyclotriphosphazene] and [(Vitamin E)$_3$(ethoxy)$_3$cyclotriphosphazene]



Scheme 2. Synthesis of [(Vitamin L$_1$)$_6$cyclotriphosphazene] and [(Vitamin B$_6$)$_6$cyclotriphosphazene]



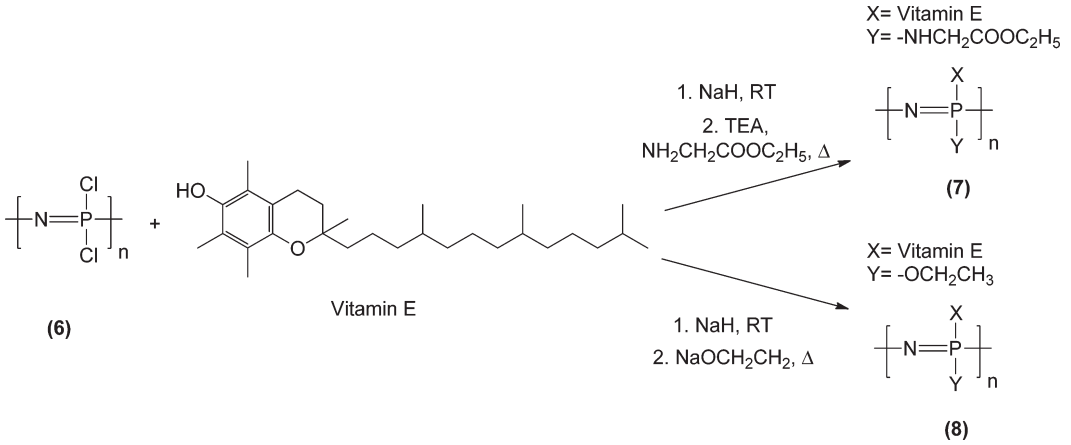
hydroxyl groups to allow only one reactive site to remain. This is an important factor for macromolecular substitution reactions to prevent cross-linking. Traditionally, synthesis of an O-linked phosphazene requires the prior formation of an alkali metal salt and use of the precipitation of the metal chloride to drive the process to completion. However, during the synthesis of **5a**, the sodium salt of the protected form of vitamin B$_6$ was insoluble. Therefore, a solution of **1** was added to the insoluble salt. During the addition, the salt became soluble, probably due to a facile reaction with the cyclic trimer. Full halogen replacement was complete within 24 h at 70 °C. After substitution, the ketal protecting group was removed with concentrated trifluoroacetic acid in methanol to form **5b**. Complete deprotection was accomplished by this technique, and the different products were separated using Sephadex-G25 with deionized water as the mobile phase. There was no evidence of hydrolysis during the separation, as determined by mass spectrometry.

Synthesis and Characterization of Polymers 7−12. Following similar synthesis procedures to those employed for the small molecule analogues, macromolecular substitution reactions were carried out for polymers **7**−**12** in a two-step process. First, the reactive intermediate, poly(dichlorophosphazene), was synthesized by the ring-opening polymerization of hexachlorocyclotriphosphazene at 250 °C under vacuum. Second, replacement of the chlorine atoms was attempted using either the sodium salt of the corresponding vitamin or the ester-protected vitamin. In each case, varying amounts of the vitamin units became covalently linked to the polymer backbone. The physical and structural data are shown in Table 3. Information obtained during the synthesis of the small molecule analogues **2** and **3** suggested that it was unlikely that fully substituted polymers containing vitamin E could be synthesized due to the bulk of the reagent. Thus, during the synthesis of polymers **7** and **8**, the bulky side group was introduced first by a slow dropwise addition to a solution of $(NPCl_2)_n$ to favor random distribution of these groups along the polymer chain. Then an excess of the second nucleophile was added to complete the substitution, as shown in Scheme 3. The maximum amount of vitamin E linked to the polymer was 47% in the case of polymer **8**. Polymers **7** and **8** were yellow-orange elastomeric solids that were soluble in THF and chloroform.

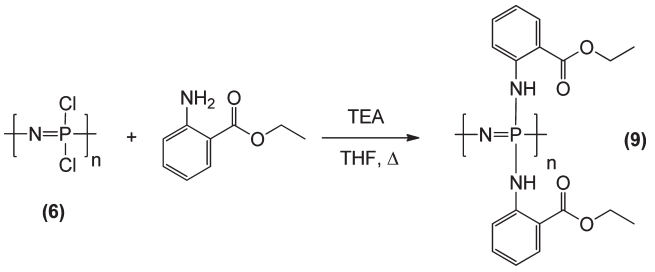
Linkage of vitamin L$_1$ to poly(dichlorophosphazene) required an extended reaction time (96 h) at 70 °C to complete the

dx.doi.org/10.1021/ma1027406 |*Macromolecules* 2011, 44, 1355–1364

Scheme 3. Synthesis of Vitamin E-Containing Polymers



Scheme 4. Synthesis of a Vitamin L_1-Containing Polymer

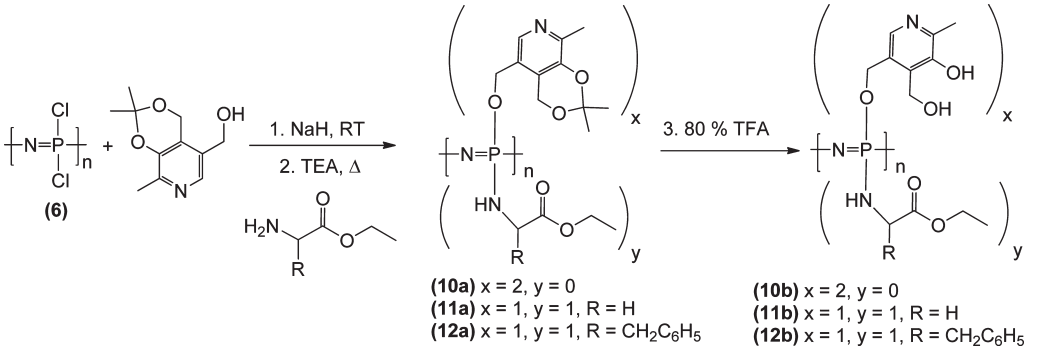


substitution (Scheme 4). The molecular weight of the resultant polymer **9** was low, as shown in Table 1. This result is attributed to the long reaction time and the release of hydrogen chloride during the substitution reaction. Hydrogen chloride normally reacts with excess triethylamine present in solution to form an insoluble salt. However, the hydrogen chloride could also react with the nitrogen atoms in the phosphazene backbone, resulting in P−N bond cleavage and a decrease in molecular weight.[32] The synthesis of polymers **10**−**12** first required the protection of two of the three hydroxyl groups to prevent cross-linking, and as in the case of the small molecule analogue, the $(NPCl_2)_n$ solution was added to the salt solution because the salt was insoluble in THF. Full substitution of polymer **10** occurred within 48 h. Polymer **10** proved to be resistant to hydrolytic degradation which was evident from the initial hydrolysis−pH study presented in Figure 2. Hence, polymers **11** and **12** were designed to incorporate a hydrolytically labile linkage between the skeleton and the side groups by incorporating glycine ethyl ester and/or phenylalanine ethyl ester as cosubstituents (Scheme 5). The amino acid ester units were introduced second to complete the substitution to minimize the liberation of hydrogen chloride and reduce its effect on chain cleavage. Polymers **10**−**12** were brittle light brown solids that were soluble in various solvents ranging from water to THF.

All these reactions were monitored by ^{31}P NMR spectroscopy to determine the extent of substitution, and the polymers were further characterized by ^{1}H NMR, GPC, and DSC, as shown in Table 1. Polymers **7**−**12** had various side group distribution patterns which were evident from different ^{31}P shifts. For example, polymer **7** gave two chemical shifts at −1.65 and 5.64 ppm. The shift at −1.65 ppm is from a phosphorus atom bearing two glycine ethyl ester units. The second shift at 5.64 is attributed to a phosphorus atom bearing a glycine ethyl ester unit and a vitamin E unit. Each of the remaining polymers with two different substituents (polymers **8**, **11**, and **12**) gave similar spectra. The relative percentages of each side group were estimated by ^{1}H NMR spectroscopy. For polymers **7** and **8** the side group ratios were 0.66:1.34 and 0.94:1.06, respectively. To ensure full replacement of chlorine atoms by glycine ethyl ester units, a smaller percentage of vitamin E was first linked to the backbone. If a larger percentage of vitamin E was first introduced, glycine ethyl ester would not complete the substitution presumably because of limited steric access to the P−Cl units. Polymers **11** and **12** contained a 1:1 ratio of the substituents within a 5% error.

Deprotection of Polymers 10a−12a. In order to remove the ketal protecting groups on polymers **10a**−**12a** to yield the deprotected vitamin unit, the polymers were treated with 80% trifluoroacetic acid in methanol. All the polymers were deprotected successfully under mild acidic conditions. Polymer **10** is stable under acidic conditions and was deprotected within 48 h at 50 °C, but polymers **11a** and **12a** bear amino acid side groups that are sensitive to acidic conditions and degrade faster at lower pH values than at higher pH.[33] However, both polymers **11a** and **12a** could be deprotected under mild conditions (room temperature, 60 h), and there was no evidence of chain cleavage detectable by ^{31}P NMR spectroscopy. Thus, no peaks at ∼0 ppm were detected which would have indicated the presence of phosphate units. The structures were confirmed by ^{1}H NMR by the disappearance of the methyl proton peaks at 1.23−1.35 ppm. Molecular weights could not be determined by GPC analysis due to the insolubility of the polymers in THF (Table 3). Polymers **10b** and **11b** were soluble in dilute acetic acid, and polymer **12b** was soluble in dimethyl sulfoxide. The limited solubility of polymers **10b**−**12b** could be due to extensive hydrogen bonding between the side groups which prevents the polymer from redissolving once dried. Unlike polymer **11b**, polymer **12b** is not soluble in acetic acid. This may be due to the presence of the hydrophobic amino acid−phenylalanine ethyl ester, instead of a hydrophilic amino

Scheme 5. Synthesis of Vitamin B_6-Containing Polymers



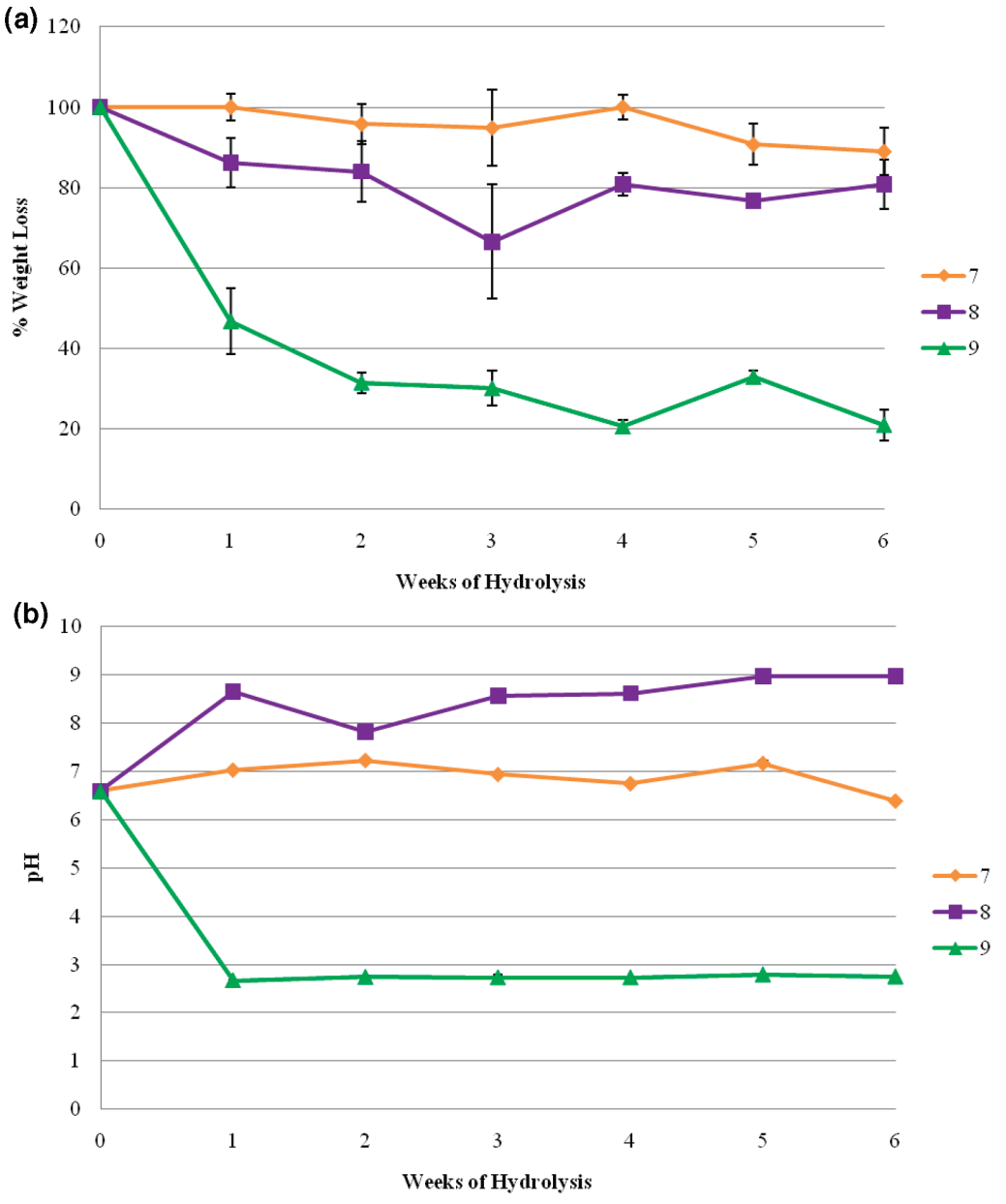


Figure 1. (a) Percent weight loss of polymers **7**−**9**. (b) pH of polymers **7**−**9**.

dx.doi.org/10.1021/ma1027406 |*Macromolecules* 2011, 44, 1355–1364

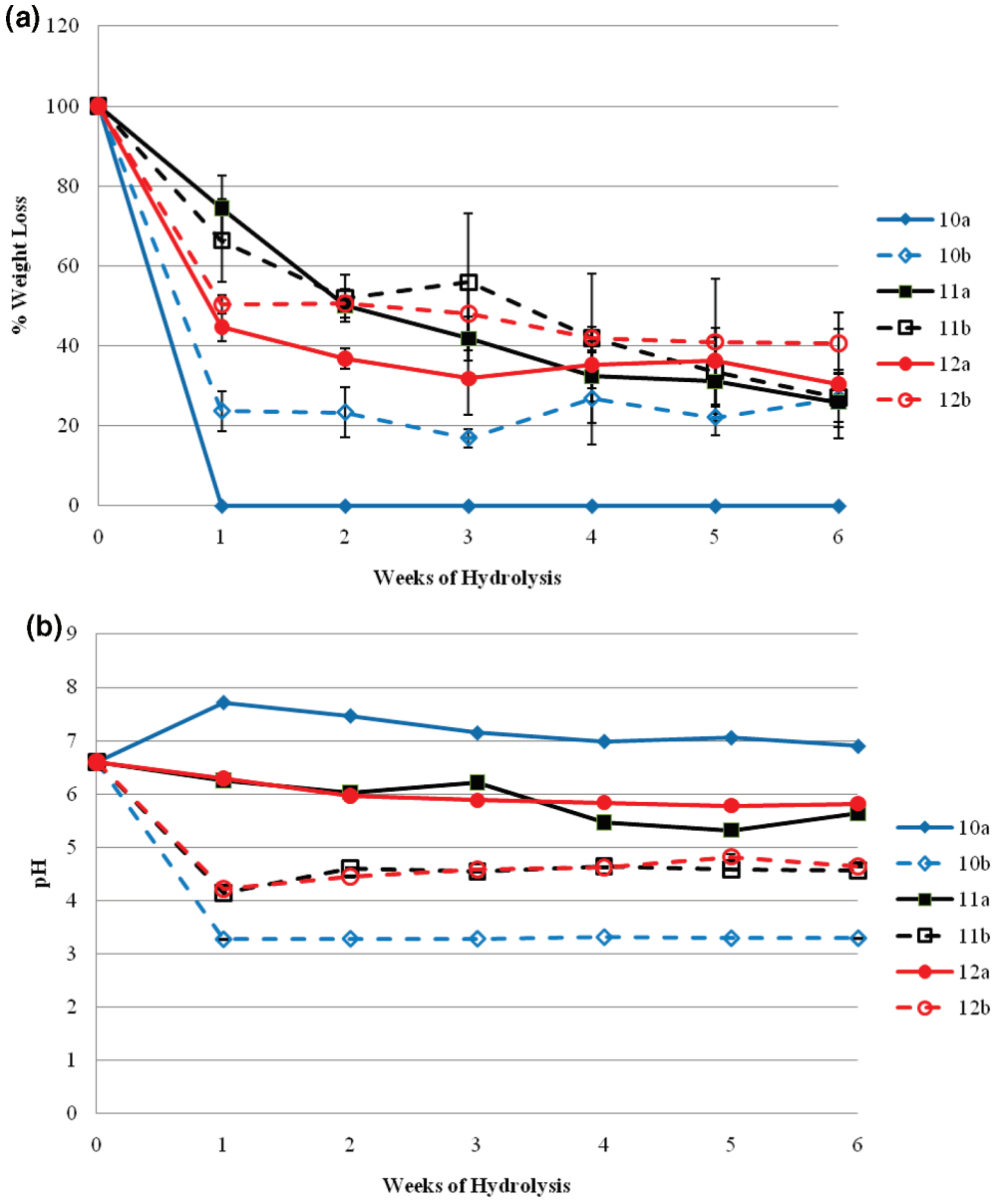


Figure 2. (a) Percent weight loss of polymers **10**−**12**. (b) pH of polymers **10**−**12**.

acid−glycine ethyl ester. Because of the amphiphilicity of the polymers, a less polar solvent is required to solubilize both the hydrophobic amino acid and hydrophilic vitamin unit.

Thermal Characterization of Polymers 7−**12.** The glass transition temperatures (T_g) of polymers **7**−**12** vary from −25 to 44 °C and are shown in Table 1. Vitamin E-containing polymers (**7** and **8**) have low and broad glass transition temperatures at −25 and −20 °C, respectively. This is surprising because the steric hindrance of the bulky vitamin unit might be expected to decrease the torsional mobility of the backbone. However, the long aliphatic chain may create significant free volume which disrupts polymer chain packing and lowers crystallinity and T_g. Polymer **9** showed a higher T_g ~25 °C probably due to the ability of the bulky phenyl rings to restrict backbone motion. This trend is similar to that for poly[bis(2-amino 4-picoline)phosphazene] with a T_g at 27 °C.[34] The protected vitamin B_6 polymers (**10a**−**12a**) had glass transition temperatures ranging from 30 to 44 °C. For polymers **11a** and **12a**, the primary transition increased as the cosubstituent glycine ethyl ester was replaced by phenylalanine ethyl ester. This follows a trend in which the T_g increases as the size of the α-carbon substituent on the amino acid increases in a series from hydrogen to a benzyl group. Polymer **11a** showed a secondary transition at 44.0 °C which could be due to an interaction between an amino proton on the amino acid ester with the basic nitrogen atom on the protected vitamin. This secondary transition was not evident in **12a** due to the steric hindrance at the α-carbon of the amino acid ester preventing this interaction. After deprotection of polymers **10a**−**12a**, each T_g was raised by several degrees, the highest being polymer **12b** at 40 °C. This is believed to be due to extensive hydrogen bonding between the polymer chains, which restrict skeletal motion. This evidence is also consistent with the low solubility of these polymers in common organic solvents after being dried.

Hydrolysis Behavior of Polymers 7−**12.** All of the polymers synthesized in this work were sensitive to hydrolysis—a key

requirement for tissue engineering materials. The relative hydrolytic degradation trends of polymers **7**−**12** in deionized water were monitored over a 6 week period at 37 °C with a starting pH of 6.60 (Figures 1 and 2). The hydrolysis varied from 10 to 100% over a 6 week period with the order of highest to lowest weight loss being **10a** > **9** > **10b** = **11a** = **11b** > **12a** > **12b** > **8** > **7**. Polymers containing vitamin E (**7** and **8**) hydrolyzed at a slow rate. Polymer **8**, co-substituted with ethoxy groups, lost ∼20% of its weight after 6 weeks, whereas polymer **7**, co-substituted with glycine ethyl ester, lost only 10% after 6 weeks. The faster hydrolysis of polymer **8** may be connected with the ability of ethoxy groups to undergo a rearrangement in which ethyl groups shift to the adjacent nitrogen atoms to form N−CH_2CH_3 and P═O units. These phospha*zane* sites in the backbone would sensitize the system to rapid hydrolysis of the backbone.[35,36] One product of hydrolysis is a phosphate detectable by a silver nitrate test and the formation of a yellow silver phosphate precipitate. Polymer **7** degrades at a slower rate than **8** presumably due the inability of water to access the polymer backbone to displace the amino acid ester. Vitamin E in combination with glycine ethyl ester renders the backbone less accessible than when vitamin E is present accompanied by ethoxy groups.

Polymer **9** degrades much faster than polymers **7** and **8** possibly due to the acidic media that are formed (pH ∼ 2.5). The low pH may result from the presence of a small amount of hydrogen chloride complexed with the nitrogen atoms of the backbone which was released into the aqueous media during hydrolysis. Hydrogen chloride would normally be removed through complexation with triethylamine. However, if a small amount remained complexed with the backbone, this would cause a decrease in pH.[37] Another explanation of this low pH could be the formation of 2-aminobenzoic acid through the cleavage of the ethyl ester group. After the formation of this acid, the side group could be displaced from the polymer, thus resulting in degradation. This mechanism has also been proposed for the degradation of poly(amino acid ester)phosphazenes.[17] It is plausible that the pH measured could be due to the dissolved 2-aminobenzoic acid present. This has also been observed for other polyacid systems, such as the dissolution of 4-hydroxybenzoic acid from poly[di(carboxylatophenoxy)phosphazene] in deionized water.[38] The pH for the hydrolysis media for polymers **7**−**9** was in the range of ∼9−2.5 with the order of highest to lowest pH being **8** > **7** > **9**.

The hydrolysis behavior of polymers **10**−**12** is shown in Figure 2. It was expected that polymers **10a** and **10b** would be water-soluble because of the amphiphilic character of vitamin B_6.[39] However, polymer **10a** was soluble in water and **10b** was not. After 6 weeks there was a 75% weight loss for **10b**. The lack of complete water solubility suggests that the hydrogen bonding between the polymer chains inhibits water from solvating the chains. This proposed explanation is supported by the DSC results. Here, the weight loss was attributed to polymer water solubility and not to polymer degradation. This was confirmed by ^{1}H and ^{31}P spectroscopies. Samples were taken from the deionized water from weeks 1 and 6, and there was no evidence of any degradation or shift in the ^{31}P peak.

Polymers **11a** and **11b** lost respectively 75% and 73% of their weight by week 6. To incorporate hydrolytic degradation, polymers **11a** and **11b** were co-substituted with an amino acid ester linkage. In doing so, the solubility of the resulting polymers in water decreased. After 6 weeks, polymers **11a** and **11b** degraded or water solubilized at similar rates, regardless of their protected/deprotected form. The hydrolysis products were tested for the presence of free amino acids using the ninhydrin test. There was a clear difference in the intensity of the violet color produced from weeks 1−6 for **11a** and **11b**, suggesting that a larger quantity of free amino acid was present in week 6. The trend for polymers **12a** and **12b** is similar to that of **11a** and **11b**, whereas **12a** and **12b** lost 70% and 60% by week 6, respectively. Polymer **11b** loses more weight than **12b** because of the decreased steric hindrance of the α-carbon on the amino acid ester. The pH for the hydrolysis media for polymers **10**−**12** was in the range of ∼7.7−3.3 with the order of highest to lowest pH being **10a** > **11a** = **12a** > **11b** = **12b** > **10b**. Of the protected polymers, polymer **10a** has a higher pH (∼7) than polymers **11a** and **12a** (∼5.5), which are co-substituted with an amino acid. This is to be expected because during hydrolysis, free amino acid is released into the aqueous media, causing a slight decrease in pH. When comparing the deprotected and protected polymers, the deprotected form of vitamin B_6 (**10b**−**12b**) generated a much lower pH (∼3.3−4.5) than when it is in it is protected form (**10a**−**12a**; ∼5.5−7). This could be due to small amounts of trifluoroacetic acid trapped within the hydrophobic matrix or complexed with either the basic nitrogen of vitamin B_6 or the basic nitrogen atoms in the backbone, to be subsequently released during the hydrolysis. Although traces of trifluoroacetic acid could explain the difference in pH, nevertheless, the hydrolysis media from **12b** showed no residue of trifluoroacetic acid by fluorine NMR spectroscopy and yet still yielded a low-pH media. Trace amounts of trifluoroacetic acid were detected by fluorine NMR for sample **11b** with a weak signal at −75.9 ppm. However, the pH of polymers **11b** and **12b** were the same during the 6 week hydrolysis study. An alternative explanation of this difference in pH could be due to the release of the acidic hydroxyl site on position 3 of vitamin B_6.[39] A comparison of the protected polymers **11b** and **12b** (pH ∼4.5) to the unprotected polymers **11a** and **12a** (pH ∼5.5) suggests that the release of the acidic hydroxyl site may cause the decrease in pH.

CONCLUSIONS AND BIOMEDICAL POTENTIAL

These polymers have considerable advantages compared to previously synthesized polymers because they contain biomolecules that have the potential to stimulate cellular responses and thus could be used in a variety of tissue engineering applications. This is the first reported synthesis of polyphosphazenes that contain vitamin side groups. The small molecule model reactions indicated both the feasibility and the synthetic challenges of these systems. Polymer synthesis was achieved by macromolecular replacement of the chlorine atoms of poly(dichlorophosphazene) by vitamin units and other cosubstituents. The glass transition temperatures ranged from −24 to 44 °C. Preliminary heterophase hydrolysis experiments were performed in deionized water for a 6 week time period at 37 °C. Hydrolyses varied from 10 to 100%, with the order of highest to lowest weight loss being **10a** > **9** > **10b** = **11a** = **11b** > **12a** > **12b** > **8** > **7**. For hard tissue engineering, polymers **11b** and **12b** appear to be the best candidates based on their physical properties and hydrolytic behavior.

AUTHOR INFORMATION

Corresponding Author

*E-mail: hra@chem.psu.edu.

■ ACKNOWLEDGMENT

This work was supported by the National Institutes of Health Grant RO1 EB004051.

■ REFERENCES

(1) Lakshmi, S.; Katti, D. S.; Laurencin, C. T. *Adv. Drug Delivery Rev.* **2003**, *55*, 467.
(2) Yamamoto, H.; Hayakawa, T. *Polymer* **1978**, *19*, 1115.
(3) Wyrsta, M. D.; Cogen, A. L.; Deming, T. J. *J. Am. Chem. Soc.* **1999**, *127*, 2026.
(4) Gunatillake, P. A.; Adhikari, R. *Eur. Cells Mater.* **2003**, *5*, 1.
(5) Grodzinski-Jagur, J. *Polym. Adv. Technol.* **2006**, *17*, 395.
(6) Nair, L. S.; Laurencin, C. T. *Prog. Polym. Sci.* **2007**, *32*, 762.
(7) Verrier, S.; Boccaccini, A. R. In *Advances in Tissue Engineering*; Polak, J., Mantalaris, S., Harding, S. E., Eds.; Imperial College Press: London, 2008; p 279.
(8) Bostman, O.; Pihlajamaki, H. *Biomaterials* **2000**, *21* (24), 2615.
(9) Shoichet, M. S. *Macromolecules* **2010**, *43*, 581.
(10) Kim, I.-Y.; Seo, S.-J.; Moon, H.-Y.; Yoo, M.-K.; Park, I.-Y.; Kim, B.-C.; Cho, C.-S. *Biotechnol. Adv.* **2008**, *16*, 1.
(11) Cho, S.-Y.; Allcock, H. R. *Macromolecules* **2007**, *40*, 3115.
(12) Nakavarapu, S. P.; Kumbar, S. G.; Brown, J. L.; Krogman, N. R.; Weikel, A. L.; Hindenlang, M. D.; Nair, L. S.; Allcock, H. R.; Laurencin, C. T. *Biomacromolecules* **2008**, *9*, 1818.
(13) Allcock, H. *Chemistry and Applications of Polyphosphazenes*; John Wiley & Sons: Hoboken, NJ, 2003; Chapter 8, p 268.
(14) Weikel, A. L.; Owens, S. G.; Fushimi, T.; Allcock, H. R. *Macromolecules* **2010**, *43*, 5205.
(15) Allcock, H. R.; Singh, A.; Ambrosio, A. M. A.; Laredo, W. R. *Biomacromolecules* **2003**, *4*, 1646–1653.
(16) Krogman, N. R.; Weikel, A. L.; Nguyen, N. Q.; Nair, L. S.; Laurencin, C. T.; Allcock, H. R. *Macromolecules* **2008**, *41*, 7824–7828.
(17) Allcock, H. R.; Pucher, S. R.; Scopelianos, A. G. *Macromolecules* **1994**, *27* (5), 1071.
(18) Deng, M.; Nair, L. S.; Nukavarapu, S. P.; Kumbar, S. G.; Jiang, T.; Krogman, N. R.; Singh, A.; Allcock, H. R.; Laurencin, C. T. *Biomaterials* **2008**, *29*, 337.
(19) Sethuraman, S.; Nair, L. S.; Al-Amin, S.; Farrar, R.; Nguyen, M-T.N.; Singh, A.; Allcock, H. R.; Gerish, Y. E.; Brown, P. W.; Laurencin, C. T. *J. Biomed. Mater. Res. 77A* **2006**, 679.
(20) O'Niel, M. J., Smith, A., Heckelman, P. E., Eds.; *The Merck Index*, 13th ed.; Merck & Co., Inc.: Whitehouse Station, NJ, 2001; p 2607.
(21) *Vitamin, The Columbia Encyclopedia*, 6th ed. [online]; Columbia University Press: posted 2008, http://www.encyclopedia.com/doc/1E1-vitamin.html (accessed June 29, 2010).
(22) Vitamin B complex http://www.cancer.org/docroot/ETO/content/ETO_5_3x_Vitamin_B_Complex.asp (accessed March 17, 2010).
(23) Lucy, J. A.; Dingle, J. T. *Nature* **1964**, *204*, 156.
(24) Turan, B.; Balcik, C.; Akkas, N. *Clin. Rheumatol.* **2007**, *16*, 441.
(25) Arjmandi, B.; Juma, S.; Beharka, A.; Bapna, M.; Akhter, M.; Meydani, S. *J. Nutr. Biochem.* **2002**, *13*, 543.
(26) Misra, S. K.; Philip, S. E.; Chrzanowski, W.; Nazhat, S. N.; Roy, I.; Knowles, J. C.; Salih, V.; Boccaccini, A. R. *J. R. Soc. Interface* **2009**, *6*, 401.
(27) Cushnie, E. K.; Khan, Y. M.; Laurencin, C. T. *J. Biomed. Mater. Res., Part A* **2010**, *94*, 568–575.
(28) Pangborn, A.; Giardello, M.; Grubbs, R.; Rosen, R.; Timmers, F. *Organometallics* **1996**, *15*, 1518.
(29) Allcock, H. R.; Kugel, R. L. *J. Am. Chem. Soc.* **1965**, *87*, 4216.
(30) Yang, D.-Y.; Shih, Y.; Liu, H.-W. *J. Org. Chem.* **1991**, *56*, 2940.
(31) Klemenc, S. *Forensic Sci. Int.* **2002**, *129*, 194–199.
(32) Singh, A.; Krogman, N. R.; Sethuraman, S.; Nair, L. S.; Sturgeon, J. L.; Brown, P. W.; Laurencin, C. T.; Allcock, H. R. *Biomacromolecules* **2006**, *7*, 914–918.
(33) Weikel, A. W.; Krogman, N. R.; Ngyyen, N. Q.; Nair, L. S.; Laurencin, C. T.; Allcock, H. R. *Macromolecules* **2009**, *42*, 636–639.
(34) Allcock, H. R.; Austin, P. E.; Neenan, T. X. *Macromolecules* **1982**, *15*, 689.
(35) Ferrar, W. T.; DiStefano, F. V.; Allcock, H. R. *Macromolecules* **1980**, *13*, 1345.
(36) Fitzsimmons, B. W.; Hewlett, C.; Shaw, R. A. *J. Am. Chem. Soc.* **1964**, 4459.
(37) Krogman, N. R.; Hindenlang, M. D.; Nair, L. S.; Laurencin, C. T.; Allcock, H. R. *Macromolecules* **2008**, *41*, 8467–8472.
(38) DeCollibus, D. P.; Marin, A.; Andrianov, A. *Biomacromolecules* **2010**, *11*, 2033.
(39) Gregory, J. F. In *Food Chemistry*; Fennema, O. R., Ed.; Marcel Dekker Inc.: New York, 1996; pp 580−581.