NEW MATERIALS FOR ADVANCED BIOMATERIAL APPLICATIONS

A Dissertation in
Chemistry
by
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Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

August 2010
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ABSTRACT

The work described in this thesis focuses on the design, synthesis, and characterization of novel polyphosphazenes for advanced biomedical applications. In addition, the fabrication of polyphosphazene / poly(lactic-co-glycolic acid) (PLGA) blends were examined for their physical properties as hard tissue engineering scaffolds. Chapter 1 outlines the basic fundamentals of polymer chemistry, along with phosphazene chemistry and its application to biomedical materials.

Chapter 2 discusses the synthesis of the dipeptides alanyl-glycine ethyl ester, valinyl-glycine ethyl ester, and phenylalanyl-glycine ethyl esters. These dipeptides were synthesized using mixed anhydride solution-phase peptide reactions. The free N-terminus was used as a reactive site for nucleophilic replacement of the chlorine atoms in poly(dichlorophosphazene). The C-terminus was protected with an ethyl ester to prevent side reactions and crosslinking. The alanyl-glycine ethyl ester replaced all the chlorine atoms in poly(dichlorophosphazene). However, replacement of all the chlorine atoms in poly(dichlorophosphazene) by valinyl-glycine ethyl ester or phenylalanyl-glycine ethyl ester polyphosphazenes was prevented by the insolubility of the partially substituted intermediates. To circumvent this problem, co-substitution was carried out using the valinyl- or phenylalanyl esters with glycine ethyl ester or alanine ethyl ester in a 1:1 ratio. Co-substituted polyphosphazenes with alanyl glycine ethyl ester and glycine ethyl ester or alanine ethyl ester were also synthesized with a side group ratio of 1:1. The polymer structures and physical properties were studied using multinuclear NMR, DSC, and GPC techniques. Heterophase hydrolysis experiments in aqueous media at different pH values were carried out to estimate the hydrolytic sensitivity of these polymers. All the polymers were less sensitive to hydrolysis under neutral or basic (pH, 10.0) conditions than at pH 4.0, where rapid hydrolysis occurred.
Chapter 3 outlines the preparation of phosphazene tissue engineering scaffolds with bioactive side groups using the biological buffer choline chloride. Mixed-substituent phosphazene cyclic trimers (as model systems) and polymers with choline chloride and glycine ethyl ester, alanine ethyl ester, valine ethyl ester, or phenyl alanine ethyl ester were synthesized. Two different synthetic protocols were examined. A sodium hydride mediated route resulted in polyphosphazenes with a low choline content, while a cesium carbonate mediated process produced polyphosphazenes with higher choline content. The phosphazene structures and physical properties were studied using multinuclear NMR, differential scanning calorimetry (DSC), and GPC techniques. The resultant polymers were then blended with PLGA (50:50) or PLGA (85:15) and characterized by DSC analysis and scanning electron microscopy (SEM). Polymer products obtained via the sodium hydride route produced miscible blends with both ratios of PLGA, while the cesium carbonate route yielded products with reduced blend miscibility. Heterophase hydrolysis experiments in aqueous media revealed that the polymer blends hydrolyzed to near-neutral pH values (~5.8 to 6.8). The effect of different molecular structures on cellular adhesion showed osteoblast proliferation with an elevated osteoblast phenotype expression compared to PLGA over a 21-day culture period.

Chapter 4 describes the preparation of phosphazenes that possess reversible cross-linking groups to control mechanical stability and hydrolysis 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.

Chapter 5 evaluates the first reported synthesis of a completely hydrolysable polyphosphazene-containing block co-polymer. The synthesis of poly(lactic acid)-co-poly[(bis-alanine ethyl ester phosphazene)], poly(lactic acid)-co-poly[(bis-valine ethyl ester phosphazene)], and poly(lactic acid)-co-poly[(bis-phenylalanine ethyl ester phosphazene)] has been accomplished. These block co-polymers were used as blend compatibilizers to form composites of PLAGA (50:50) or PLAGA (85:15) with poly[(bis-alanine ethyl ester phosphazene)], poly[(bis-valine ethyl ester phosphazene)], or poly[(bis-phenylalanine ethyl ester phosphazene)]. The effect of the block copolymers on the composites formed from previously immiscible blends was studied. The resultant composites were characterized using differential scanning calorimetry (DSC) and scanning electron microscopy (SEM) to investigate systems miscibility. The rates of hydrolysis and pH of the hydrolysis media were also investigated.

Chapter 6 discusses a unique polymer erosion process for biodegradable biomaterials through which the polymer changes from a solid coherent film to an assemblage of microspheres with interconnected porous structures. The polymer system was developed on the highly versatile platform of self-neutralizing polyphosphazene-polyester blends. Co-substituting a polyphosphazene backbone with both glycylglycine dipeptide and with side groups that can retard the polymer degradation, such as hydrophobic 4-phenylphenoxy, generated a unique polymer with strong hydrogen bonding ability and a slow degradation rate. During the blend hydrolysis in aqueous media, the relatively fast degradation rate of the polyester favored 3D void space formation characterized by macropores (10-100 µm) between polyphosphazene spheres as well as micro and nanopores on the sphere surface. The blend degradation was further investigated in
vivo using a rat subcutaneous implantation model. A 12-week degradation resulted in a 3D porous structure with 82-87% porosity and with 100% interconnectivity. This *in situ*-formed 3D interconnected porous structure enabled cell infiltration and collagen tissue in-growth. Thus, the dynamic pore formation process accompanying the matrix erosion provides a new strategy in regenerative medicine for developing solid matrices that allows tissue integration as the matrix degrades.

Chapter 7 describes a series of closely related polyphosphazenes with propoxy, pentoxy, hexoxy, octoxy, isostearyloxy, and 2-(2-methoxyethoxy)ethoxy (MEE) side groups, together with co-substituent species with both the alkoxy and MEE side chains. These were studied for their morphology and miscibility with oligoisobutylene (OIB). All the pure polymers except one had a single glass transition temperature. The exception was the species with both isostearyloxy and MEE side groups, which underwent two low temperature second order transitions, even though 31P NMR spectra indicated the absence of a block-type structure. For the single-substituent macromolecules, the solubility at 80 °C in OIB increased as the length of the unbranched alkoxy side groups rose from propoxy to octoxy (from 1 to 11 wt/wt%). However, the polymer with two isostearyloxy side chains per repeat unit had a low solubility in OIB (3 wt/wt%) and the species with the two MEE side groups on every repeat unit was totally insoluble. When both alkoxy and MEE side groups were present, the solubility in OIB was also low (0-3%), except for the species with both isostearyloxy and MEE side groups, which was soluble in OIB at a level of 21 wt/wt% at 80 °C, and showed $T_g$ evidence of polymer/oligomer miscibility even at -80 °C. Explanations are suggested for the unusual behavior of this polymer.
# TABLE OF CONTENTS

LIST OF FIGURES........................................................................................................xii

LIST OF TABLES...........................................................................................................xvii

PREFACE............................................................................................................................xviii

ACKNOWLEDGEMENTS.................................................................................................xix

Chapter 1 Introduction to Polymer Chemistry .................................................................1

1.1 History....................................................................................................................1
1.2 Polymer Definition and Architecture.................................................................2
1.3 Polymer Blends.......................................................................................................7
1.4 Polymer Synthesis..................................................................................................7
  1.4.1 Step-Growth Polymerization........................................................................8
  1.4.2 Chain-Growth Polymerization......................................................................10
  1.4.3 Ring-Opening Polymerizations (ROPs).........................................................12
1.5 Hybrid Inorganic-Organic Polymers ....................................................................13
1.6 Polyphosphazenes................................................................................................14
  1.6.1 Significance....................................................................................................14
  1.6.2 Synthesis........................................................................................................15
  1.6.3 Macromolecular Substitution ........................................................................16
  1.6.4 Polyphosphazene Architecture ....................................................................17
  1.6.5 Applications ..................................................................................................18
1.7 Biomaterials..........................................................................................................20
1.8 Polyphosphazene Biomaterials .............................................................................21
1.9 References.............................................................................................................24

Chapter 2 Polyphosphazenes that Contain Dipeptide Side Groups: Synthesis, Characterization, and Sensitivity to Hydrolysis.................................................................29

2.1 Introduction............................................................................................................29
2.2 Experimental.........................................................................................................30
  2.2.1 Reagents and Equipment ..............................................................................30
  2.2.2 Synthesis of Boc-alanyl-glycine ethyl ester ..................................................31
  2.2.3 Synthesis of Boc-valinyl-glycine ethyl ester ..................................................32
  2.2.4 Synthesis of Boc-phenylalanyl-glycine ethyl ester .......................................32
  2.2.5 Deprotection of Boc-alanyl-glycine ethyl ester* ..........................................32
  2.2.6 Deprotection of Boc-valinyl-glycine ethyl ester ..........................................33
  2.2.7 Deprotection of Boc-phenylalanyl-glycine ethyl ester .................................33
  2.2.8 Synthesis of Cyclic Trimmers 2-4 ..................................................................33
  2.2.9 Synthesis of Polymer 6 ................................................................................34
  2.2.10 Synthesis of Polymers 7-12 .........................................................................34
  2.2.11 Controlled pH hydrolysis studies .................................................................35
2.3 Results and Discussion............................................................................................35
Chapter 3 Choline-Substituted Polyphosphazenes as Tissue Regeneration Scaffolds: Blend Compatibility with PLGA and Effects on Osteoconductivity ........................................ 45

3.1 Introduction ................................................................. 45
3.2 Experimental .................................................................. 47
  3.2.1 Reagents and Equipment ........................................ 47
  3.2.2 Synthesis of Model Cyclic Trimers 1-4 ....................... 48
  3.3.3 Synthesis of Polymers 5-8 ........................................... 49
  3.3.4 Synthesis of Polymers 9-12 ......................................... 50
  3.3.5 Formation of Polyphosphazene Blends with PLGA (50:50) and PLGA (85:15) .................................................. 51
  3.3.6 Hydrolysis Studies of Polymers 5-12 and blended polymer samples ................................................................. 51
  3.3.7 In Vitro Osteocompatibility Studies of Polymer Blends ........................................................................................................ 52
3.4 Results and Discussion ..................................................... 53
  3.4.1 Synthesis of Trimers 1-4 .............................................. 53
  3.4.2 Synthesis of Polymers 5-12 .......................................... 55
  3.3.3 Thermal characterization of polymers 5-12 .................... 59
  3.3.4 Hydrolysis of Polymers 5-12 ........................................ 60
  3.4.5 Compatibility of 5-12 with PLGA (50:50) and PLGA (85:15) ................................................................. 61
  3.4.6 Hydrolysis of polymers 5-12 blended with PLGA (50:50) and PLGA (85:15) ................................................................. 65
  3.4.7 In vitro osteocompatibility of polymers 6-8 blended with PLGA (85:15) ...... 66
3.5 Conclusions .................................................................. 68
3.6 Acknowledgment ............................................................ 69
3.7 References ..................................................................... 70

Chapter 4 Synthesis and Characterization of Methionine- and Cysteine-Substituted Phosphazenes ................................................................. 73

4.1 Introduction ................................................................. 73
4.2 Experimental .................................................................. 74
  4.2.1 Reagents and Equipment ........................................ 74
  4.2.2 Synthesis of ethyl ethanethiolsulfinate (CH3CH2S(=O)SCH2CH3) .......... 75
  4.2.3 Protection of cysteine ethyl ester hydrochloride with ethyl ethanethiolsulfinate ........................................................................................................ 76
  4.3.4 Synthesis of hexa(methionine ethyl ester)cyclotriphosphazene ........ 76
  4.3.5 Synthesis of hexa(cysteine ethyl disulfide ethyl ester)cyclotriphosphazene ... 77
  4.3.6 Deprotection of hexa(cysteine ethyl disulfide ethyl ester)cyclotriphosphazene with β-mercaptoethanol ................................................................. 78
Chapter 5 Hydrolysable Polylactide-Polypophosphazene Block Copolymers for Biomedical Applications: Synthesis, Characterization, and Composites with Poly(lactic-co-glycolic acid)

5.1 Introduction .................................................................................................................. 95
5.2 Experimental ............................................................................................................... 97
  5.2.1 Materials .................................................................................................................. 97
  5.2.2 Equipment ............................................................................................................... 98
  5.2.3 Polymerization of L-Lactide (PLA-Boc) ................................................................ 99
  5.2.4 Deprotection of Amino-polylactide (PLA-NH$_2$) .................................................. 99
  5.2.5 Polylactide Functionalization with Br(CF$_3$CH$_2$O)$_2$P=NSiMe$_3$ ........................ 99
  5.2.6 Synthesis of Polylactide-Alanine Polypophosphazene Block Copolymer
      (PLA-Ala) 4 ................................................................................................................ 100
  5.2.7 Synthesis of Polylactide-Valine Polypophosphazene Block Copolymer
      (PLA-Val) 5 .............................................................................................................. 100
  5.2.8 Synthesis of Polylactide-Phenyl alanine Polypophosphazene Block
      Copolymer (PLA-PheAla) 6 ...................................................................................... 101
  5.2.9 Synthesis of Polymers 7-9 .................................................................................... 101
  5.2.10 Fabrication of polymer blends via solution casting .............................................. 102
  5.2.11 pH hydrolysis studies ......................................................................................... 102
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.2.6 Solubility testing</td>
<td>160</td>
</tr>
<tr>
<td>7.3 Results and Discussion</td>
<td>161</td>
</tr>
<tr>
<td>7.3.1 Synthesis of polyphosphazenes</td>
<td>161</td>
</tr>
<tr>
<td>7.3.2 Glass transition temperatures</td>
<td>164</td>
</tr>
<tr>
<td>7.3.3 Solubility and Miscibility of Polymers 3-13 in Oligoisobutylene (OIB)</td>
<td>167</td>
</tr>
<tr>
<td>7.3.4 Thermal Decomposition of Polymers 3-13 and OIB</td>
<td>171</td>
</tr>
<tr>
<td>7.4 Conclusions</td>
<td>174</td>
</tr>
<tr>
<td>7.5 Acknowledgement</td>
<td>174</td>
</tr>
<tr>
<td>7.6 References</td>
<td>175</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 1-1. Common Organic Polymers. ....................................................................................... 2
Figure 1-2. Various Polymer Architectures. .................................................................................. 4
Figure 1-3. Different types of block copolymer structures. ......................................................... 6
Figure 1-4. Condensation polymerization of bisphenol-A and phosgene to form a polycarbonate. ......................................................................................................................... 9
Figure 1-5. Synthesis of A) polyetherketone and B) polysulfides. ................................................ 10
Figure 1-6. Monomers used in chain-growth polymerizations. .................................................... 11
Figure 1-7. Reaction sequence for chain-growth polymerization of vinyl chloride. ............... 12
Figure 1-8. Ring opening polymerization of various cyclic monomers. ...................................... 14
Figure 1-9. Synthesis of poly(dichlorophosphazene) and subsequent macromolecular substitution of organic groups along the polymer backbone .............................................. 17
Figure 1-10. Possible architectures of phosphazenes. ................................................................. 18
Figure 1-11. Applications of various polyphosphazenes. .............................................................. 19
Figure 1-12. Examples of bio-absorbable FDA approved polymers. ........................................... 21
Figure 1-13. Hydrolysis mechanism of poly(amoio acid ester phosphazenes) ......................... 23
Figure 2-1. Synthesis of the Boc-protected dipeptides alanyl-glycine ethyl ester, valinyl-glycine ethyl ester, and phenylalanyl-glycine ethyl ester. The N-terminus was then deprotected under acidic conditions to yield the free amino dipeptides. ......................... 36
Figure 2-2. Synthesis of dipeptide substituted cyclic trimers 2-4. ................................................ 37
Figure 2-3. Synthesis of dipeptide substituted polyphosphazenes 6-12..................................... 38
Figure 3-1. Synthesis of [(amino ethyl ester)_3(choline chloride)_3 cyclotriphasphenazenes]....... 54
Figure 3-2. (a) Synthesis of poly[(amino ethyl ester)_3(choline chloride)_3phosphazene] using sodium hydride (NaH); (b) Synthesis of poly[(amino ethyl ester)_3(choline chloride)_3phosphazene] using cesium carbonate (Cs_2CO_3). .................................................... 56
Figure 3-3. DSC analysis of polymer blends. (a) Blends with 6 that demonstrate the plasticization effect with polymer blends of 5-8. (b) Blends of 5 and 9 that display the differences between synthetic history vs. blend compatibility with PLGA (50:50) and PLGA (85:15) ................................................................................................................................. 64
Figure 3-4. The percent mass loss of 6 blended with (a) PLGA (50:50) and (c) PLGA (85:15). The pH values of the hydrolyzed media for (b) blends with PLGA (50:50) and (d) blends with PLGA (85:15). .......................... 66

Figure 3-5. In vitro osteocompatibility of polymer matrices. (a): Cell proliferation by protein assay. (b) ALP activity for osteoblastic phenotype expression over 21 days. (*) indicates significant decrease and (**) indicates significant increase. p< 0.05. The gradual increase in the total protein amount indicated that the blend matrices were able to support the growth and proliferation of PRO. It was found that ALP activity of the cells on Blend 7A and Blend 8A was significantly enhanced compared to PLAGA at day 14 whereas the significant increase was found on Blend 8A at day 2. ALP activity of the cells on Blend 6A was comparable to PLAGA throughout 21 days of cell culture. .............................................................................. 68

Figure 4-1. Synthesis of (1) hexa(methionine ethyl ester)cyclotriphosphazene] and (2) hexa(cysteine ethyl disulfide ethyl ester)cyclotriphosphazene. ...................................................... 83

Figure 4-2. Synthesis of poly[bis-(methionine ethyl ester)phosphazene] (3). ......................... 85

Figure 4-3. Attempted synthesis of (a) poly[bis-(cysteine ethyl ester)phosphazene] (4), and synthesis of (b) poly[bis-(cysteine ethyl disulfide ethyl ester)phosphazene] (5) ...... 87

Figure 4-4. Deprotection of poly[bis-(cysteine ethyl disulfide ethyl ester)phosphazene] using (A) dithiothreitol, (B) β-mercaptoethanol, and (C) Zinc / hydrochloric acid. .......... 89

Figure 5-1. Structure of the Initiator of l-Lactide Polymerization. ........................................ 103

Figure 5-2. 1H NMR spectrum of polylactide bearing-NHBoc end group in CDCl₃. ................. 104

Figure 5-3. Synthesis of Amino-polylactide. ....................................................................... 104

Figure 5-4. Synthesis of Polylactide-Alanine Substituted Polyphosphazene Block Copolymer ........................................................................................................ 106

Figure 5-5. 1H NMR spectrum of Ala-PLA block copolymer (4) in CDCl₃. ......................... 107

Figure 5-6. Chemical structures of poly(amino acid ester phosphazenes) 7-9. ................. 109

Figure 5-7. DSC traces of polymer blends composed of (a) 5 wt% or 2.5 wt% of 5 with 8 and PLAGA (85:15) and (b) 5 wt% or 2.5 wt% of 6 with 9 and PLAGA (85:15). ........ 111

Figure 5-8. SEM images of PLGA (85:15) with (a) 5 wt% of 5 with 8, (b) 2.5 wt% of 5 with 8, (c) 7.5 wt% 6 with 9, and (d) 5 wt% 6 with 9 .................................................. 112

Figure 5-9. Hydrolysis of PLGA blended with poly(amino ethyl ester phosphazene) using corresponding PLA-block-PPhos (Ala = alanine ethyl ester, Val = valine ethyl ester, and Phe = Phenylalanine ethyl ester substituted polyphosphazenes) blend compatibilizers: (a) pH of hydrolysis media and (b) percent mass remaining with
PLAGA (50:50) and (c) pH of hydrolysis media and (d) percent mass remaining with PLAGA (85:15).

**Figure 6-1.** Schematic illustration of different types of polymer erosion a) bulk erosion; b) surface erosion; c) a unique polymer erosion through which the polymer changes from a solid coherent film to an assemblage of microspheres with an interconnected porous structure.

**Figure 6-2.** High-resolution solid-state $^{13}$C NMR spectra for PLAGA, PPHOS, and the blend matrices demonstrating the hydrogen bonding interactions between the dipeptide units of PPHOS and the carbonyl groups in PLAGA. Thermal ring opening polymerization of (NPCl$_2$)$_3$, followed by nucleophilic substitution reactions of the resultant poly(dichlorophosphazene) produced the yellow polymers of PPHOS.

**Figure 6-3.** Surface morphologies of the blend matrices incubated in aqueous media at 37°C over 12 weeks as a function of time. Top row: SEM images showing surface morphologies of Matrix1 following 0, 4, 7, and 12 weeks of *in vitro* degradation. The insets of (e) and (g) show the detailed 3D spherical structures. Bottom row: SEM images showing surface morphologies of Matrix2 following 0, 4, 7, and 12 weeks of *in vitro* degradation. The unique polymer erosion of the blend system resulted in the change of matrix morphology from a solid coherent film to an assemblage of microspheres with interconnected porous structures characterized by macropores (10-100 µm) between polyphosphazene spheres as well as micro/nanopores on the sphere surface.

**Figure 6-4.** *In vitro* degradation profiles of the blend matrices in pH 7.4 aqueous media at 37°C over 12 weeks: a) Percentage of PLAGA molecular weight remaining in the pristine PLAGA and blend matrices over 12 weeks; b) Percentage of mass remaining; c) Percentage of polyphosphazene molecular weight remaining in the blend matrices; d) pH change of degradation media with the pristine PLAGA and blend matrices. The blend matrices showed slower degradation rate than pristine PLAGA. The changes of molecular weight for both the PLAGA and polyphosphazene components in the matrix further confirmed that the two components degraded in a similar pattern.

**Figure 6-5.** Surface morphologies of blend matrices as a function of subcutaneous implantation time. Top row: SEM images showing surface morphologies of Matrix1 following 2, 7, and 12 weeks of implantation. Bottom row: SEM images showing surface morphologies of Matrix2 following 2, 7, and 12 weeks of implantation. The polymer spheres were less than 100 µm in diameter. Furthermore, the size of spheres increased significantly with the increase of polyphosphazene ratio in the blend system.

**Figure 6-6.** Representative SEM image of sphere cross-sections showing the intricate pore structure within the formed polymer spheres of Matrix2 after 10-week implantation. Such continuous porous structure within the polymer spheres provides additional surface area and space for promoting cell-material interactions.
Figure 6-7. *In vivo* degradation profiles of blend matrices and pristine PLAGA over 12 weeks: (a) Percentage of mass remaining; (b) Percentage of PLAGA molecular weight remaining in the pristine PLAGA and blend matrices during 12 weeks of implantation; (c) Percentage of polyphosphazene molecular weight remaining in the blend matrices during 12 weeks of implantation. The mass loss profiles during the implantation suggested the degradation rate to be PLAGA > Matrix1 > Matrix2. The molecular weight for both PLAGA and polyphosphazene components in the blend decreased in a similar trend throughout the 12-week implantation period.

Figure 6-8. Micro-CT analysis for the blend matrices during in vivo implantation where a-h) Representative 2D micro-CT images illustrating the progression of morphological and structural changes within the blend system; i) In-situ porosity ((total volume-polymer volume)/total volume) of the matrix based on the 3D micro-CT reconstructions during the polymer degradation. Since the PLAGA component served as a “dynamic” porogen, the interconnectivity of the resultant porous structures was 100% as indicated in the 2D micro-CT images. In addition, it was shown that the blend composition had no significant effect on the porosity of the blend matrices. Both the blend matrices had a porosity of 82-87% after 12 weeks of implantation.

Figure 6-9. Histology images illustrating the formation of polymer spheres with pore system that is capable of accommodating cell infiltration and tissue in-growth within the blend matrices. (a,b, H&E): The arrows indicate the polymer sphere formation within Matrix1 and Matrix2 after 7 weeks of implantation, respectively; (c,d, H&E): The arrows indicate the polymer sphere formation within Matrix1 and Matrix2 after 12 weeks of implantation, respectively. The insets of c) and d) with TRI show robust collagen tissue infiltration within the matrix through the *in situ* formed pores after 12 weeks of implantation. It demonstrated that the *in situ* formed 3D interconnected porous structure enabled accommodation of cell infiltration and collagen tissue in-growth.

Figure 7-1. Structural similarity between poly(isobutylene) and polyphosphazenes.

Figure 7-2. General synthesis of polydichlorophosphazene and subsequent substitution with alkoxide (OR). Chemical structures of polymers 3-13.

Figure 7-3. DSC traces of poly(di(2-(2-methoxyethoxy)ethoxy)phosphazene), polymer 12, and polymer 13.

Figure 7-4. One possible orientation of the side group structure in polymer 13.

Figure 7-5. DSC traces of polymer 6, oligoisobutylene, and a blend of polymer 6 with OIB.

Figure 7-6. DSC traces of polymer 13, oligoisobutylene, and a blend of 13 with OIB.

Figure 7-7. Possible interactions between 13 and oligoisobutylene that leads to increased solubility compared to 12.
Figure 7-8. Thermal characteristics of (a) poly(dialkoxyphosphazenes) polymers 3-6 and 12; (b) poly(alkoxy)(2-(2-methoxyethoxy)ethoxy)phosphazene) polymers 8-11 and 13. The thermal characteristics of oligoisobutylene is included in both (a) and (b).
LIST OF TABLES

Table 2-1. Structural and physical properties of polymers 6-12 .................................................. 39

Table 3-1: Characterization data for small molecule model [(amino ethyl ester)_x(choloxyl chloride)_y)cyclotriphosphazenes]. ........................................................................................................ 54

Table 3-2: Structural properties of poly[(amino ethyl ester)_x(choloxyl chloride)_yphosphazenes]. The molar percent (mol%) choline was determined by integration of the H NMR spectra for each polymer. .................................................................................. 57

Table 3-3. Physical properties of poly[(amino ethyl ester)_x(choloxyl chloride)_yphosphazenes] (a) using the NaH synthetic route and (b) using the Cs_2CO_3 synthetic route. ........................................................................................................ 58

Table 3-4. DSC analysis of polymer blends of PLGA with polyphosphazenes synthesized using the (a) sodium hydride route and (b) the cesium carbonate route. Blends A-C and G-I describe blends of polymers 5-12 with PLGA 50:50. Blends D-F and J-L describe blends with polymers 5-12 with PLGA 85:15. Blends A, D, G, and J contained 25% polyphosphazene, Blends B, E, H, and K contain 50% polyphosphazene, and Blends C, F, I, and L contain 75% polyphosphazene. ........................................... 63

Table 4-1. Structural and physical properties of polymers 3-5. *The GPC-derived molecular weight approximate to Mw. ........................................................................................................ 88

Table 5-1. Characterization of Polylactide-Polyphosphazene Block Copolymers ..................... 108

Table 7-1. Characterization Data for Polymers 3-13, OIB and PIB ................................. 162
PREFACE

ACKNOWLEDGEMENTS

I would like to thank my advisor, Professor Harry R. Allcock, for the opportunity to conduct research under his guidance and support throughout my graduate career. The skills that I developed under his tutelage will undoubtedly prove invaluable throughout my scientific career. I would like to thank my graduate committee Prof. Karl Mueller, Prof. Alan Benesi, and Prof. James Runt for their guidance throughout my graduate work. I would also like to thank our group secretary Noreen Allcock for her help throughout my graduate career. I am also thankful to The Pennsylvania State University, The National Institute of Health, Penreco, and Johnson & Johnson for funding my research.

I would also like to thank my undergraduate research advisor, Dr. John Richardson, of Shippensburg University who gave me the initial experience in conducting research. I would like to express my gratitude towards collaborators who proved to be valuable during my graduate career: Meng Deng, Dr. Syam Nukavarapu, Dr. Lakshmi S. Nair, and Dr. Cato T. Laurencin in the Department of Chemical, Materials and Biomolecular Engineering at the University of Connecticut, Dr. Curt Omiecinski, Denise Weyant, and Dr. Seong Kim at The Pennsylvania State University. I would also like to acknowledge and thank past and present group members who have helped me during my graduate career particularly Dr. Nicholas Krogman, Dr. Song Yun Cho, Dr. Dan Welna, David Lee, Steven Owens, and Nicole Morozowich. My research would have been less fulfilling and meaningful without their help, guidance, scientific discussions, and friendship.

Finally, I would like to thank my family and friends for their never-ending love and support throughout my studies. I would like to express my gratitude towards my wife Mindi Weikel for her eternal love and for always believing in me. Without you this would not have been possible. I would also like to thank my parents, Karen and William Krohn, for their
continued support and guidance throughout my entire academic career. I would also like to thank family and friends, especially Harold and Eleanor Lenker, for their immeasurable support.
Chapter 1

Introduction to Polymer Chemistry

1.1 History

The first polymers processed by mankind were by the Egyptians and Chinese who used natural polymers such as wool, cotton, and silk as clothes to protect them from the elements. However, the materials used by early man were utilized as they were found in nature. It was not until the 19th century that humans acquired the knowledge to alter natural polymers for use as commercial products. The vulcanization of rubber by Charles Goodyear in 1839 was the first successfully commercialized polymer. The remainder of the 19th century was focused on further modifications of natural polymers to change their chemical and physical properties such as the nitration or acetylation of cellulose. The first synthetic polymer was discovered shortly after the turn of the 19th century by Leo Baekeland. In 1907 he synthesized a heat resistant phenol-formaldehyde polymer resin that he named Bakelite. All polymers, including Bakelite, that predate the 1930s were believed to be aggregates of small molecules. Then in the 1920s, Nobel Laureate Hermann Staudinger advanced polymer science by hypothesizing that polymers were composed of long chain-like molecules held together by covalent bonds. During the 1930s, Carothers work on polyesters and polyamides verified Staudinger’s hypothesis through the synthesis of low molecular weight polymers, that eventually led to the development of Nylons. During the 1930s-1940s the development of several polymers such as polystyrene, poly(methyl methacrylate), and poly(vinyl chloride) yielded several commercialized products (Figure 1-1). With the outbreak of World War II, significant shortages of traditional materials such as natural rubber, glass, wood, and steel accelerated the development of polymers as alternatives for these...
traditional materials. This need for alternative materials led to the development of polymer science as we know it today.

\[
\{\text{CH}_2\text{CH}_2\}_n \quad \{\text{CH}_2\text{CH}_3\}_n
\]

Polyethylene (PE)  Polypropylene (PP)

\[
\{\text{CF}_2\text{CF}_2\}_n \quad \{\text{CH}_2\text{CH}_2\}_n
\]

Poly(tetrafluoroethylene) (Teflon)  Poly(vinylchloride) (PVC)

\[
\text{Polystyrene (PS)} \quad \text{Poly(methyl methacrylate) (PMMA)}
\]

**Figure 1-1.** Common Organic Polymers.

### 1.2 Polymer Definition and Architecture

The word polymer is derived from the Greek words *poly* (many) and *meres* (units).\(^6\) Polymers are defined as large macromolecules composed of many repeat units, also known as *monomers*, linked together by covalent bonds. The chemical and structural nature of the repeat unit has significant influence over the chemical and physical properties of the resultant polymer. Polymers are typically depicted as linear structures. In reality, polymers exist as random coils.
with many conformations or are organized into crystalline domains. A polymer synthesized from a single type of monomer is referred to as a homopolymer, while copolymers are formed when two or more different monomers are used. The physical representation of a polymer is based on the polymer’s skeleton. Polymer skeletons consist of linear, straight-chain macromolecules or as more complicated architectures such as stars, dendrimers, combs, and cross-linked systems (Figure 1-2). The polymer architecture has a direct effect on the physical properties of the polymer.

Linear homopolymers are the most common architecture that is studied in polymer science. Unlike the more complex architectures, linear polymers are generally soluble in organic solvents. In addition, increased viscosities are produced as the length of the skeleton increases due to increased entanglements between polymer chains. Comb type polymers have a similar trend in viscosity as the length of the combs increases. However, the branched segments of comb polymers inhibit the formation of ordered domains, which decreases the amount of micro-crystallinity. The solubility of these polymers is dependent on the density of the branches. Increased branch density results in more chain entanglements that may limit solvation of the polymer.
Figure 1-2. Various Polymer Architectures.
Star and dendrimer polymers are characterized by a core with arms that extend outward. The two main techniques used to form these architectures are the chain growth of the arm from the central core or the attachment of the fully grown arm onto the central core post polymerization. Dendrimers are grown symmetrically from a core with equal chain lengths and with the number of branches doubling after each generation. Whereas, star polymers may have varying chain lengths, and the number of branches is controlled by the initial number of growth sites in the core. The reduction in chain entanglements with these architectures lowers the viscosity while increasing the solubility in organic solvents compared to linear or comb polymers.

Unlike the previously mentioned architectures, cross-linked polymers are markedly different because the covalent linkages between chains render the polymers insoluble in all solvents. The average number of monomers between the intermolecular cross-links is referred to as the cross-link density. The physical properties of cross-linked polymers can be tuned by varying the cross-link density. For example, a light cross-link density of 1-2% can result in elastomeric properties or hydrogels (after absorption of liquids). On the other hand, a large cross-link density of 30% can result in a rigid or brittle polymer.

The physical and chemical properties of all architectures are further influenced by the structure of the monomers used to produce the homopolymers. However, some homopolymers have limitations that may be overcome by co-polymerization with more than one monomer to produce copolymers. Many different copolymers have been synthesized that include random and alternating structures, block copolymers, and graft copolymers. (Figure 1-3).

Random copolymers are produced by the random addition of two monomers during a synthesis process that lacks any particular monomer addition sequence. Random polymers are used to control the melting temperature \(T_m\) of a copolymer without affecting the glass transition temperature \(T_g\). One the other hand, alternating block copolymers are formed by the controlled
addition of each monomer to produce alternating monomer regularity along the polymer chain. Block copolymers are formed when a linear chain of one homopolymer is connected to a different homopolymer to produce di-block, tri-block, etc structures. Block copolymers can act as blend compatibilizers in polymer by insertion of each block into a different polymer domain. Graft copolymers are another class of copolymers, where the main chain is composed of one polymer and the side chains (called grafts) are composed of different polymer. Small amounts of grafts along a polymer chain can decrease the polymers crystallinity and melting points without affecting the glass transition temperature, which allows for easier processing of crystalline polymers.

Figure 1-3. Different types of block copolymer structures.
1.3 Polymer Blends

Many homopolymers have useful properties that allow them to be applied in a wide range of applications. However, some of these polymers used for applications do not possess all the necessary properties. Therefore, the physical mixing of two or more polymers to meet the required characteristics is a method used to attain more appropriate property combinations. Moreover, it is much easier to mix two polymers than to overcome the synthetic challenges associated with the formation of block copolymers. The physical mixing of two polymers can result in miscible, partially miscible, or immiscible blends. The degree of mixing is directly related to how strong the interactions are between the two polymers. Miscible polymer blends require a strong intermolecular interaction to overcome entropy that is produced when mixing two high molecular weight polymers. The two most common interactions are ionic interactions or hydrogen bonding. Many polymers do not form miscible combinations. For example, the physical mixing of polyethylene with polystyrene will result in immiscible phase-separated domains due to the lack of adhesive forces between the polymers. The degree of miscibility is also dependent on the molecular weight of each individual polymer. Blend miscibility is easier to achieve when low molecular weight polymers are used.

1.4 Polymer Synthesis

Thousands of polymers have been synthesized to produce the architectures mentioned previously. These polymers can be classified into three main groups based on the mechanism of polymerization: 1) step-growth polymerization, 2) chain-growth polymerization, or 3) ring-opening polymerization. Some polymers can be synthesized using more than one of these
polymerization techniques. The type of polymerization used is determined by the amount and types of functional groups that each monomer possesses.

1.4.1 Step-Growth Polymerization

Step-growth polymerizations occur when bifunctional or multifunctional monomers react to first form dimers, and then trimers, followed by short chain oligomers, and then high molecular weight polymers. The reaction between the monomer molecules results in the release of small molecules during polymerization called condensates (usually water or another small molecule). Polyesters, polyamides, polycarbonates, polyurethanes and natural polymers such as wool and silk are all examples of step-growth polymers. In general, step-growth polymerizations produce low to medium molecular weight polymers due to the long reaction times that are necessary to achieve high molecular weight materials. For example, the reaction of bisphenol-A with phosgene results in the synthesis of a polycarbonate (Figure 1-4). Another example of a step-growth polymerization involves the loss of salts that can be used to synthesis polyetherketones or polysulfides (Figure 1-5).
Figure 1-4. Condensation polymerization of bisphenol-A and phosgene to form a polycarbonate.
1.4.2 Chain-Growth Polymerization

Chain-growth polymerization occurs when the polymer grows by the addition of one monomer at a time onto the end of the growing polymer chain. The distinction between chain-growth and step-growth is that step-growth monomers are bifunctional and a small molecule side product is produced, whereas chain-growth requires the formation of a radical or ion at the chain end for polymerization to occur.\textsuperscript{2,7} The monomers used for this type of polymerization are characterized by unsaturation. Examples of monomers used for chain-growth polymerization are styrene, ethylene, and methyl methacrylate (Figure 1-6). Chain-growth polymerization is initiated through the use of initiators such as an \textit{azo} compound, peroxides, or an organometallic species to form the initiation intermediate. The initiated species can then react with monomer to initiate chain propagation. The last step is chain termination that occurs by combination with other chain ends, by disproportionation, or by reaction with terminates. Some chain-growth polymerizations do not have a termination step and are referred to as living polymers. Living polymers can resume polymerization with the addition of more monomer. Unlike step-growth polymerization, high molecular weights can be achieved with low concentrations of monomer.
converting to polymer. An example of the chain growth mechanism is the polymerization of vinyl chloride through initiation using peroxides (Figure 1-7).\textsuperscript{12-14}

**Figure 1-6.** Monomers used in chain-growth polymerizations.
1.4.3 Ring-Opening Polymerizations (ROPs)

Many cyclic monomers can undergo ring-opening polymerization to form linear or cross-linked polymers. \(^1,^7\) Ring-opening polymerizations progress in a unique manner compared to step-growth or chain-growth polymerizations. Ring-opening polymerizations are driven by the release of ring strain when the cyclic monomers are ring-opened. Insufficient steric strain, depending on the size of the ring, requires the use of a catalyst such as metal complexes, heat, acids or bases to perform ring-opening polymerizations. \(^1,^2,^7\) Many types of organic polymers have been synthesized using ring-opening polymerizations including polyesters, polylactones, and polylactams along with inorganic-organic polymers such as polysiloxanes and polyphosphazenes (Figure 1-8).
1.5 Hybrid Inorganic-Organic Polymers

The majority of polymers used today are based on organic monomers that are derived from petroleum or, more recently, from renewable sources like corn or soy beans. The widespread use of these polymers is traced back to the low costs of the monomers along with the well-defined synthetic, processing, and fabrication protocols that have been developed. As a direct result, traditional materials such as glass, cotton, and metals are being replaced by organic polymers. However, organic polymers are based on the element carbon. Carbon based polymers have inherent problems with low thermal, oxidative, and chemical stability. In addition, few organic polymers maintain their flexibility and mechanical stability over large temperature ranges, which limits their utility.

Hybrid inorganic-organic polymers have been developed to meet the increasing demand for novel polymer based materials and to circumvent the limitations of organic polymers. The incorporation of inorganic elements into the organic polymer backbone allows the fabrication of macromolecules with increased thermal, oxidative, and chemical stability. The two most common inorganic-organic macromolecules are polysiloxanes and polyphosphazenes (Figure 1-8).
1.6 Polyphosphazenes

1.6.1 Significance

Polyphosphazenes are hybrid inorganic-organic macromolecules in which the properties are determined by both the alternating phosphorus-nitrogen inorganic backbone and the organic side groups (Figure 1-9). Polyphosphazenes are unique because the properties can be directly controlled via macromolecular substitution of the chlorine atoms by various organic groups. The use of this technique to produce the final polymer is markedly different from organic polymers.
where the final polymer structure and chemical properties are determined by the monomers used. In addition, the polyphosphazene backbone imparts properties such as fire resistance, thermo-oxidative stability, flexibility, and low glass transition temperatures, while the side groups control solubility and many other properties. Several hundred different polyphosphazenes are now known, each with a combination of different properties and solubilities. They can be used as ion conductors, fire retardants, and biomedical materials.\textsuperscript{15,16}

### 1.6.2 Synthesis

Phosphazene chemistry dates back to the discovery and characterization of the small-molecule ring compound hexachlorocyclotriphosphazene by Liebig\textsuperscript{17}, Rose\textsuperscript{18}, Gerhardt\textsuperscript{19}, Gladstone\textsuperscript{20}, and Wichelhaus\textsuperscript{21} during the 1830s to 1870s. Then, in the 1890s, Stokes demonstrated that hexachlorocyclotriphosphazene could undergo thermal ring opening polymerization to form an insoluble inorganic rubber that decomposed under atmospheric conditions.\textsuperscript{22-25} The major breakthrough in phosphazene chemistry occurred in 1964 when Allcock and Kugel discovered a technique to prepare soluble polyphosphazenes.\textsuperscript{26-28} This technique involves the purification of hexachlorocyclotriphosphazene by re-crystallization from n-heptanes followed by sublimation. The hexachlorocyclotriphosphazene is then sealed in an evacuated glass tube and heated to 250°C for 6 to 36 hours, which causes thermal ring opening polymerization of the trimer to poly(dichlorophosphazene). Viscosity is monitored to determine when to terminate the polymerization. A conversion of about 60-75\% trimer to polymer is standard without allowing cross-links to form. This melt polymerization does not allow control of molecular weight or dispersity, but it can produce poly(dichlorophosphazenes) with very high molecular weights.\textsuperscript{15}
Poly(dichlorophosphazene) can also be synthesized using a controlled living cationic polymerization that was developed by Allcock, Manners et al. in the 1990s.\textsuperscript{15,29,30} This route involves the reaction of the monomer, trichloro(trimethylsilyl)phosphoraneamine, in the presence of a cationic initiator such as phosphorus pentachloride (PCl$_5$). The initiator displaces the trimethylsilyl group to create an active site at which chain growth proceeds with the addition of more monomer (Figure 1-9). The advantage of this route is that it occurs at 25°C, can provide control of polydispersities and molecular weight. Moreover, the living chain end enables poly(dichlorophosphazene) to be linked to other polymers to produce more complex structures.

1.6.3 Macromolecular Substitution

The different macromolecular substitution by nucleophiles on poly(dichlorophosphazene) is a unique phenomenon within polymer chemistry. The replacement of the labile chlorine atoms by alkoxides, aryloxides, and amines determines the final polymer properties (Figure 1-9).\textsuperscript{15} This substitution technique has resulted in more than 700 different polyphosphazenes many of which contain a single type of side group. However, there are many more polymers that contain multiple side groups, attached sequentially or simultaneously to the polymer backbone, that allow fine tuning of the properties for many applications. In addition, the chemical nature of the side group determines the bulk properties of the resultant polymer. Some examples are polyphosphazenes that contain high levels of fluorinated side groups to produce super hydrophobic surfaces, or contain amino acids that impart hydrolytic instability for use as tissue engineering scaffolds.\textsuperscript{15} The main advantage of polyphosphazenes over organic polymers is in the ease of tunability via macromolecular substitution. As discussed, organic polymers rely on the use of new monomers for each new polymer followed by the study of the conditions required for polymerization to tune the properties, which is time-consuming and costly.
1.6.4 Polyphosphazene Architecture

Many different side groups can be attached to the polyphosphazene skeleton using the macromolecular substitution technique. This substitution technique combined with the living cationic polymerization route allows for many polymer architectures to be produced. These include linear polymers, star polymers, graft polymers, dendrimers, and block copolymers (Figure 1-10). Polyphosphazene-block-polyphosphazene copolymers, polyphosphazene-block-polystyrene copolymers, poly(methyl methacrylate)-graft-polyphosphazene copolymer, polyphosphazene-block-poly(ethylene oxide) copolymers are a few examples. Cyclo-linear polymers have also been developed that are based on the polymerization of substituted phosphazene trimers. Cyclic trimeric phosphazenes have also been attached to organic polymers to alter their properties.

![Figure 1-9](image)

**Figure 1-9.** Synthesis of poly(dichlorophosphazene) and subsequent macromolecular substitution of organic groups along the polymer backbone.
1.6.5 Applications

The large number of different side groups that can be linked to the polyphosphazene backbone allows access to the properties needed for many different applications (Figure 1-11). Phosphazenes that contain etheric side groups like 2-(2-methoxyethoxy)ethoxy are useful as lithium ion conductors for batteries.46-49 The high ion conductivity is due to the low glass transition temperature ($T_g$) of -80°C that solvates lithium salts.15 Proton conduction polymers have been developed by incorporation of sulfonic acid containing side groups.50-53 These polymers were fabricated into membranes for use in proton and direct methanol fuel cells.
Highly fluorinated phosphazenes based on trifluoroethoxy side groups are resistant to ultraviolet and gamma irradiation, and have been fabricated into super-hydrophobic surfaces\(^{54-55}\). Co-substituted trifluoroethoxy polymers with other fluorinated side groups produced polymers that are used as low and high temperature elastomers by the US military\(^{56-57}\). Highly fluorinated cross-linked matrices based on the cyclic phosphazene trimer have demonstrated the ability of these species to be used as optical wave guide materials\(^{58}\). Other applications for phosphazenes are as fire retardants\(^{59-62}\), high refractive index glasses\(^{63-64}\), electroluminescent materials\(^{65}\), and as biomaterials\(^{68-76}\).

\[
\begin{align*}
\text{Poly[bis(2-(2-methoxyethoxy)ethoxy)phosphazene]} & & \text{Poly[bis(2-trifluoroethoxy)phosphazene]} \\
\text{Lithium Ion Conductor / Hydrogels} & & \text{Superhydrophobic Surfaces}
\end{align*}
\]

\[
\begin{align*}
\text{Poly[(3-methylphenoxy)x(3-methyl4-sulfurylphenoxy)phosphazene]} & & \text{Poly[bis( amino acid ester)phosphazene]} \\
\text{Proton / Methanol Fuel Cell Membranes} & & \text{Tissue Engineering Scaffolds / Drug Delivery Vehicles}
\end{align*}
\]

**Figure 1-11.** Applications of various polyphosphazenes.
1.7 Biomaterials

Numerous biomaterials have been developed over the past 80 years that are used to fabricate devices to correct for imperfections within or on the outside of the human body. These biomaterials can be separated into two distinct categories: bio-stable and bio-absorbable materials. The most common bio-stable materials are metals, ceramics, and non-biodegradable polymers. Gold, stainless steel and titanium are used in tooth restoration, orthopedic pins and screws, and in artificial hips because of their inertness, strength, corrosion resistance, and, in the case of titanium, light weight. In general, ceramics are used in fillings for teeth and as bone repair materials. Oxide ceramics, like calcium hydroxyapatite, are more commonly used rather than their non-oxide ceramic counterparts. Non-biodegradable polymers are currently the most widely used bio-stable materials. Polymers such as poly(methyl methacrylate), polyamides, and poly(dimethylsiloxanes) are used in contact lenses, as hydrophobic elastomers, and sutures. The underlying requirement for a bio-stable material is that it does not generate a detrimental biological response.

Bio-absorbable or bio-degradable materials can also be placed on, but usually within, the human body without causing an unfavorable biological response. Most, bio-absorbable materials will degrade by hydrolysis over time, be metabolized, and then excreted from the body. These materials are exclusively made from polymers. Poly(glycolic acid) (PGA), poly(lactic acid) (PLA), poly(lactic-co-glycolic acid) (PLGA), poly(caprolactone), polydioxanone, polytrimethylene carbonate, and polyanhydrides have been studied since the 1960s and have gained FDA approval for medical applications together with naturally occurring polymers such as collagen, alginates, and chitosan (Figure 1-12). The majority of these polymers are used as drug delivery vehicles and as biodegradable sutures. However, materials for tissue engineering scaffolds, bone cements, liquid band-aids, and many other applications have been developed.
Polyphosphazenes are another bio-absorbable polymer that has received attention as implantable materials.\textsuperscript{2,15,66}

**Figure 1-12.** Examples of bio-absorbable FDA approved polymers.

1.8 Polyphosphazene Biomaterials

There is a pressing need for newly developed biomaterials for use as implantable materials that hydrolyze to benign small molecules. Polyphosphazenes have been gaining
attention in the biomedical field due to the deficiencies of the current organic polymer implants based on poly(lactic-co-glycolic acid) (PLGA) or other polyesters. These hydrolyze to acidic products that cause tissue necrosis or irritation around the implant site.\textsuperscript{67} Biomaterials based on polyphosphazenes have the advantage over polyesters because they hydrolyze into benign products with neutral pH values. Polyphosphazenes that contain amino acid ester\textsuperscript{68-72}, glucosyl\textsuperscript{71}, glycerol\textsuperscript{74}, imidazole\textsuperscript{75}, lactide and glycolide\textsuperscript{76} side groups hydrolyze in aqueous media. All of these side groups cause the polyphosphazene to hydrolyze using a similar hydrolysis mechanism. For example, the hydrolysis mechanism of poly(amine acid ester phosphazenes) proceeds by the cleavage of the amino acid side group from the skeleton to produce a P-OH linkage (Figure 1-13). Hydrolysis is followed by a proton shift from the oxygen to the nitrogen in the polymer backbone to form the unstable phosphazane.\textsuperscript{69} This causes rapid hydrolysis of the polymer backbone to phosphates, ammonium, and the original organic side group, with near-neutral pH values. In addition, the rate of hydrolysis is controlled by the steric hindrance along the polymer backbone.\textsuperscript{69} The introduction of P-O organic side group linkages retards the rate of hydrolysis. Bioerodible phosphazenes have been investigated as controlled drug delivery vehicles and as tissue engineering scaffolds.

In this thesis the use of blends between polyphosphazenes and polyesters as tissue engineering scaffolds is discussed. The main focus is on the design, synthesis, and characterization of polyphosphazenes used in the fabrication of blends with polyesters. Biocompatibility, hydrolytic sensitivity, blend miscibility, and osteoconductivity were examined for the polyphosphazenes and for blends between polyphosphazenes and polyesters, namely PLGA. The end goal of the project was to produce novel polyphosphazenes that can form miscible blends with PLGA, buffer the acidic hydrolysis products from PLGA, increase the mechanical properties through intermolecular hydrogen bonding or cross-linking, and improve osteoconductivity. The remaining chapters in this thesis will cover the use of dipeptide esters and
the biological buffer, choline, to increase the mechanical stability and pH values of the resultant hydrolysis media in blended PLGA composites. The synthesis of a reversible cross-linking polyphosphazene using cysteine ethyl ester was also examined. The first reported synthesis of a 100% hydrolysable polyphosphazene block copolymer and its application as a blend compatibilizer is reported. Finally, a unique hydrolysis mechanism is reported in which a poly[(glycylglycine ethyl ester)(4-phenylphenoxy)phosphazene] blended with PLGA (50:50) hydrolyzes from a solid film to form micro-spheres.

Figure 1-13. Hydrolysis mechanism of poly(amino acid ester phosphazenes).
1.9 References


   **1997**, *30*, 2213.


   5824.


   2870.

   3896.

   3563.


Chapter 2

Polyphosphazenes that Contain Dipeptide Side Groups: Synthesis, Characterization, and Sensitivity to Hydrolysis

2.1 Introduction

The need for new bioerodible or biostable biomedical materials is a driving force for the development of advanced synthetic macromolecules. Polymeric materials with backbones based on synthetic polypeptides have generated a great deal of interest because new synthetic methods have led to the development of controllable molecular architectures and functionalities that can subsequently be tuned for specific applications. These polymers have potential uses as scaffolds for tissue engineering, membranes, surgical adhesives, and antimicrobial materials. However, the insolubility of synthetic polypeptides and their sensitivity to thermal decomposition has limited the usefulness of biomaterials based on these polymers. Thus, the incorporation of peptides into other polymer systems is an alternative approach that might overcome these complications.

Polyphosphazenes are highly tunable via the macromolecular substitution synthetic route that is often employed. This method allows facile changes in the polymer side groups, so that different polyphosphazenes have markedly different properties. Polymers for ionic conduction, fire retardance, superhydrophobicity, and various levels of hydrolytic sensitivity have been developed. Hydrolytically sensitive polyphosphazenes have recently received a great deal of attention for applications as biomaterials. Hydrolytic sensitivity in a polyphosphazene can be achieved with the use of amino acid ester side groups that are linked to the polymer backbone via the amino terminus. The hydrolytic sensitivity of these polymers is controlled by the molecular structure at the α-carbon of the amino acid as well as by co-substitution with other organic groups along the polyphosphazene backbone. The hydrolytic sensitivity of
polyphosphazenes is also pH-dependent. For example, when poly[(ethyl glycinato)\(_2\)(p-methyl phenoxy)\(_3\) phosphazene] is blended with poly(lactide-co-glycolic acid) (PLGA), the hydrolysis rate of the phosphazene is increased due to the presence of an acid environment created by the hydrolysis of PLGA\(^\text{22}\). The amino terminus of peptides can also be used for chlorine replacement with poly(dichlorophosphazene).

In this study, we have synthesized three dipeptide ethyl esters to give alanyl-glycine ethyl ester, valinyl-glycine ethyl ester, and phenylalanyl-glycine ethyl ester, and these have been linked to the polyphosphazene chain together with glycine or alanine ethyl ester co-substituents to yield new bioerodible polymers. Heterophase, pH-dependent hydrolysis experiments of these polymer derivatives were carried out with the use of buffered aqueous media at pH 4.0, pH 7.0, and pH 10.0.

### 2.2 Experimental

#### 2.2.1 Reagents and Equipment

All synthetic reactions were carried out under a dry argon atmosphere using standard Schlenk line techniques. Tetrahydrofuran and triethylamine (EMD) were dried using solvent purification columns\(^\text{23}\). Chloroform (EMD), isobutyl chloroformate (Aldrich), Boc-phenylalanine, Boc-valine, Boc-alanine (Aroz Technologies), alanine ethyl ester hydrochloride (Chem Impex) and glycine ethyl ester hydrochloride (Alfa Aesar) were used as received. 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 use of a Bruker 360 WM instrument operated at 145 MHz 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 tetrabutylammonium nitrate in THF. The elution times were calibrated with polystyrene standards.

2.2.2 Synthesis of Boc-alanyl-glycine ethyl ester

This compound was synthesized by a modification of a published procedure.\textsuperscript{24} Specifically, triethylamine was used as a base in place of N-methylmorpholine, and the purification procedure was modified. Chloroform (135 mL) was cooled to -15 °C, then Boc-alanine (25.0 g, 132 mmol) and triethylamine (36.8 mL, 264 mmol) were added sequentially. This mixture was stirred at 15 °C for five minutes before the addition of isobutyl chloroformate (IBCF) (17.23 g, 132 mmol). The mixture was then stirred at -15 °C for two minutes, followed by the addition of glycine ethyl ester hydrochloride (18.5 g, 132 mmol). The reaction mixture was stirred for four hours at -15 °C, and then allowed to warm slowly to room temperature, while stirred overnight. The solvent was removed under reduced pressure and an oily residue remained. The residue was dissolved in ethyl acetate and was washed sequentially with deionized water, 20% citric acid (aq), deionized water, saturated sodium bicarbonate, and deionized water. The organic layer was dried with magnesium sulfate and the solvent was removed under reduced pressure, to yield a viscous oil. The yield was approximately 80%. \textsuperscript{1}H NMR (D\textsubscript{2}O), ppm: δ 1.16 (t, 3H, CH\textsubscript{3}), 1.34 (m, 12H, CH\textsubscript{3}), 3.80 (m, 4H, CH\textsubscript{2}), 4.04 (q, 1H, CH). m/z = 275.
2.2.3 Synthesis of Boc-valinyll-glycine ethyl ester

The synthesis of this dipeptide follows the procedure used for Boc-alanyl-glycine ethyl ester dipeptide. \(^1\)H NMR (D\(_2\)O), ppm: \(\delta 0.87\) (d, 3H, CH\(_3\)), 0.90 (d, 3H, CH\(_3\)), 1.21 (t, 3H, CH\(_3\)), 1.38 (s, 9H, CH\(_3\)), 2.10 (m, 1H, CH), 3.96 (m, 4H, CH\(_2\)), 4.13 (q, 1H, CH). m/z = 292. The yields were typically 82%.

2.2.4 Synthesis of Boc-phenylalanyll-glycine ethyl ester

The synthesis of this dipeptide follows the procedure used for Boc-alanyl-glycine ethyl ester dipeptide. \(^1\)H NMR (D\(_2\)O), ppm: \(\delta 1.20\) (t, 3H, CH\(_3\)), 1.38 (s, 9H, CH\(_3\)), 2.98 (d, 2H, CH\(_2\)), 3.82 (m, 4H, CH\(_2\)), 4.86 (t, 1H, CH), 7.05 (m, 5H, aromatic). m/z = 350. The yields were typically 85%.

2.2.5 Deprotection of Boc-alanyl-glycine ethyl ester\(^{25}\)

Boc-alanyl glycine ethyl ester (48.0 g, 0.221 mol) was dissolved in 200mL of ethyl acetate, and 100 mL of 6 M HCl in ethyl acetate were added to this solution. The solution was stirred for four hours at room temperature. The solvent was removed under reduced pressure and an adhesive oil remained. The oil was tritrated with diethyl ether and was dried under reduced pressure. The final product was recovered as a white, crystalline product. The final yield was 87%. \(^1\)H NMR (D\(_2\)O), ppm: \(\delta 1.02\) (d, 3H, CH\(_3\)), 1.12 (t, 3H, CH\(_3\)), 3.40 (q, 1H, CH), 3.92 (s, 2H, CH\(_2\)), 4.03 (q, 2H, CH\(_2\)). m/z = 162.
2.2.6 Deprotection of Boc-valinyl-glycine ethyl ester

$^1$H NMR (D$_2$O), ppm: δ 0.89 (d, 6H, CH$_3$), 1.10 (t, 3H, CH$_3$), 2.09 (m, 1H, CH), 3.69 (d, 1H, CH), 4.05 (m, 4H, CH$_2$). m/z = 190. The yield was 83%.

2.2.7 Deprotection of Boc-phenylalanyl-glycine ethyl ester

$^1$H NMR (D$_2$O), ppm: δ 1.12 (t, 3H, CH$_3$), 2.90 (d, 2H, CH$_2$), 3.80 (m, 1H, CH), 3.90 (m, 4H, CH$_2$), 7.12 (m, 5H, aromatic). m/z = 238. The yield was 85%.

2.2.8 Synthesis of Cyclic Trimers 2-4

The synthesis of the model compound cyclic trimers 2-4 followed similar procedures. The synthesis of 4 is given as a representative example. Hexachlorocyclotriphosphazene (1.00 g, 2.88 mmol) was dissolved in toluene (100 mL). Phenylalanyl glycine ethyl ester hydrochloride (8.25 g, 28.8 mmol) and triethylamine (10.0 mL, 71.9 mmol) were added to the solution. The mixture was stirred at room temperature for 24 hours. $^{31}$P NMR spectroscopy detected some degree of substitution: however complete chlorine replacement was not achieved. Therefore, the solution was refluxed for 24 hours. The absence of a $^{31}$P NMR signal indicated that the product had precipitated from solution. The recovered precipitate was insoluble and no further characterization was attempted. The cyclic trimers 2 and 3 also had some degree of substitution after mixing at room temperature, but the products became insoluble after reflux.
2.2.9 Synthesis of Polymer 6

Poly(dichlorophosphazene) (2.00 g, 17.3 mmol) was dissolved in THF (200 mL). Alanyl-Glycine ethyl ester hydrochloride (8.18 g, 36.2 mmol) was suspended in 200 mL of THF, and triethylamine (12.1 mL, 86.5 mmol) was added. This suspension was refluxed for 24 hours, and then filtered and added to the polymer solution. The resultant solution was stirred at room temperature for 24 hours, and then refluxed for 48 hours. The solvent was removed under reduced pressure to yield a yellow solid. This was dialyzed against ethanol for 3 days. The yield was 73% based on the amount of poly(dichlorophosphazene) used.

2.2.10 Synthesis of Polymers 7-12

Polymers 7-12 were synthesized using similar procedures. Polymer 7 is described as a representative example. Poly(dichlorophosphazene) (2.00 g, 17.3 mmol) was dissolved in 200 mL of THF. Alanyl-Glycine ethyl ester hydrochloride (3.90 g, 17.3 mmol) was suspended in 150 mL of THF, and triethylamine (7.23 mL, 51.9 mmol) was added. This suspension was refluxed for 24 hours, then filtered and added to the polymer solution. Glycine ethyl ester hydrochloride (7.25 g, 51.9 mmol) was suspended in 150 mL of THF and triethylamine (24.1 mL, 173 mmol) was added. This suspension was refluxed for 24 hours, then filtered and added drop-wise to the polymer solution, which was then stirred at room temperature for 24 hours, and refluxed for a further 48 hours. The solvent was removed under reduced pressure to yield a yellow solid. The polymer was purified by dialysis versus methanol for 3 days. The yields were in the range of 70-80%.
2.2.11 Controlled pH hydrolysis studies

Polymers 6-12 were dissolved in chloroform (100 mg/1 mL) and were solution cast into films. These were air dried for 24 hours and then vacuum dried for another seven days. The dried films were cut into squares (10 mm x 10 mm) and placed in specific pH buffered aqueous media. Aqueous media with pH 4.0, 7.0, and 10.0 were used. Three samples were removed from each medium after 1, 2, 3, and 4 weeks to measure weight loss. The pH of each hydrolysis medium was monitored throughout the study. GPC analysis of the polymers during the hydrolysis was precluded by the insolubility of the polymers in THF after hydrolysis was initiated.

2.3 Results and Discussion

2.3.1 Synthesis of alanyl glycine ethyl ester, valinyl glycine ethyl ester, and phenylalanyl glycine ethyl ester

The mixed anhydride solution-phase synthetic route was utilized for the synthesis of alanyl glycine ethyl ester, valinyl glycine ethyl ester, and phenylalanyl glycine ethyl ester. Isobutyl chloroformate was chosen to synthesize the mixed anhydride. The appropriate Boc-protected amino acid and glycine ethyl ester were added in stoichiometric amounts to form the protected N-terminus dipeptide. Deprotection of the N-terminus yielded the desired dipeptide ethyl ester. The complete synthesis of the dipeptides is outlined in Figure 2-1. Proton NMR techniques confirmed the removal of the Boc-protection group by the disappearance of the proton shift at approximately 1.3 ppm for all dipeptides. Mass spectrometry characterization confirmed the final mass of the dipeptides with free amino termini.
Figure 2-1. Synthesis of the Boc-protected dipeptides alanyl-glycine ethyl ester, valinyl-glycine ethyl ester, and phenylalanyl-glycine ethyl ester. The N-terminus was then deprotected under acidic conditions to yield the free amino dipeptides.

2.3.2 Synthesis of model cyclic trimers 2-4

Small molecule model reactions were attempted with the use of hexachlorocyclotriphosphazene (1). A stoichiometric excess of each dipeptide ethyl ester was added to a solution of (1) in toluene or THF in the presence of excess triethylamine, as described in Figure 2-2. The reaction mixture was stirred at room temperature for 24 hours. The $^{31}$P NMR spectra revealed that the unexpected geminal di-substituted cyclic trimer was formed with valinyl glycine ethyl ester (3) or phenylalanyl glycine ethyl (4) substituents. The cyclic trimeric species partially substituted with the alanyl glycine ethyl ester (2) reacted with (1) to form a mixture of products including those formed by geminal di-substitution to tetra-substitution. However, by the time the reaction mixtures had been heated at reflux for 24 hours, the products had precipitated from solution as incompletely substituted species. These experiments revealed that some chlorine replacement did occur at the small molecule model level, but that the degree of substitution depended on the dipeptide ethyl ester that was used. This information was used as a basis to plan the substitution reactions of poly(dichlorophosphazene) with the dipeptide ethyl esters.
Figure 2-2. Synthesis of dipeptide substituted cyclic trimers 2-4.

2.3.3 Synthesis of polymers 6-12

Initial attempts to synthesize polyphosphazenes that contained only valinyl-glycine ethyl ester or phenylalanyl-glycine ethyl ester as side groups were unsuccessful. Approximately 50% of the chlorine atoms were replaced after 24 hours at room temperature, as indicated by $^{31}$P NMR spectra. These partly substituted polymers precipitated from a refluxing solution in THF. Subsequent syntheses of polymers with valinyl-glycine ethyl ester or phenylalanyl-glycine ethyl ester side groups were therefore carried out after half the chlorine atoms in poly(dichlorophosphazene) had been replaced by the dipeptide, followed by the replacement of the remaining chlorine atoms by glycine ethyl ester or alanine ethyl ester to yield polymers 9-12. The synthesis of polymers 9-12 is described in Figure 2-3. Polymers 9-12 were yellow, brittle solids that were soluble in THF, chloroform, and methanol.

Alanyl-glycine ethyl ester was the only dipeptide ethyl ester able to replace 100% of the chlorine atoms in poly(dichlorophosphazene) while remaining soluble in THF during the synthesis process (polymer 6). Two polyphosphazenes were also co-substituted with both alanyl-glycine ethyl ester and glycine ethyl ester or alanine ethyl ester in a 1:1 ratio (Polymers 7 and 8). Polymers 6-8 were isolated as yellow, brittle solids that were soluble in the same solvents as polymers 9-12.
Molecular characterization of all the polymers was by multinuclear NMR and IR spectroscopy, as shown in Table 2-1. $^{31}$P NMR experiments suggested that complete replacement of all the chlorine atoms had occurred. It is known that polyphosphazenes that have 100% dipeptide and/or amino acid ester side groups give a broad $^{31}$P NMR signal at approximately 0 ppm. $^1$H NMR shifts were used to determine the percentages of substitution for each side group. For polymers 6-12, a 1:1 ratio was attempted and each polymer was within 3% of the target ratio. The infrared spectra of polymers 6-12 were used to confirm the side group and polymer backbone structural characteristics. The P-N bonds were evident from absorbances at 1214 cm$^{-1}$, C=O bonds were obvious at 1738 cm$^{-1}$, and N-H bonds showed stretching modes at 3200 cm$^{-1}$.

**Figure 2-3.** Synthesis of dipeptide substituted polyphosphazenes 6-12.
Table 2-1. Structural and physical properties of polymers 6-12.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>$^{31}$P NMR (ppm)</th>
<th>$^1$H NMR (ppm)</th>
<th>$T_g$ (°C)</th>
<th>$M_w$ (g/mol)</th>
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<td>6</td>
<td>-0.8</td>
<td>0.8 (6H), 1.2 (6H), 3.0 (2H), 4.1 (8H)</td>
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<td>198 000</td>
</tr>
<tr>
<td>7</td>
<td>-3.8</td>
<td>1.2 (9H), 3.7 (1H), 4.1 (8H)</td>
<td>23.4</td>
<td>165 000</td>
</tr>
<tr>
<td>8</td>
<td>-2.8</td>
<td>1.4 (12H), 3.2 (2H), 4.7 (6H)</td>
<td>25.2</td>
<td>178 000</td>
</tr>
<tr>
<td>9</td>
<td>-1.2</td>
<td>1.2 (6H), 1.3 (6H), 1.8 (1H), 3.1 (1H), 3.6 (2H), 3.8 (2H), 4.1 (4H)</td>
<td>29.8</td>
<td>231 000</td>
</tr>
<tr>
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<td>-2.2</td>
<td>1.2 (6H), 1.4 (9H), 1.8 (1H), 3.1 (1H), 3.6 (1H), 3.9 (2H), 4.1 (4H)</td>
<td>36.9</td>
<td>220 000</td>
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<tr>
<td>11</td>
<td>-3.1</td>
<td>1.2 (6H), 3.2 (2H), 4.1 (1H), 4.2 (4H), 4.4 (4H), 7.3 (5H)</td>
<td>31.5</td>
<td>242 000</td>
</tr>
<tr>
<td>12</td>
<td>-1.1</td>
<td>1.3 (9H), 3.3 (1H), 4.0 (2H), 4.2 (4H), 4.3 (3H), 7.1 (5H)</td>
<td>43.7</td>
<td>261 000</td>
</tr>
</tbody>
</table>
2.3.4 Thermal characterization of polymers 6-12

The glass transition temperatures ($T_g$) for polymers 6-12 are shown in Table 2-1. Polymer 12 has the highest $T_g$ at 43.7º C. For comparison, poly[bis(ethyl phenylalanato)phosphazene] has a $T_g$ of 68º C. The presence of glycine ethyl ester as the co-substituent together with phenylalanyl-glycine ethyl ester (polymer 11) causes a small $T_g$ decrease, presumably because of the lower steric hindrance characteristics of the glycine unit. A similar trend exists for polymers 7 and 8, and polymers 9 and 10. The $T_g$ decreased as the size of the $\alpha$-carbon substituent decreased from an isopropyl function to the methyl group of the dipeptide ethyl ester. Polymer 6 is unique because this polymer contains 100% of the dipeptide ethyl ester and the $T_g$ of 28.2º C is higher than for polymers 7 and 8. This could be a consequence of stronger hydrogen bonding within the amide linkage of the dipeptide, which would cause additional restriction of the polymer backbone tensional mobility.

2.3.5 Heterophase hydrolysis of polymers 6-12 under different pH conditions

The weight loss of films immersed in pH 4, 7, and 10 aqueous media was used as a preliminary criterion of hydrolytic stability, and this was followed by an analysis of molecular weight changes. At pH 4, the molecular weight of polymers 6-8 declined drastically within one week due to the high acidity and lack of steric bulk at the $\alpha$-carbon that helps to retard hydrolysis. The introduction of steric hindrance via the isopropyl and phenyl group in polymers 9-12 retarded the hydrolysis in this acidic environment, resulting in weight losses of 67% for polymer 9, 55% for polymer 10, 35% for polymer 11, and 32% for polymer 12 during the 4 weeks of hydrolysis.
The pH 7 data provides a preliminary estimate of the hydrolysis behavior of these polymers if implanted in mammals. At pH 7, the heterophase hydrolysis rates were slower than the rates at pH 4. Polymers 6-8 had 90% weight loss during the 4 weeks of hydrolysis. Polymers 9, 10, 11, and 12 lost 67%, 50%, 20%, and 5% weight respectively during the same period of hydrolysis. At pH 10, polymers 6-8 lost 75% of their weight during the 4 weeks of hydrolysis. Once again, the steric bulk in the valine- and phenylalanine ethyl ester side groups retards the hydrolysis of polymers 9, 10, 11, and 12 resulting in weight losses of 40%, 20%, 19%, and 14% respectively. Overall, the rates of hydrolysis for polymers 6-12 decrease as the pH increases from 4 to 10. This reduction in the rate of hydrolysis as the pH increases is due to the loss of protons that help to increase the rate of hydrolysis. All of the dipeptide polymers hydrolyzed slower than their amino acid counterparts at neutral pH 7.

The differences in hydrolytic stability reflect the influence of several different factors associated with the dipeptide and amino acid side groups. The presence of the phenyl- and isopropyl groups on the α-carbon in the phenylalanine and valine dipeptide residues provide steric hindrance that blocks hydrolytic access to the polymer backbone and results in retardation of the rate of hydrolysis compared to the alanine dipeptide. Furthermore, for polymers 7-12 the steric hindrance of the co-substituent glycine- or alanine ethyl ester helps to control the rate of hydrolysis associated with the alanine co-substituent, and provides a 5-20% retardation in hydrolysis compared to when glycine is the co-substituent. One conclusion is clear, fine-tuning of the substituent ratios has the potential to allow control of the hydrolysis behavior to satisfy the diverse properties needed for different biomedical applications such as in tissue engineering and controlled drug release. Moreover, the potential exists for further control of both physical properties and hydrolysis behavior through the formation of composites of these polymers with other biomedical polymers such as poly(lactic-co-glycolic acid) or polycaprolactone.
2.4 Conclusions

Polyphosphazenes that contain the side groups alanyl-glycine ethyl ester, valinyl-glycine ethyl ester, and phenylalanyl-glycine ethyl ester with the co-substituents glycine ethyl ester or alanine ethyl ester were synthesized. After deprotection of the N-terminus, the dipeptides were used for nucleophilic replacement of the chlorine atoms in poly(dichlorophosphazene). The final polymeric products were characterized structurally by multinuclear NMR techniques, and by GPC. Glass transitions were identified by DSC. The pH-dependent heterophase hydrolyses of polymers 6-12 were analyzed and showed that aqueous acidic pH media cause rapid hydrolysis. As the medium becomes more alkaline, the polymers are more stable. The weight loss and hydrolysis behavior of the polymers reflect both the hydrophobicity and the steric protection provided by the group on the α-carbon atom of the amino acid esters. Thus, alanine-linked units hydrolyzed more rapidly than the valine-based species, which degraded faster than the phenylalanine-substituted polyphosphazenes.

2.5 Acknowledgment

This work was supported by the National Institute of Health, through grant number RO1EB004051.
2.6 References


Chapter 3

Choline-Substituted Polyphosphazenes as Tissue Regeneration Scaffolds: Blend Compatibility with PLGA and Effects on Osteoconductivity.

3.1 Introduction

A large fraction of biomaterials research is focused on the use of long existing polymers such as polylactic acid (PLA), polylactic-co-glycolic acid (PLGA), and polycapro lactones (PCLs). These polymers are used mainly for their biodegradability and biocompatibility that allows them to be employed as hard tissue engineering scaffolds, drug delivery vehicles, bioerodible sutures, and fixation devices. The primary concern is that no single polymer can address all the required properties needed for these applications. For example, biomaterials based solely on PLA, PLGA, or PCLs degrade into acidic byproducts that can cause tissue necrosis and cellular delamination at or around the implant site. To address this problem, incorporation of polymers that buffer the acidic hydrolysis products of polyesters in order to obtain biomaterials that generate the required properties. In addition, polymer blends have the potential to produce materials that have mechanical stability greater than the individual polymers.

Biological buffers are an important class of molecules that perform many essential functions necessary for normal cellular activity. However, existing polymers that contain or are derived from biological buffers are low molecular weight water-soluble materials used for drug or gene delivery. These compounds could be useful in tissue engineering materials if they were components of high molecular weight water-insoluble polymers. Choline is one such biological buffer that could be useful if incorporated into a high molecular weight polymer. Choline is a naturally-occurring quaternary amino alcohol that has many important functions such as a source
for methyl groups used in biological methylation\textsuperscript{10,11}. It is a nutritional supplement\textsuperscript{12,13}, and is also a significant structural component in cell walls.\textsuperscript{14,15} In addition, choline chloride has been shown to promote cell growth.\textsuperscript{16} In the case of hard tissue engineering, the positive charge would provide a nucleation site for hydroxyapatite formation. It could also be used as an ionic cross-linking site in the presence of phosphates to improve the mechanical performance, and should be able to buffer the acidic hydrolysis products of polyesters when used in tissue engineering scaffolds.

Polyphosphazenes are highly tunable polymers that in principle could serve as a platform to produce novel choline biomaterials. The high degree of molecular tunability of phosphazenes allows for facile changes in the side group structure. Different side groups can generate properties that are appropriate for drug delivery\textsuperscript{17}, hydrophobic surfaces\textsuperscript{18}, or biomaterials\textsuperscript{19-23}. These changes in side group structure allow polyphosphazenes to form miscible blends with polyesters and can buffer the acidic hydrolysis products of polyesters.\textsuperscript{28, 30} For example, polyphosphazenes that contain dipeptide esters have better blend miscibility with PLGA (50:50) than to poly(amino acid ester phosphazenes)\textsuperscript{26, 27}, due to the extra hydrogen bonding sites provided by the amide linkage in the dipeptide ester side groups.\textsuperscript{24, 25, 30} However, these polymers cause rapid osteoblast cellular delamination and necrosis due the acidity generated when the polyphosphazene / PLGA blended system is hydrolyzed \textit{in vivo} over a period of 21 days. Therefore, alternative polymers developed from choline should help to promote cell growth by buffering the hydrolysis products of PLGA in tissue engineering scaffolds.

Here, we describe the synthesis of a series of small-molecule model cyclic-trimeric phosphazene species with choline co-substituted with glycine ethyl ester, alanine ethyl ester, phenylalanine ethyl ester, or valine ethyl ester. This was followed by the synthesis of the corresponding polyphosphazenes using two different synthetic pathways: (1) the use of sodium hydride to reduce the choline chloride to the choloxide before addition to
poly(dichlorophosphazene) and (2) the in situ reaction with cesium carbonate. The hydrolytic stability and unique thermal properties of the resultant polymers were then examined. These choline-based polyphosphazenes were then blended with PLGA (50:50) and PLGA (85:15) via solution-casting techniques. The blends were studied by differential scanning calorimetry (DSC), scanning electron microscopy (SEM), and FT-IR spectroscopy to determine the extent of blend miscibility. The hydrolysis rates and products were determined along with the osteoconductivity of the blended polymer materials.

3.2 Experimental

3.2.1 Reagents and Equipment

All synthetic reactions were carried out under a dry argon atmosphere using standard Schlenk line techniques. Tetrahydrofuran and triethylamine (EMD) were dried using solvent purification columns. Alanine ethyl ester hydrochloride (Chem Impex), valine ethyl ester hydrochloride (Bachem), phenylalanine ethyl ester hydrochloride (Chem Impex), glycine ethyl ester hydrochloride (Alfa Aesar), 60% sodium hydride dispersion in mineral oil (Sigma Aldrich), Sephadex G-25 (VWR), PLGA (50:50) (Ethicon Division of Johnson and Johnson; weight-average molecular weight 2,000,000), PLGA (85:15) (Ethicon Division of Johnson and Johnson; weight-average molecular weight 4,800,000), and anhydrous diglyme (Sigma Aldrich) were used as received. Cesium carbonate (Sigma Aldrich) and choline chloride was dried under vacuum at 100°C for 1 week before being stored in an inert atmosphere glove box before use. 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 the use of a
Bruker 360 WM instrument operated at 145 MHz and 360 MHz, respectively. Glass transition temperatures were measured with a TA Instruments Q10 differential scanning calorimetry (DSC) 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 calibrated with polystyrene standards. Mass spectrometric analysis data were collected using turbo spray ionization technique on an Applied Biosystems API 150EX LC/MS mass spectrometer. Scanning electron microscopy (SEM) was obtained using a Philips FEI Quanta 200 Environmental Scanning Electron Microscope. The SEM samples were prepared by placement of a polymer sample onto carbon tape, followed by insertion into the SEM equipment. The use of low vacuum mode was used for imaging under the following conditions: 20KeV source voltage, pressure approx. 0.88 Torr, and a working distance of approx. 10 mm. ATRIR scans of the films were analyzed with a Digilab (Randolph, MA) FTS 7000 spectrometer with a zinc selenide ATR crystal with 32 scans per sample. pH values were measure using a VWR Symphony SB70P pH meter.

3.2.2 Synthesis of Model Cyclic Trimers 1-4

The preparation of the model compound cyclic trimers 1-4 followed similar procedures. The synthesis of 1 is given as a representative example. Hexachlorocyclotriphosphazene (1.00 g, 2.88 mmol) was dissolved in tetrahydrofuran (150 mL) and cesium carbonate (9.18 g, 28.2 mmol) was added to the hexachlorocyclotriphosphazene solution. Glycine ethyl ester hydrochloride (1.20 g, 8.63 mmol) and triethylamine (1.24 mL, 8.92 mmol) in THF (100 mL) were refluxed for 24 hrs, filtered, and then added to the chlorophosphazene solution drop-wise
over a period of 1 hr and the mixture was stirred for 24 hours at room temperature. Choline chloride (2.41 g, 17.3 mmol) and diglyme (100mL) were added to the reaction mixture, which was stirred for 48 hrs at room temperature, 24 hrs at 40°C, and was then refluxed for 24 hrs. The absence of a $^{31}$P NMR signal after this sequence indicated that the product had precipitated from the THF solution. The recovered precipitate was soluble in de-ionized water. Purification by column chromatography using Sephadex G-25 (size exclusion chromatography) was completed. Cyclic trimers 1-4 contained equal molar amounts of amino acid ester and choline, and were soluble in de-ionized water. Physical and structural characterization data are presented in Table 1. The yields were 40-55% based on the initial amount of hexachlorocyclotriphosphazene.

3.3.3 Synthesis of Polymers 5-8

The preparation of the polyphosphazenes 5-8 using sodium hydride to form the sodium salt of choline followed similar procedures. The synthesis of 5 is given as a representative example. Poly(dichlorophosphazene) (5.00 g, 43.1 mmol) was dissolved in THF (500 mL). Glycine ethyl ester hydrochloride (6.02 g, 43.1 mmol) was suspended in THF (200 mL), and triethylamine (24.0 mL, 172 mmol). This suspension was refluxed for 24 hours, filtered, and then added to the poly(dichlorophosphazene) solution over a 1 hour period. The polymer mixture was stirred for 24 hours at room temperature. Choline chloride (18.1g, 129 mmol) was reacted with sodium hydride (3.45g, 86.2 mmol) in diglyme (250 mL) at 75°C for 48 hours. This suspension was added to the polymer solution. The resultant solution was stirred at room temperature for 24 hours, and was then refluxed for an additional 48 hours. The solution was concentrated and dialyzed against methanol for 3 days in 12-14,000 MWCO dialysis tubing. The solution was removed from the dialysis tubing and was dried under reduced pressure for 1 week to produce a
yellow-orange powder. The yield was 41-50% based on the amount of poly(dichlorophosphazene) used.

3.3.4 Synthesis of Polymers 9-12

The preparation of the polyphosphazenes 9-12 made use of cesium carbonate to prepare the sodium salt of choline. The synthesis of 12 is given as a representative example. Poly(dichlorophosphazene) (5.00 g, 43.1 mmol) was dissolved in THF (400 mL). Cesium carbonate (Cs₂CO₃) (61.9 g, 190 mmol) was added to the poly(dichlorophosphazene) solution. Phenylalanine ethyl ester hydrochloride (9.912 g, 43.1 mmol) was suspended in THF (300 mL), and was treated with triethylamine (6.62 mL, 47.5 mmol). This suspension was refluxed for 24 hours, filtered, and then added to the polymer solution over a 1 hour period, before the polymer solution was stirred for 24 hours at room temperature. Choline chloride (18.1 g, 129 mmol) and diglyme (200 mL) were added to the polymer solution. The resultant solution was stirred at room temperature for 24 hours, followed by 24 hours at 40°C, and then refluxed for an additional 24 hours, at which point the polymer precipitated from solution. All the solvent was removed from the polymer solution and the polymer was re-dissolved in methanol (200 mL). The polymer solution was dialyzed against methanol for 3 days in 12-14,000 MWCO dialysis tubing. The polymer was removed from dialysis and dried under reduced pressure for 1 week to produce a yellow-orange solid. The yield was 49-54% based on the amount of poly(dichlorophosphazene) used.
3.3.5 Formation of Polyphosphazene Blends with PLGA (50:50) and PLGA (85:15)

Three compositions of blends were fabricated for each polyphosphazene/polyester system. The compositions contained 25%, 50%, and 75%, by weight of the polyphosphazene relative to PLGA (50:50) and PLGA (85:15). Each pair of component polymers (0.1g) was individually dissolved in 1 mL of chloroform : dimethylformamide (50:50) solution. The polyphosphazene solution (0.1g/1mL) was added to the PLGA solution (0.1g/1mL) and the mixture was stirred overnight. The combined solution of the two polymers was allowed to stand undisturbed for one hour to confirm that solution phase miscibility existed. The solutions were then poured into film casting trays, air dried for 48 hours, and then vacuum dried for one week. Each polymer blend was cast as a single replicate film that was analyzed by DSC. SEM imaging was used to confirm the results of the DSC analysis.

3.3.6 Hydrolysis Studies of Polymers 5-12 and blended polymer samples

Polymers 5-12 were dissolved in chloroform : dimethylformamide (50:50) (100 mg/1 mL) and were solution-cast into films. These were air dried for 48 hours and then vacuum dried for another seven days. The dried films were cut into squares (10 mm x 10 mm or approx. 10mg) and placed in test tubes containing de-ionized water (5 mL). The tubes were secured in a shaker bath at 37°C. Three samples were removed from each medium after 1, 2, 3, 4, 5, and 6 weeks to measure weight loss. The pH of each hydrolysis medium was monitored throughout the study. GPC analysis of the polymers during hydrolysis was not possible due to the insolubility of the materials in THF after hydrolysis was initiated.
3.3.7 In Vitro Osteocompatibility Studies of Polymer Blends

Primary rat osteoblast (PRO) cells were isolated from calvarias of 2-day-old neonatal Sprague-Dawley rats according to a standard procedure\textsuperscript{32-33}. Prior to cell seeding, polymer films including 6A, 7A, 8A and PLGA (85:15) were sterilized with ultraviolet (UV) light for 15 min on each side after being treated with 70% ethanol for 30 min and distilled water twice for 15 min. The polymer films were pre-incubated in Ham’s F-12 media (Gibco) for 1 h prior to cell seeding. PRO cells were seeded onto the polymer films at a density of $6 \times 10^4$ cells/cm² after removing the pre-incubation media. The cells were cultured at 37°C in a 95% humidified air and 5% CO₂ for various periods of time after adding 1 mL supplemented mineralized media (Ham’s F-12 media supplemented with 12% FBS, 1% penicillin/streptomycin, 3 mM of β-glycerophosphate (Sigma) and 10 μg/ml of ascorbic acid (Fisher)) to each well. The media were changed every 2 days. The cultures were maintained for 21 days. The proliferation of PRO cells on the polymer films was analyzed by quantifying the total protein content. In brief, at days 7, 14, and 21, the media were removed, the cells were washed with PBS, and then lysed with 1 mL of 1% Triton X-100 solution for 30 min. The cell lysates were collected and stored in a -70°C freezer. Three freeze-thaw cycles were performed before analysis. The protein concentration of cell lysates was determined with a BCA Protein Assay Reagent kit (Pierce) which is based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. Proteins of the cell suspension in an alkaline medium can reduce the cupric ions to cuprous ions which can form a chelation complex with BCA. The purple color of this complex is directly proportional to the protein concentration and the absorbance is read at 562 nm using TECAN. Alkaline phosphatase (ALP) activity of PRO cells on the polymer films was evaluated as an early marker of the retention of osteoblastic phenotype using an ALP substrate kit (Bio-Rad)\textsuperscript{34}. In brief, at days 7, 14, and 21, 100 µL sample was added to 400 µL of P-NPP substrate and buffer solution mixture and
incubated at 37°C for 30 min. At the end of the incubation time, the reaction was stopped by adding 500 µL of 0.4 N of sodium hydroxide. The optical density of the solution was determined by the absorbance at 405 nm using the TECAN. The intensity of the color formed is proportional to ALP activity. The results for ALP activity were further normalized by the amount of proteins as determined in the companion protein assay.

3.4 Results and Discussion

3.4.1 Synthesis of Trimers 1-4

Initial attempts to synthesize cyclic trimeric phosphazenes with 100% choline chloride side groups were unsuccessful. The small-molecule phosphazenes became insoluble following the addition of choline chloride. Therefore, co-substitution of cyclic trimeric phosphazenes with amino acid esters and choline groups was attempted to increase the solubility during the synthesis protocol (Scheme 3-1). The amino acid esters were added drop-wise to a dilute solution / suspension of hexachlorocyclotriphosphazene and cesium carbonate. This procedure yielded an amino acid ester tri-substituted cyclic trimer, as determined by $^{31}$P NMR spectroscopy. The remaining P-Cl bonds were then replaced by the addition of choline chloride. However, this resulted in precipitation from the THF / diglyme solution after the completion of full substitution. The precipitate was re-dissolved in de-ionized water and purified using Sephadex-G25 with de-ionized water as the mobile phase. It was determined through $^1$H NMR spectroscopy and mass spectrometry that the products contained equal molar amounts of the two side groups as shown in Figure 3-1. The physical and structural data are given in Table 3-1.
Figure 3-1. Synthesis of [(amino ethyl ester)$_3$(choline chloride)$_3$] cyclotriphosphazenes.

Table 3-1: Characterization data for small molecule model [(amino ethyl ester)$_3$(choloxyl chloride)$_3$] cyclotriphosphazenes.

<table>
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<tr>
<th>Trimer</th>
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<th>$^1$H (ppm)*</th>
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<td>1.21 (3H, t, gly), 3.43 (9H, s, chol), 3.53 (2H, t, chol), 3.61 (2H, s, gly), 3.86 (2H, t, chol), 4.09 (2H, q, gly)</td>
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<tr>
<td>Ala/Choline</td>
<td>1.8</td>
<td>1.18 (3H, t, ala), 1.30 (3H, d, ala), 3.43 (9H, s, chol), 3.51 (2H, t, chol), 3.59 (1H, q, ala), 3.83 (2H, t, chol), 4.06 (2H, q, ala)</td>
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</tr>
<tr>
<td>Val/Choline</td>
<td>2.3</td>
<td>0.91 (6H, d, val), 1.21 (3H, t, val), 3.34 (1H, m, val), 3.51 (9H, s, chol), 3.60 (2H, t, chol), 3.66 (1H, d, val), 3.83 (2H, t, chol), 4.12 (2H, q, val)</td>
<td>292</td>
</tr>
<tr>
<td>Phe/Choline</td>
<td>3.3</td>
<td>0.93 (3H, t, phe), 3.05 (2H, d, phe), 3.39 (9H, s, chol), 3.62 (2H, t, chol), 3.72 (1H, t, phe), 3.86 (2H, t, chol), 4.24 (2H, q, phe), 7.09 (5H, m, phe)</td>
<td>340</td>
</tr>
</tbody>
</table>

*All NMRs were taken in D$_2$O. *Gly refers to glycyl ethyl ester, Ala refers to alanyl ethyl ester, Val refers to valinyl ethyl ester, and Phe refers to phenylalanyl ethyl ester.
3.4.2 Synthesis of Polymers 5-12

Initial attempts to synthesize high polymeric phosphazenes that contained only choline chloride side groups were also unsuccessful. As monitored by $^{31}$P NMR spectroscopy, only 10% of the chlorine atoms were replaced before precipitation of the polymer occurred from THF. A $^{31}$P NMR peak was detected at -9.9ppm before precipitation occurred. Therefore, the synthesis of polyphosphazenes that contain choline chloride co-substituted with various amino acid esters was attempted using two alternative synthetic routes. To ensure solubility of the final compounds in THF, the first step in both routes involved the replacement of 50% of the chlorine atoms in poly(dichlorophosphazene) by glycine ethyl ester, alanine ethyl ester, valine ethyl ester, or phenylalanine ethyl ester as monitored by $^{31}$P NMR spectroscopy. Subsequently, the remaining chlorine atoms were replaced by choline chloride without precipitation from the THF / diglyme solution using the two different synthetic procedures.

In the first route, choline chloride was treated with sodium hydride in diglyme to form the sodium choloxy reagent. The mixture was then added to the amino ethyl ester partially-substituted phosphazene to produce polymers 5-8 shown in Figure 3-2 (a). The structural and physical properties of polymers 5-8 are shown in Tables 3-2 and 3-3 (a). The poly[(amino ethyl ester)$_x$(choline chloride)$_y$phosphazene] synthesized using the sodium hydride route contained 20-34 mole percent choline chloride, with the highest percentage achieved when glycine ethyl ester was the amino acid ester co-substituent. It was expected that the phenylalanine ethyl ester polymer should have the highest percentage of choline chloride, since the steric hindrance helps to retard the acidic hydrolysis. Overall, the low mole percent of choline chloride is due to the length of time it takes for the relatively insoluble choloxy side group to complete the substitution. This is the probable reason for the low molecular weights produced through this synthetic route. However, the steric hindrance generated by certain amino acid esters can increase reaction times
that favor acidic degradation of the polymer backbone during the macromolecular substitution process.\textsuperscript{17, 20, 30}

\begin{figure}
\centering
(a) \hspace{1cm} (b)
\end{figure}

\textbf{Figure 3-2.} (a) Synthesis of poly[(amino ethyl ester)$_x$(choline chloride)$_y$phosphazene] using sodium hydride (NaH); (b) Synthesis of poly[(amino ethyl ester)$_x$(choline chloride)$_y$phosphazene] using cesium carbonate (Cs$_2$CO$_3$).
Table 3-2: Structural properties of poly[(amino ethyl ester), (choloy chloride), phosphazenes]. The molar percent (mol%) choline was determined by integration of the $^1$H NMR spectra for each polymer.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>$^{31}$P (ppm)$^*$</th>
<th>$^1$H (ppm)$^*$</th>
<th>mol% Choline NaH Route</th>
<th>mol% Choline Cs$_2$CO$_3$ Route</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly/Choline</td>
<td>2.3</td>
<td>1.21 (3H, gly), 3.43 (9H, chol), 3.53 (2H, chol), 3.61 (2H, gly), 3.86 (2H, chol), 4.09 (2H, gly)</td>
<td>28 (5)</td>
<td>40 (9)</td>
</tr>
<tr>
<td>Ala/Choline</td>
<td>-0.42</td>
<td>1.18 (3H, ala), 1.30 (3H, ala), 3.43 (9H, chol), 3.51 (2H, chol), 3.59 (1H, ala), 3.83 (2H, chol), 4.06 (2H, ala)</td>
<td>26 (6)</td>
<td>42 (10)</td>
</tr>
<tr>
<td>Val/Choline</td>
<td>0.83</td>
<td>0.91 (6H, val), 1.21 (3H, val), 3.34 (1H, val), 3.51 (9H, chol), 3.60 (2H, chol), 3.66 (1H, val), 3.83 (2H, chol), 4.12 (2H, val)</td>
<td>21 (7)</td>
<td>41 (11)</td>
</tr>
<tr>
<td>Phe/Choline</td>
<td>-1.7</td>
<td>0.93 (3H, phe), 3.05 (2H, phe), 3.39 (9H, chol), 3.62 (2H, chol), 3.72 (1H, phe), 3.86 (2H, chol), 4.24 (2H, phe), 7.09 (5H, phe)</td>
<td>20 (8)</td>
<td>53 (12)</td>
</tr>
</tbody>
</table>

$^*$All NMR spectra were for compounds in CDCl$_3$. $^\dagger$Gly refers to glycyl ethyl ester, Ala refers to alanyl ethyl ester, Val refers to valinyl ethyl ester, and Phe refers to phenylalanyl ethyl ester.
Table 3-3. Physical properties of poly[(amino ethyl ester)$_x$ (choloxy chloride)$_y$ phosphazenes] (a) using the NaH synthetic route and (b) using the Cs$_2$CO$_3$ synthetic route.

(a)

<table>
<thead>
<tr>
<th>Polymer</th>
<th>PDI$^\alpha$</th>
<th>$M_w$ (g / mol)</th>
<th>$T_g$ ($^\circ$C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NaH Route</td>
<td>NaH Route</td>
<td>NaH Route</td>
</tr>
<tr>
<td>5</td>
<td>1.79</td>
<td>26252</td>
<td>22.6</td>
</tr>
<tr>
<td>6</td>
<td>2.27</td>
<td>49674</td>
<td>30.9</td>
</tr>
<tr>
<td>7</td>
<td>2.14</td>
<td>36560</td>
<td>33.8</td>
</tr>
<tr>
<td>8</td>
<td>2.15</td>
<td>31892</td>
<td>26.5</td>
</tr>
</tbody>
</table>

(b)

<table>
<thead>
<tr>
<th>Polymer</th>
<th>PDI$^\alpha$</th>
<th>$M_w$ (g / mol)</th>
<th>$T_g$ ($^\circ$C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cs$_2$CO$_3$ Route</td>
<td>Cs$_2$CO$_3$ Route</td>
<td>Cs$_2$CO$_3$ Route</td>
</tr>
<tr>
<td>9</td>
<td>2.36</td>
<td>230000</td>
<td>-30.8</td>
</tr>
<tr>
<td>10</td>
<td>1.67</td>
<td>183000</td>
<td>-8.07</td>
</tr>
<tr>
<td>11</td>
<td>N/A</td>
<td>N/A</td>
<td>-25.2</td>
</tr>
<tr>
<td>12</td>
<td>N/A</td>
<td>N/A</td>
<td>-20.1</td>
</tr>
</tbody>
</table>

$^\alpha$Gly refers to glycyl ethyl ester, Ala refers to alanyl ethyl ester, Val refers to valinyl ethyl ester, and Phe refers to phenylalanyl ethyl ester. $^\beta$PDI was determined by $M_w$/M$_n$.

The alternative route involved a similar first step as used in the sodium hydride route. Triethylamine was allowed to react with the amino acid ester hydrochloride to remove the hydrochloride salts. However, the amount of triethylamine was limited to the amount needed only to remove the hydrochloride salt from the amino acid esters. Cesium carbonate was the proton acceptor for the linkage of the alcohol function of choline during macromolecular substitution with poly(dichlorophosphazene). Specifically, the amino acid ester reaction mixture
was added drop-wise to a solution of poly(dichlorophosphazene) to produce a partially-substituted polyphosphazene. Then choline chloride in diglyme was added to the polymer solution to complete the halogen replacement to produce polymers 9-12 as shown in Figure3-2 (b). The structural and physical properties of polymers 9-12 are shown in Table 3-2 and Table 3-3 (b). The resulting poly[(amino ethyl ester)x(choline chloride)yphosphazene] synthesized by the cesium carbonate route contained 38-53 mole percent choline chloride. The reaction times to achieve full substitution were shorter than when the sodium hydride route was employed. The cesium carbonate route gave molecular weights 4-9 times higher than via the sodium hydride route, along with an increase in the choline content as shown in Table 3-3. Molecular weight data could not be obtained when valine ethyl ester or phenylalanine ethyl ester was the second side group due to the insolubility of the polymers in THF. This is probably due to the higher concentration of choline in these polymers.

**3.4.3 Thermal characterization of polymers 5-12**

The glass transition temperatures (T_g) of polymers 5-12 are shown in Table 3-3. A significant difference existed between the T_g values of polymers 5-8 and those of 9-12. Polymers 5-8 had T_g’s in the range of 22°C to 35°C and polymers 9-12 between -8°C to -30°C. The lower values can be attributed to the higher choline content. The low glass transition temperatures in 9-12 provide a clue to the T_g that might be expected for a fully-substituted choline phosphazene polymer. However, a fully substituted choline polymer has not yet been synthesized.
3.4.4 Hydrolysis of Polymers 5-12

The weight loss of polymers 5-12 in water was used to estimate the initial hydrolytic sensitivity. Polymers 5 and 6 underwent 100% mass loss by week 3 of hydrolysis. This is attributed to the lack of steric hindrance at the α-carbon of the amino acid ester side group, which sensitizes these polymers to hydrolytic attack. Polymers 7 and 8 underwent 90% mass loss by week 6 of hydrolysis as shown in Figure 1 (a). The pH of the resulting hydrolysis media for polymers 5-8 was in the range of 6.3-6.8 with the order of highest to lowest pH being 5>6>8>7.

On the other hand, polymers 9-12 appeared to have hydrolyzed completely within the first week of hydrolysis. This suggests that choline side groups cause rapid hydrolysis or that polymers 9-12 become soluble in the aqueous media as hydrolysis occurs. The role of the hydrolysis medium was explored by removal of water to determine the cause of rapid hydrolysis. The remaining solid was dissolved in deuterated water and analyzed by $^{31}$P NMR spectroscopy. The results from polymer 9 are used as an example. The $^{31}$P NMR spectra contained a peak at 2.3 ppm from polymer 9, another peak at 0.8 ppm that corresponds to a phosphorus-glycine ethyl ester linkage, and a peak at -9.9 ppm derived from the phosphorus-choline linkage. These results suggest that polymers 9-12 dissolve in the hydrolysis medium as the hydrophobic amino acid ester groups hydrolyze first from the polymer backbone. This solubility effect was not detected in 5-8 and may be retarded due to the increased molar abundance of amino groups that required longer times of hydrolysis before the polymer becomes soluble in the aqueous media. The pH of the hydrolysis media after 6 weeks of hydrolysis of polymers 9-12 was in the range of 6.1-6.5 with the order of highest to lowest pH being 5>6>8>7.
3.4.5 Compatibility of 5-12 with PLGA (50:50) and PLGA (85:15)

Polymers 5-12 and PLGA (50:50) or PLGA (85:15) were individually dissolved in chloroform : dimethylformamide (DMF) (50:50), mixed, cast by solvent evaporation, and dried for one week under vacuum to ensure the removal of all solvent. The micro-scale miscibility of the films was examined by DSC and SEM techniques. Polymer 5 was found to be partially miscible with PLGA (50:50). However, 6-8 were completely miscible with PLGA (50:50), and yielded a single glass transition in the DSC traces, as shown in Table 3-4. In addition, the formation of miscible blends was supported by ATR-FTIR data that indicated a strong carbonyl hydrogen bonding interaction from the absorption at 1734 cm\(^{-1}\), compared to the C=O vibrational peak at 1750 cm\(^{-1}\) for pristine polyphosphazenes and PLGA. Scanning electron microscopy (SEM) images showed smooth surfaces with no phase-separated domains, thus confirming the DSC results.

On the other hand, polymer blends of 5-8 with PLGA (85:15) showed a distinct decrease in compatibility. Although, polymers 6 and 8 formed miscible blends with PLGA (85:15) and PLGA (50:50) regardless of blend composition, polymers 5 and 7 showed a tendency for immiscibility, perhaps reflecting steric hindrance constraints. Representative DSC traces of 6 with PLGA are shown in Figure 3-3(a). The resultant composite materials showed evidence of plasticization when 6 was blended with PLGA. This plasticization is due to the low molecular weights of 5-8. Similar trends were observed for 7 blended with PLGA (50:50) and when polymer 8 was blended with PLGA (50:50) or PLGA (85:15).

An unexpected result was that blends of 9 with PLGA showed an increase in blend miscibility. It might be expected that blends of 9 with PLGA would be partially miscible due to the structural similarity of 9 with 5. Although, polymer 9 formed miscible blends with PLGA (50:50), blends with PLGA (85:15) were miscible only when 50% or 75% of 9 was used. The
increased blend miscibility is thought to be due to the increased molecular weight of 9 that encouraged chain entanglements between 9 and PLGA. The lack of miscibility of 5 with PLGA can be explained by a strong intramolecular interaction between the abundant N-termini in glycine ethyl ester units that will bind tightly with the quaternary amino unit in choline. This interaction would cause the low molecular weight polymer to form a collapsed conformation that would hinder interactions with PLGA and cause phase separation. However, the increased choline content in 9 together with higher molecular weight interferes with this intermolecular interaction and facilitates intermolecular interactions with PLGA to produce blends with 9. DSC traces of 5 and 9 blended with PLGA (50:50) and PLGA (85:15) are shown in Figure 3-3(b). The glass transitions in blends A and F are indicative of partially miscible materials because the transitions of 5 and PLGA are shifted from the values of the pure polymers. On the other hand, blends between 9 and PLGA (50:50) or PLGA (85:15) produced single glass transition temperatures at 36°C and 30°C respectively.

Polymers 10-12 showed decreased blend compatibility with both PLGA (50:50) and PLGA (85:15) compared to 6-8. Polymer 11 formed partially miscible blends with both ratios of PLGA and 12 formed miscible blends only when high loadings were used. Polymer 10 remained miscible with PLGA (50:50) regardless of the component ratio. However, the miscibility of 10 with PLGA (85:15) was reduced, and only formed miscible blends when 75% of 10 was used. The combination of increased choline content and higher molecular weights of 10-12 are believed to be the reason for the reduction in blend miscibility when compared to 6-8. Polymers with higher molecular weights require stronger interactions between 10-12 and PLGA to overcome the enthalpy of mixing, in order to produce miscible systems.³¹ The increased choline content does not provide the necessary interactions with PLGA to compensate for the increased molecular weights.
Table 3-4. DSC analysis of polymer blends of PLGA with polyphosphazenes synthesized using the (a) sodium hydride route and (b) the cesium carbonate route. Blends A-C and G-I describe blends of polymers 5-12 with PLGA 50:50. Blends D-F and J-L describe blends with polymers 5-12 with PLGA 85:15. Blends A, D, G, and J contained 25% polyphosphazene, Blends B, E, H, and K contain 50% polyphosphazene, and Blends C, F, I, and L contain 75% polyphosphazene.

(a)

<table>
<thead>
<tr>
<th>Blend</th>
<th>Polymer</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Partial</td>
<td>Yes (18 °C)</td>
<td>Yes (13 °C)</td>
<td>Yes (18 °C)</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Partial</td>
<td>Yes (27 °C)</td>
<td>Yes (23 °C)</td>
<td>Yes (35 °C)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Partial</td>
<td>Yes (31 °C)</td>
<td>Yes (26 °C)</td>
<td>Yes (34 °C)</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>Partial</td>
<td>Yes (32 °C)</td>
<td>Partial</td>
<td>Yes (30 °C)</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>Partial</td>
<td>Yes (31 °C)</td>
<td>Partial</td>
<td>Yes (34 °C)</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>Partial</td>
<td>Yes (45 °C)</td>
<td>Partial</td>
<td>Yes (38 °C)</td>
<td></td>
</tr>
</tbody>
</table>

(b)

<table>
<thead>
<tr>
<th>Blend</th>
<th>Polymer</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>Yes (36 °C)</td>
<td>Yes (19 °C)</td>
<td>Partial</td>
<td>Partial</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>Yes (38 °C)</td>
<td>Yes (16 °C)</td>
<td>Partial</td>
<td>Partial</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>Yes (45 °C)</td>
<td>Yes (22 °C)</td>
<td>Partial</td>
<td>Yes (22 °C)</td>
<td></td>
</tr>
<tr>
<td>J</td>
<td>Partial</td>
<td>Partial</td>
<td>Partial</td>
<td>Partial</td>
<td></td>
</tr>
<tr>
<td>K</td>
<td>Yes (30 °C)</td>
<td>Partial</td>
<td>Partial</td>
<td>Partial</td>
<td></td>
</tr>
<tr>
<td>L</td>
<td>Yes (38 °C)</td>
<td>Yes (30 °C)</td>
<td>Partial</td>
<td>Yes (22 °C)</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3-3. DSC analysis of polymer blends. (a) Blends with 6 that demonstrate the plasticization effect with polymer blends of 5-8. (b) Blends of 5 and 9 that display the differences between synthetic history vs. blend compatibility with PLGA (50:50) and PLGA (85:15).
3.4.6 Hydrolysis of polymers 5-12 blended with PLGA (50:50) and PLGA (85:15)

The hydrolysis in aqueous media of polymers 5-12 blended with PLGA (50:50) or PLGA (85:15) was accomplished. Percent mass loss and the resultant pH of the media were monitored throughout the study. Similar trends were found for all the blend ratios with 5-12 that resulted in similar percent mass loss and similar pH values. The blend miscibility of 6 is used as a representative example with both compositions of PLGA. This allowed a direct comparison to be made of the rates of hydrolysis and the changes in pH that occur with PLGA (50:50) and PLGA (85:15). The mass loss was found to be 78-93% after 6 weeks when PLGA (50:50) was blended with 6 as shown in Figure 2 (a). The fast hydrolysis is typical of blends of other polyphosphazenes with PLGA (50:50).\textsuperscript{27, 29} In previous work with dipeptide based polyphosphazenes, this generated pH values below 3\textsuperscript{30}. However, the pH values from the hydrolysis of 6 were higher than pH of 4, shown in Figure 3-4(b). These increased pH values are due both to the release of choline and the hydrolysis of the polyphosphazene backbone with its inherent buffering capacity. This buffering effect was more noticeable when 6 was combined with PLGA (85:15). The pH of the resultant hydrolyzed medium was found to be near neutral with values of 5.8 to 6.8 (Figure 3-4(d)). The increased pH values can be correlated to the lower mass loss of the PLGA (85:15) blends that release less acidic hydrolysis products. In addition, blends with PLGA (85:15) or PLGA (50:50) showed intrinsic trends in percent mass loss and pH values. Blends that were composed of high percentages of 5-12 had increased mass loss that was due to the faster hydrolysis of 5-12 over PLGA (50:50) and PLGA (85:15), as shown in Figure 3-4 (a) and (c). However, the increased rate of hydrolysis released a larger percentage of buffering components that result in increased media pH when higher percentages of 5-12 were blended as shown in Figure 3-4 (b) and (d).
3.4.7 In vitro osteocompatibility of polymers 6-8 blended with PLGA (85:15)

To assess the osteocompatibility of the blends for bone tissue engineering, PRO cells isolated from neonatal rat calvaria were cultured on blends using PLGA as controls. Figure 3-5a shows the total amount of protein over 21 days of culture, which correlates with the number of cells on different polymer matrices. The gradual increase in the total protein amount indicated that the blend matrices were able to support the growth and proliferation of PRO. The protein content was significantly lower on the blend matrices than on PLGA after 14 days. However, there were no significant differences among the different blend matrices. ALP expression of PRO...
cells was measured as a marker of osteoblast differentiation. Figure 3-5b shows the ALP activity of cells on different matrices normalized to the total protein content. Throughout the culture period, PRO cells maintained their phenotype on the blend matrices. At day 7, cells secreted similar level of ALP on all the polymer matrices. At day 14, dramatic increase in ALP expression was found on Blend 7A and Blend 8A, resulting in a statistical increase compared to PLGA. After 21 days of culture, the cells maintained higher level of ALP expression on Blend 7A and Blend 8A than PLGA. Throughout the culture, no significant differences in ALP activity were detected between the osteoblasts grown on Blend 6A and on PLGA. It is known that ALP activity is indicative of an inverse relationship of cell proliferation and differentiation. During the 21-day culture period, the elevated ALP expressions of osteoblast cells on Blend 7A and Blend 8A suggested the progression of cell maturity, whereas osteoblasts on PLGA were proliferating throughout the later time points. These experiments suggest that the blend matrices (7A and 8A) supported osteoblast proliferation with an elevated osteoblast phenotype expression.
Figure 3-5. *In vitro* osteocompatibility of polymer matrices. (a): Cell proliferation by protein assay. (b) ALP activity for osteoblastic phenotype expression over 21 days. (*) indicates significant decrease and (**) indicates significant increase. p< 0.05. The gradual increase in the total protein amount indicated that the blend matrices were able to support the growth and proliferation of PRO. It was found that ALP activity of the cells on Blend 7A and Blend 8A was significantly enhanced compared to PLAGA at day 14 whereas the significant increase was found on Blend 8A at day 2. ALP activity of the cells on Blend 6A was comparable to PLAGA throughout 21 days of cell culture.

3.5 Conclusions

Mixed-substituent small-molecule trimeric and polymeric phosphazenes were synthesized with choline chloride as one side group and glycine ethyl ester, alanine ethyl ester, valine ethyl ester, or phenyl alanine ethyl ester as the other. The polymeric species
were synthesized via two different synthetic routes from poly(dichlorophosphazene). Both routes involved the initial linkage of amino acid ester units to the polymer backbone to ensure solubility of the subsequent fully-substituted polymer in organic solvents. The first route involved the use of sodium hydride to form the choloxide reagent before linkage to the polyphosphazene. The resultant polymers were water insoluble with low molecular weights. Unlike the sodium hydride route, the cesium carbonate route involves an in situ linkage of choline chloride to the polymer backbone. This produced polyphosphazenes with increased choline chloride content. Moreover, the molecular weights by this route are higher due to the shorter reaction times needed to achieve full chlorine replacement. The increased choline content allowed these polymers to dissolve in aqueous media during hydrolysis. However, polymers synthesized via both synthetic routes formed miscible blends with PLGA (50:50) and PLGA (85:15). The percent mass loss and pH values when 5-12 were blended with PLGA were similar regardless of synthetic history. In vitro osteocompatibility studies demonstrated that the choline-based blends supported PRO cell growth with an elevated osteoblast phenotype expression compared to the polyester.

3.6 Acknowledgment

This work was supported by NIH RO1 EB004051.
3.7 References

15. Kharat, A.S.; Tomasz, A. Molecular Microbiology 2006, 60, 93.


Chapter 4

Synthesis and Characterization of Methionine- and Cysteine-Substituted Phosphazenes

4.1 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, biological assays, mucoadhesion, nitric oxide generation, metal ion detection, and in soft and hard tissue engineering. 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. This method allows facile changes to be made in the polymer side groups to produce materials that are useful for hydrophobicity and
hydrolytic sensitivity.\textsuperscript{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.\textsuperscript{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 analogs – in this case on cyclic trimeric phosphazenes.\textsuperscript{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.\textsuperscript{21-25}

4.2 Experimental

4.2.1 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.\textsuperscript{26} Dichloromethane (EMD), acetic acid (EM Science), hexanes (EMD), ethyl acetate (EMD, diethyl disulfide\textsuperscript{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. 31P and 1H NMR spectra were obtained with a Bruker 360 WM instrument operated at 145 MHz 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 spectrometric analysis data were collected using turbospray ionization technique on an Applied Biosystems API 150EX LC/MS mass spectrometer.

4.2.2 Synthesis of ethyl ethanethiolsulfinate (CH3CH2S(=O)SCH2CH3)

An aqueous solution of 30% hydrogen peroxide in water (18.55 g, 164 mmol) was added to 40.00 g acetic acid and the mixture was stirred for 1 hour at 0°C. Diethyl disulfide (20.00 g, 164 mmol) was mixed with 40.00 g acetic acid and cooled to 0°C in an ice bath. The acetic acid / H2O2 solution was added drop-wise to a cooled diethyl disulfide solution over a period of 10 minutes. The reaction mixture was stirred for 1 hour at 0°C, followed by 2 hours at 25°C. Potassium hydroxide (53.35g, 950 mmol) was dissolved in 150 mL distilled water and was added to the main reaction mixture to neutralize the solution. Liquid-liquid extractions of the reaction mixture using dichloromethane were completed. The organic layers were combined and dried over anhydrous sodium sulfate. The solution was filtered and the solvent removed to produce a pale yellow oil containing diethyl disulfide, ethyl ethanethiolsulfinate (CH3CH2S(=O)SCH2CH3), ethyl ethanethiolsulfonate (CH3CH2S(=O)2SCH2CH3), and acetic acid in a molar ratio of 19:54:14:14 as determined by 1H NMR spectroscopy. The yield of ethyl ethanethiolsulfinate
based on diethyl disulfide was 59%. This mixture was used in the next reaction without further purification. $^1$H NMR (CDCl$_3$), ethyl ethanethiol sulfate ppm: $\delta$ 1.43 (t, 3H, CH$_3$CH$_2$S), 1.49 (t, 3H, CH$_3$CH$_2$S(=O), 3.15 (m, 4H, CH$_3$CH$_2$S and CH$_3$CH$_2$S(=O)); ethyl ethanethiol sulfonate ppm: $\delta$ 1.43 (t, 3H, CH$_3$CH$_2$S), 1.49 (t, 3H, CH$_3$CH$_2$S(=O)$_2$), 3.18 (q, 2H, CH$_3$CH$_2$S), 3.35 (q, 2H, CH$_3$CH$_2$S(=O)$_2$).

4.2.3 Protection of cysteine ethyl ester hydrochloride with ethyl ethanethiol sulfate

Cysteine ethyl ester hydrochloride salt (10.00 g, 53.86 mmol) and an appropriate amount of the above mixture containing 0.5 molar equivalent of ethyl ethanethiol sulfate (3.723 g, 26.93 mmol) was stirred in 100 mL of absolute ethanol at 25°C for 24 hours. The solvent was removed via evaporation to leave a white solid as the crude product. The crude product was re-crystallized by dissolution in a minimal amount of dichloromethane, followed by an addition of five 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: $\delta$ 1.26 (m, 6H, OCH$_2$CH$_3$ and SCH$_2$CH$_3$), 2.70 (q, 2H, SCH$_2$CH$_3$), 3.40 (d, 2H, SCH$_2$CH), 4.26 (q, 2H, OCH$_2$CH$_3$), 4.51 (t, 1H, SCH$_2$CH), 3.40 (d, 2H, SCH$_2$CH), 8.79 (s-broad, 3H, NH$_3$Cl).

4.3.4 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 THF. This solution was refluxed for 24 hours, filtered, and added to the hexachlorocyclotriphosphazene solution. The resultant
solution was stirred at 25°C for 96 hours followed by removal of all the solvent. The precipitate was dissolved in dichloromethane and extractions against de-ionized 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 hexachlorocyclotriposphazene. $^{31}$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 (ESCl+) m/z = 1191 ([M+H]$^+$) m/z was calculated for C$_{42}$H$_{84}$N$_9$P$_3$O$_{12}$S$_6$.

### 4.3.5 Synthesis of hexa(cysteine ethyl disulfide ethyl ester)cyclotriposphazene

Hexachlorocyclotriposphazene (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 THF. This solution was refluxed for 24 hours, filtered, and added to the hexachlorocyclotriposphazene solution. The resultant solution was stirred at 25°C for 96 hours followed by removal of all solvent. The residue was dissolved in dichloromethane and extractions against de-ionized 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 hexachlorocyclotriposphazene. $^{31}$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 (ESCl+) m/z = 1384 ([M+H]$^+$) m/z was calculated for C$_{42}$H$_{84}$N$_9$P$_3$O$_{12}$S$_{12}$. 
4.3.6 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 hours at 25°C. The trimer solution was dried and re-dissolved 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 0.335 g of 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$_2$CH$_3$), 1.26 (3H, t, CH$_3$ from ethyl disulfide), 2.67 (2H, q, CH$_2$ from ethyl disulfide), 3.21 (2H, d, CH$_2$S), 3.59 (1H, t, CH), 4.18 (2H, q, OCH$_2$CH$_3$). m/z = 1023, 1083, and 1143 ([M+H]$^+$) m/z was calculated for C$_{30}$H$_{60}$N$_9$P$_3$O$_{12}$S$_6$, C$_{32}$H$_{62}$N$_9$P$_3$O$_{12}$S$_7$, and C$_{34}$H$_{64}$N$_9$P$_3$O$_{12}$S$_8$ respectively.

4.3.7 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 hours at 25°C. The trimer solution was dried and re-dissolved 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$_2$CH$_3$), 3.21 (2H, d, CH$_2$S), 3.59 (1H, t, CH), 4.18 (2H, q, OCH$_2$CH$_3$). m/z = 1023 ([M+H]$^+$) m/z was calculated for C$_{30}$H$_{60}$N$_9$P$_3$O$_{12}$S$_6$. 
4.3.8 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 12M Hydrochloric acid (260 uL, 21.7 mmol) was added to the trimer solution and allowed to react for 24 hours at 25°C. The trimer solution was dried and re-dissolved 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 0.1636 g of yellow oil for a 23% yield. $^{31}$P NMR (CDCl$_3$), ppm: $\delta$ +15.38 (3P, s). $^1$H NMR (CDCl$_3$), ppm: $\delta$ 1.27 (3H, t, OCH$_2$CH$_3$), 3.21 (2H, d, CH$_2$S), 3.59 (1H, t, CH), 4.18 (2H, q, OCH$_2$CH$_3$). $m/z = 1041$ ([M+NH$_4$]$^+$) $m/z$ was calculated for C$_{30}$H$_{60}$N$_9$P$_3$O$_{12}$S$_6$.

4.3.9 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 hours, filtered, and added to the polymer solution. The polymer solution was stirred at room temperature for 24 hours and was refluxed for another 48 hours. 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: $\delta$ -0.2255. $^1$H NMR ($d$THF), ppm: $\delta$ 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$_2$).
4.3.10 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 THF. This solution was refluxed for 24 hours, filtered, and added to the polymer solution. The solution was then refluxed for 28 hours and the product was precipitated into hexanes. The polymer was re-dissolved 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: $\delta$ -0.7220. $^1$H NMR (CDCl$_3$), ppm: $\delta$ 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$_2$).

4.3.11 Attempted deprotection of poly[bis-(cysteine ethyl disulfide ethyl ester)phosphazene] with 2.0 molar equivalents 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 hours at 40°C. It was then concentrated and purified by dialysis versus acetone for 24 hours. 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. $^1$H NMR (CDCl$_3$), ppm: $\delta$ 1.27 (3H, OCH$_2$CH$_3$) and (3H, SCH$_2$CH$_3$), 2.68 (2H, SCH$_2$CH$_3$), 3.21 (2H, CH$_2$S), 3.59 (1H, CH), 4.18 (2H, OCH$_2$CH$_3$).
4.3.12 Deprotection of poly[bis-(cysteine ethyl disulfide ethyl ester)phosphazene] with 20.0 molar equivalents 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 hours at 40°C. The polymer solution was concentrated and purified by dialysis versus acetone for 24 hours. 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. $^1$H NMR (CDCl$_3$), ppm: δ 1.26 (3H, OCH$_2$C$_3$H) and (3H, SCH$_2$C$_3$H), 2.68 (2H, SCH$_2$C$_3$H), 3.20 (2H, CH$_2$S), 3.58 (1H, CH), 4.17 (2H, OCH$_2$C$_3$H).

4.3.13 Deprotection of poly[bis-(cysteine ethyl disulfide ethyl ester)phosphazene] with 1.0 molar equivalent 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 hours 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. $^1$H NMR (CDCl$_3$), ppm: δ 1.27 (3H, OCH$_2$C$_3$H) and (3H, SCH$_2$C$_3$H), 2.68 (2H, SCH$_2$C$_3$H), 3.21 (2H, CH$_2$S), 3.59 (1H, CH), 4.18 (2H, OCH$_2$C$_3$H).
4.3.14 Deprotection of poly[bis-(cysteine ethyl disulfide ethyl ester)phosphazene] with 5.0 molar equivalents 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 hours 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. $^1$H NMR (CDCl$_3$), ppm: δ 1.26 (3H, OCH$_2$CH$_3$) and (3H, SCH$_2$CH$_3$), 2.68 (2H, SCH$_2$CH$_3$), 3.20 (2H, CH$_2$S), 3.58 (1H, CH), 4.17 (2H, OCH$_2$CH$_3$).

4.3.15 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 12M hydrochloric acid (388 uL, 32.4 mmol) were added to the polymer solution and the mixture was refluxed for 16 hours. 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.
4.4 Results and Discussion

4.4.1 Synthesis of model cyclic trimers 1 and 2

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 Figure 4-1. 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 and 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.

![Figure 4-1](image)

**Figure 4-1.** Synthesis of (1) hexa(methionine ethyl ester)cyclotriphosphazene] and (2) hexa(cysteine ethyl disulfide ethyl ester)cyclotriphosphazene.
4.4.2 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 Zn⁰ in aqueous HCl solution. 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 ¹H 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 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 thio-ethyl groups in the ¹H NMR spectra and was confirmed by mass spectroscopy at m/z = 1123. 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. Again, ¹H NMR spectroscopy was used to confirm the loss of the thio-ethyl groups, together with the mass spectrometry peak of m/z = 1141 (NH₄⁺) peak. A ³¹P NMR peak at +15.38ppm was also detected, which is evidence that acidic hydrolysis of the phosphazene ring did not occur during the deprotection.
**4.4.3 Synthesis of polymers 3-6**

Methionine is an essential amino acid that contains a thio-ether 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 Figure 4-2, methionine ethyl ester reacts cleanly with poly(dichlorophosphazene) in the presence of triethylamine to produce polymer 3. The physical and structural properties of 3 are shown in Table 4-1. 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.

![Figure 4-2. Synthesis of poly[bis-(methionine ethyl ester)phosphazene] (3).](image)

The synthesis of high polymeric phosphazenes with cysteine side groups and their bi-functional 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 4 directly. However, the initial $^{31}\text{P}$ 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 +26ppm) disappeared leaving only the phosphorus amine (P-N) signal (-1ppm). 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 non-solvents yielded a product that was thereafter insoluble in common organic solvents. It’s solubility in acetic acid or trifluoroacetic acid was accompanied by hydrolytic decomposition. Hydrolysis in strong acids is typical of amino acid substituted polyphosphazenes. 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 uncrosslinked 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 disulfide ethyl ester was allowed to react with poly(dichlorophosphazene) to produce polymer 5 as shown in Figure 4-3.

The physical and structural properties of polymers 3, 4, and 5 are shown in Table 4-1.
**Table 4-1.** Structural and physical properties of polymers 3-5. *The GPC-derived molecular weight approximate to Mw.

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<td>N/A</td>
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<tr>
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<td>160,000</td>
<td>1.81</td>
</tr>
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### 4.4.4 Deprotection of Polymer 5

Polymer 5 was deprotected via three common sulfur-sulfur bond cleavage methods. 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 Figure 4-4 (A). Solutions of DTT (2.0 and 20.0 molar equivalent) 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, as determined by $^1$H NMR spectroscopy. Higher molar equivalences than 20.0 molar 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, Figure 4-4 (B). Use of 1.0 molar and 5.0 molar equivalents 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 molar per polymer repeat unit did not increase the number of cleaved disulfide bonds. The $T_g$ of polymer 3 with 52% disulfide deprotection was lowered slightly from 22°C to 18°C with no appreciable change in the molecular weight. Again, these partially deprotected derivatives remained soluble in organic solvents.

![Deprotection process](image)

**Figure 4-4.** Deprotection of poly[bis-(cysteine ethyl disulfide ethyl ester)phosphazene] using (A) dithiothreitol, (B) β-mercaptoethanol, and (C) Zinc / hydrochloric acid.

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, $\beta$-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 5. It is also important to note that both the DTT and $\beta$-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 undergo reversible cross-linking 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, Figure 4-4 (C). 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.

4.5 Conclusions

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, relatively few skeletal cleavage reactions in a high polymer may have a significant effect on the properties, whereas the corresponding ring-cleavage reaction in a cyclic trimer would result mainly in a small decrease in yield. 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 uncross-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.

4.6 Acknowledgement

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


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Chapter 5

Hydrolysable Polylactide-Polyphosphazene Block Copolymers for Biomedical Applications: Synthesis, Characterization, and Composites with Poly(lactic-co-glycolic acid)

5.1 Introduction

Composites of poly(amino acid ester phosphazenes) with poly(lactic-co-glycolic acid) (PLGA) to form composites have been utilized as osteoconductive biomaterials for hard tissue engineering scaffolds.\(^1\)\(^-\)\(^3\) However, some polyphosphazenes, specifically those that contain alanine ethyl ester, valine ethyl ester, and phenyl alanine ethyl ester side groups, caused the composites to undergo phase-separation. This can lead to a reduction in mechanical stability from the inability to transfer stress across phase domains.\(^4\) There are many examples where block copolymers have been used to compatibilize phase-separated composites. The block copolymers concentrate at the domain interface which improves inter-domain adhesion.\(^5\)\(^-\)\(^10\) The increased phase adhesion allows transfer of the mechanical load from one phase to the other in the composite materials.

Polyphosphazene block copolymers can be synthesized via a living cationic polymerization to yield polymers with well-defined molecular weights.\(^11\) Separate routes have been developed, with both routes starting with an amine-terminated organic polymer. In this work, a phosphoranamine-terminated organic polymer was used to terminate a preformed living poly(dichlorophosphazene) chain. Alternatively, a trifluoroethoxy-substituted phosphoranamine monomer was linked to the organic polymer via its amino terminus, and a polyphosphazene chain was then grown from this site using a chlorophosphoranamine monomer. For both routes, the final poly(dichlorophosphazene) structure may then undergo chlorine replacement by macromolecular substitution with organic nucleophiles. Polyphosphazene-\textit{block}-polyphosphazene
copolymers \textsuperscript{12-14}, polyphosphazene-\textit{block}-polystyrene copolymers \textsuperscript{15-17}, poly(methyl methacrylate)-\textit{graft}-polyphosphazene copolymers \textsuperscript{18}, and polyphosphazene-\textit{block}-poly(ethylene oxide) copolymers \textsuperscript{19-21} have been investigated for their materials properties, as micelleular drug delivery systems, and as lithium ion conductors. However, none of these developed block copolymers incorporated hydrolytic sensitivity units into either block in ways that would allow these materials to be used in tissue engineering applications. The first reported partially-hydrolytically sensitive phosphazene block copolymer was poly(lactic acid)-\textit{block}-poly(bis-trifluoroethoxy phosphazene).\textsuperscript{22} However, in this macromolecules the phosphazene block contains trifluoroethoxy side groups that impart resistance to hydrolysis. Therefore, a need existed to develop a block copolymer system that hydrolyzes completely to nontoxic products for tissue engineering applications. These block copolymers could also be used as interfacial agents to allow formation of compatible blends between previously immiscible blends of PLAGA with certain poly(amino acid ethyl ester phosphazenes).

The synthesis of poly(amino acid ester phosphazenes)-\textit{block}-poly(lactic acid) (PLA-\textit{block}-PPhos) polymers had not been attempted previously due to the inherent acidic degradation that occurs during the introduction of amino acids into polyphosphazenes. The procedure required to synthesize a block copolymer first requires PLA to be linked to poly(dichlorophosphazene). This is then followed by replacement of the chlorine atoms in the poly(dichlorophosphazene) block by the amino acid esters. The acidic media generated during introduction of the amino acid esters can cause premature degradation of the phosphazene block or cause cleavage at the phosphazene to PLA linkage.

In this work, we have synthesized and characterized the first completely degradable phosphazene-PLA block copolymers. Amine terminated poly(lactic acid) (PLA) was synthesized via the ring-opening polymerization of the \textit{l}-lactide. The amine terminated PLA was subsequently end capped with a bromophosphoranamine that was utilized as the termination
species for a preformed living poly(dichlorophosphazene) chain. Macromolecular substitution was then carried out on the phosphazene block in order to replace the chlorine atoms with alanine ethyl ester, valine ethyl ester, or phenylalanine ethyl ester. The PLA-block-PPhos species were then used as blend compatibilizers between a poly(amino ethyl ester phosphazene), where the amino ethyl esters were alanine ethyl ester, valine ethyl ester, and phenylalanine ethyl ester, and PLGA (50:50 or 85:15) systems that were previously found to be incompatible. The blended systems were studied by differential scanning calorimetry (DSC) and scanning electron microscopy (SEM) to determine the extent of miscibility. As a model for the biological response, the hydrolysis media generated from the composite materials was analyzed for percent mass loss and the pH values.

5.2 Experimental

5.2.1 Materials

Lithium bis(trimethylsilyl)amide, sodium hydride (60 %, dispersed in mineral oil), phosphorus trichloride, and sulfuryl chloride were obtained from Aldrich and were used without further purification. Phosphorus pentachloride (Aldrich) was purified by sublimation under vacuum before use. The compounds, \( \text{Cl}_3\text{P}=\text{NSiMe}_3 \), \( (\text{C}_2\text{H}_5\text{O})_3\text{P}=\text{NSiMe}_3 \), and \( (\text{CF}_3\text{CH}_2\text{O})_2\text{BrP}=\text{NSiMe}_3 \) were synthesized and purified by literature procedures.\(^{23-26}\) \( \text{l-Lactide} \) (Frinton Lab.) was used as received. Alanine ethyl ester hydrochloride, valine ethyl ester hydrochloride, and phenyl alanine ethyl ester hydrochloride were purchased from Bachem and used without further purification. PLGA (50:50) (weight-average molecular weight 2,000,000), PLGA (85:15) (weight-average molecular weight 4,800,000) were gift from the Ethicon Division of Johnson and Johnson and used without further purification. The initiator for the \( \text{l-Lactide} \)
polymerization was synthesized according to the method of Gotsche and was used immediately.\textsuperscript{27} Tetrahydrofuran (THF), n-hexane, methylene chloride, toluene, and triethylamine were dried using solvent purification columns.\textsuperscript{28} Anhydrous trifluoroacetic acid (Aldrich) was used as received. All glassware was dried overnight in an oven at 125 °C, or flame dried under vacuum before use. Reactions were carried out using standard Schlenk techniques or in an inert atmosphere glove box (Vacuum Atmospheres or M Braun) under an atmosphere of dry argon or nitrogen.

5.2.2 Equipment

\(^1\)H and \(^{31}\)P NMR spectra were obtained using a Bruker AMX-360 NMR spectrometer, operated at 360 and 146 MHz respectively. \(^1\)H NMR spectra were referenced to tetramethylsilane signals while \(^{31}\)P NMR chemical shifts are relative to 85% phosphoric acid as an external reference. Molecular weight distribution data were estimated using a Hewlett-Packard HP 1090 gel permeation chromatograph equipped with an HP-1047A refractive index detector, Phenomenex Phenogel 10 µm mixed MXL and linear (2) analytical columns, and calibrated against polystyrene standards (Polysciences). The samples were eluted at 40 °C with a 10 mM solution of tetra-n-butylammonium nitrate (Aldrich) in THF (OmniSolv). Glass transition temperatures were measured with a TA Instruments Q10 differential scanning calorimetry (DSC) apparatus with a heating rate of 10°C/min and a sample size of ca. 10 mg. Scanning electron microscopy (SEM) images were obtained using a Philips FEI Quanta 200 Environmental Scanning Electron Microscope. The images were obtained under the following conditions: 20KeV source voltage, pressure approx. 0.88 Torr, and a working distance of approx. 10 mm. pH values were measure using a VWR Symphony SB70P pH meter.
5.2.3 Polymerization of L-Lactide (PLA-Boc)

Polylactide was obtained by ring-opening polymerization of L-lactide initiated by zinc tert-butoxycarbonylaminopropoxide according to the method of Gotsche.\textsuperscript{27} L-Lactide (14.21 g, 100 mmol) in toluene (140 mL) was cannula transferred to the initiator (2 mmol) at 80 °C. The solution was stirred at 80 °C for 1 hr, and acetic acid (6.0 mL) was added to terminate the polymerization. The solvent was evaporated, and the polymer was dissolved in methylene chloride and precipitated into 2 L of methanol. The precipitated polymer was filtered and washed with methanol. A white solid was obtained for an 87% yield.

5.2.4 Deprotection of Amino-polylactide (PLA-NH\(_2\))

A large excess of anhydrous trifluoroacetic acid (14 mL) was added to a solution of polylactide (12 g) in methylene chloride (150 mL). The solution was stirred at ambient temperature (25°C) for 3 hr, washed with aqueous NaHCO\(_3\) (5%), followed by a wash with water, and finally dried over anhydrous MgSO\(_4\). After filtration and evaporation of the solvent, the polymer was recovered in 78% yield.

5.2.5 Polylactide Functionalization with Br(CF\(_3\)CH\(_2\)O)\(_2\)P=NSiMe\(_3\), 1

Amino polylactide (0.75 g, 0.1 mmol of end groups) was dissolved in THF (30 mL). Triethylamine (0.011 mg, 0.11 mmol) was added to this solution, followed by Br(CF\(_3\)CH\(_2\)O)\(_2\)P=NSiMe\(_3\) (0.044 g, 0.11 mmol). The reaction mixture was stirred at room temperature for 24 hr. The solvent was removed under vacuum and the solid product was used without further purification.
5.2.6 Synthesis of Polylactide-Alanine Polyphosphazene Block Copolymer (PLA-Ala) 4

(C$_2$H$_5$O)$_2$P=NSiMe$_3$P=NSiMe$_3$ (0.028 g, 0.11 mmol) was added to a stirred solution of PCl$_5$ (0.046 g, 0.22 mmol) in methylene chloride (30 mL) and the reagents were allowed to react for 2 hr. The monomer, Cl$_3$P=NSiMe$_3$ (2.54 g, 11.33 mmol), was added to this reaction solution which was stirred for 4 hours at room temperature. A solution of polymer 1 in methylene chloride (25 mL) was added and stirred for 4 hours at room temperature. A solution of polymer 1 in methylene chloride (25 mL) was added and stirred for 24 hours to terminate the reaction. The methylene chloride was then removed under vacuum and the residual polymer was re-dissolved in THF. In a separate reaction vessel, alanine ethyl ester was prepared by treatment of alanine ethyl ester hydrochloride (5.22 g, 33.99 mol) with triethylamine (6.88 g, 67.98 mol) in refluxing THF (100 mL). The solution was stirred for 24 hrs, filtered and then added to a stirred solution of polylactide-poly(dichlorophosphazene) block copolymer solution. The reaction mixture was stirred at reflux for 24 hrs followed by removal of the solvent. The crude copolymer was purified by dialysis against methanol using 6000-8000 MWCO tubing for four days. A light yellow solid was obtained for a 46% yield. $^1$H NMR (CDCl$_3$): δ 1.19 (br, P-NH-CH(CH$_3$)-COO-CH$_2$CH$_3$), 1.32 (br, P-NH-CH(CH$_3$)-COO-CH$_2$CH$_3$), 1.51 (d, -CO-CH(CH$_3$)-O-), 4.08 (br, P-NH-CH(CH$_3$)-COO-CH$_2$CH$_3$), 5.10 (q, -CO-CH(CH$_3$)-O-). $^{31}$P NMR (CDCl$_3$): δ -1.96 (brs).

5.2.7 Synthesis of Polylactide-Valine Polyphosphazene Block Copolymer (PLA-Val) 5

The same synthetic procedure used for polymer 4 was employed with valine ethyl ester hydrochloride to give polymer 5. The yield was 38%. $^1$H NMR (CDCl$_3$): δ 0.88 (br, P-NH-CH(CH(CH$_3$)$_2$)-COO-CH$_2$CH$_3$), 1.19 (br, P-NH-CH(CH(CH$_3$)$_2$)-COO-CH$_2$CH$_3$), 1.51 (d, -CO-CH(CH$_3$)-O-), 1.98 (br, P-NH-CH(CH(CH$_3$)$_2$)-COO-CH$_2$CH$_3$), 3.57 (br, P-NH-
5.2.8 Synthesis of Polylactide-Phenyl alanine Polyphosphazene Block Copolymer (PLA-PheAla) 6

The same synthetic procedure employed for polymer 4 was used with phenyl alanine ethyl ester hydrochloride to give polymer 6. The yield was 36%. $^1$H NMR (CDCl$_3$): $\delta$ 0.94 (br, P-NH-CH(CH$_2$-Ar)-COO-CH$_2$H$_2$CH$_3$), 1.51 (d, -CO-CH(CH$_3$)-O-), 2.92 (br, P-NH-CH(CH$_2$-Ar)-COO-CH$_2$CH$_3$), 3.87 (br, P-NH-CH(CH$_2$-Ar)-COO-CH$_2$H$_2$CH$_3$), 5.10 (q, -CO-CH(CH$_3$)-O-), 7.08 (br, P-NH-CH(CH$_2$-Ar)-COO-CH$_2$CH$_3$). $^{31}$P NMR (CDCl$_3$): $\delta$ -2.22 (brs).

5.2.9 Synthesis of Polymers 7-9

The synthesis of polymers 7-9 was completed using a previously published technique. Polymer 7 is described as an example. Poly(dichlorophosphazene) (5.00 g, 43.1 mmol) was dissolved in THF (500 mL). Alanine ethyl ester hydrochloride (26.4 g, 17.2 mmol) and triethylamine (60.1 mL, 431 mmol) were suspended in THF (300 mL). This suspension was refluxed for 24 hours, filtered, and then added to the polymer solution. The polymer solution was stirred for 24 hours at room temperature, followed by another 24 hours under reflux. The polymer solution was concentrated, dialyzed against methanol for 3 days in 12-14,000 MWCO dialysis tubing, and dried under reduced pressure for 1 week. The yields were 75-82% based on the amount of poly(dichlorophosphazene) used, with molecular weights ranging from 350,000 to 425,000. The glass transition temperatures for 7, 8, and 9 were -16°C, 21°C, and 44°C respectively.
5.2.10 Fabrication of polymer blends via solution casting

Blends were prepared using a mutual solvent approach. Equal masses (0.1g) of polymers 7, 8, or 9 were co-dissolved with PLAGA (50:50) or PLAGA (85:15) in chloroform (2mL). PLA-block-PPhos (0.1g) was dissolved in chloroform (1mL). The specific block copolymer used matched the polyphosphazene block in the copolymer. For example, if polymer 7 was blended with PLAGA, then block co-polymer 4 was used as the compatibilizer. The corresponding block co-polymers 4-6 were added to the respective blends. The combined solution was stirred for 1 hour and then allowed to stand undisturbed for one hour to confirm that solution-phase miscibility existed. The solutions were then cast into trays lined with Bytac®, air dried for 24 hours, and then vacuum dried for one week. Each polymer blend was analyzed by DSC and SEM techniques. After confirmation of blend miscibility, the amount of PLA-block-PPhos added to the mixture of 7, 8, or 9 with PLAGA was progressively reduced until blend compatibility was no longer detected in the DSC spectrum.

5.2.11 pH hydrolysis studies

The blended polymers were cut into squares (10 mm x 10 mm) and placed in aqueous media at 37°C in a shaker bath. Three samples were removed from each medium after 1, 2, 3, 4, 5, and 6 weeks to measure the pH of the hydrolysis media and to determine the percent mass loss.
5.3 Results and Discussion

5.3.1 Synthesis of Amine-Functionalized Poly(l-lactide)

Amine-functionalized poly(l-lactide) was synthesized according to Höcker’s method through ring opening polymerization of l-lactide via a zinc alkoxide (Figure 5-1) containing a protected amine group. The zinc initiator was synthesized from diethylzinc and Boc-aminoethanol and was used immediately after the synthesis. Addition of the initiator to an l-lactide solution produced Boc-protected PLA. The molecular weight of the polymer was determined by comparing the molecular weight obtained by GPC with the molecular weight determined from $^1$H NMR. Molecular weight determination from $^1$H NMR spectroscopy was accomplished by analysis of the ratio of the end group signals (a, c, or d) compared to the signals from lactide units (f or g) (Figure 5-2). In all cases, the actual number of repeat units (103 r.u.) was in agreement with the target number of units (100 r.u.).

![Figure 5-1. Structure of the Initiator of l-Lactide Polymerization.](image-url)
**Figure 5-2.** $^1$H NMR spectrum of polylactide bearing-NHBoc end group in CDCl$_3$.

**Figure 5-3.** Synthesis of Amino-polylactide.
The deprotection of the Boc group to generate the amine functional group was completed by reaction of Boc-PLA with trifluoroacetic acid (TFA). This reaction (Figure 5-3) deprotected the primary amine function at the end of the polymer without lowering the molecular weight of the polymer. This was confirmed by the GPC chromatogram and by $^1$H NMR spectroscopy. The signal corresponding to the Boc group at 1.4 ppm disappeared, and that from the -CH$_2$-N unit shifted from 3.15 to 2.78 ppm. It might be anticipated that the loss of the Boc group should lead to a decrease in the molar mass of the corresponding polymer. However, the modification of the end group has little effect on the overall molecular weight of the polymer.

### 5.3.2 Synthesis of Block Copolymers

Polylactide-polyphosphazene (PLA-PPhos) block copolymers were synthesized via the controlled cationic polymerization of Cl$_3$P=NSiMe$_3$ at ambient temperature using polylactide-phosphoranimine as a macro-terminator (Scheme 3). The amino-functionalized polylactide was treated with Br(CF$_3$CH$_2$O)$_2$P=NSiMe$_3$ to yield the polylactide-phosphoranimine 1, which served as the macromolecular terminator for the controlled cationic polymerization of Cl$_3$P=NSiMe$_3$. The addition of 1 to solutions of [(CF$_3$CH$_2$O)$_2$P=N-(Cl$_2$P=N)$_m$-PCl$_3$]$^+[PCl_6]^-$(2), yielded polylactide-poly(dichlorophosphazene) block copolymers (3) with a controlled phosphazene block length.$^4$
The macromolecular substitution reactions of poly(dichlorophosphazenes) were performed with three different amino acid esters in the presence of triethylamine as a proton acceptor. The molar composition ratios of the repeating units of polylactide (PLA) to polyphosphazene (PPhos) in 4, 5 and 6 were 1.0 to 0.36, 1.0 to 0.26, and 1.0 to 0.36 respectively. The molar ratios between the PLA and PPhos block showed, by $^1$H NMR spectroscopy, a lower

**Figure 5-4. Synthesis of Polylactide-Alanine Substituted Polyphosphazene Block Copolymer**
PPhos block ratio compared to the feed ratio. This is due to the degradation of phosphazene block by hydrogen chloride generated during the macromolecular substitution reactions. The molecular weights were determined by comparing the $^1$H NMR spectroscopy peak integration ratio of the polyphosphazene –CH$_3$ protons (1.0 – 1.3 ppm) to the -CH- protons (5.1ppm) of polylactide (Figure 5-5). Gel permeation chromatography was used to estimate average molecular weights and polydispersity values. These molecular weights were compared to the values calculated using $^1$H NMR spectroscopy (Table 5-1).

Figure 5-5. $^1$H NMR spectrum of Ala-PLA block copolymer (4) in CDCl$_3$. 
Table 5-1. Characterization of Polylactide-Polyphosphazene Block Copolymers.

<table>
<thead>
<tr>
<th>Block copolymer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Yield</th>
<th>$M_n$ (1H NMR)</th>
<th>Block ratio&lt;sup&gt;b&lt;/sup&gt; (PLA:PPhos)</th>
<th>$M_n$ ($M_w$ / $M_n$)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>$T_g$ (CMT)&lt;sup&gt;*&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLA-Ala (4)</td>
<td>46%</td>
<td>17 700</td>
<td>1.0:1.0</td>
<td>12 300 (1.12)</td>
<td>36°C (156°C)</td>
</tr>
<tr>
<td>PLA-Val (5)</td>
<td>38%</td>
<td>15 700</td>
<td>1.0:1.0</td>
<td>14 800 (1.17)</td>
<td>35°C (156°C)</td>
</tr>
<tr>
<td>PLA-PheAla (6)</td>
<td>36%</td>
<td>22 500</td>
<td>1.0:1.0</td>
<td>16 000 (1.18)</td>
<td>35°C (152°C)</td>
</tr>
</tbody>
</table>

<sup>a</sup> All the samples were prepared by using PLA with Mn of ~ 7500 by 1H NMR (103 lactic acid repeating units). <sup>b</sup>Calculated from 1H NMR by comparing -CH- protons (5.1 ppm) on PLA block to –CH3 protons (1.0 - 1.3 ppm) on PPhos block. <sup>c</sup>Measured by GPC. * CMT = crystalline melting transition.

5.3.3 Blend compatibility effect of block co-polymers 4-6 on poly(lactide-co-glycolide) (PLAGA) / poly(amino acid ester phosphazene) composites

Equal masses of polymers 7, 8, or 9 (Figure 5-6) and PLAGA (50:50) or PLAGA (85:15) were individually dissolved in chloroform and then mixed together. Corresponding block co-polymers 4-6 were then added to the respective blends. Initially, high loadings (0.1 : 0.1 : 0.1) of block co-polymers were used to promote blend miscibility between the previously incompatible blends.<sup>31-32</sup> The amount of block co-polymer was sequentially reduced by 0.05 g after confirmation of micro-scale blend miscibility by DSC and SEM techniques for each blend ratio. The procedure was repeated until immiscible systems were produced. Miscible blends were recognized by a single glass transition temperature detected for the blended matrix.
Figure 5-6. Chemical structures of poly(amino acid ester phosphazenes) 7-9.

DSC results indicated that 5 wt% of block co-polymer 4 produced miscible composite materials when added to the corresponding polyphosphazene 7 blended with PLGA (50:50) or PLGA (85:15) and were characterized by glass transition temperatures of 33°C and 49°C respectively. Alternatively, 5 wt% of block co-polymer 5 also produced miscible composite materials when composite materials were fabricated with 8 and PLGA (50:50) or PLGA (85:15) and were characterized by glass transition temperatures of 24°C and 31°C respectively. The miscible blend of composites composed of 5, 8 and PLGA (85:15) are shown in Figure 5-7(a). Reduction of 4 or 5 to 2.5 wt% resulted in the formation of immiscible blends. An example of this is shown in Figure 5-7(a) where the 2.5 wt% of 5 produced immiscible blends with two glass transitions associated with the parent polymers at 31°C and 38°C. Scanning electron microscope (SEM) images of blends fabricated from 8 and PLGA (50:50) (not shown) or PLGA (85:15) (shown) with 5wt% of 5 show a smooth surface with no phase separation, Figure 5-8(a). However, phase separation is visible when 2.5wt% of 5 was used as the compatibilizer as shown in Figure 5-8(b). Similar results were observed when 4 was used to compatibilize blends fabricated with PLGA (85:15) and PLGA (50:50).
Composites of 9 and PLGA (50:50) or PLGA (85:15) required higher percentages of block copolymer 6 to maintain compatibility. Addition of 7.5wt% of 6 was needed to achieve miscibility when blends were fabricated with PLGA (50:50) or PLGA (85:15) and these resulted in glass transitions of 28°C and 29°C respectively. The transitions for 6, 9, PLGA (85:15) and the miscible blend with 7.5 wt% of 6 are shown in Figure 5-7(b). In addition, Figure 5-7(b) also indicates an immiscible blend when 5wt% of 6 was used. The DSC results for the 7.5wt% blend and the 5 wt% blend are confirmed by the lack of or confirmation of phase separation in the SEM images shown in Figure 5-8(c) and Figure 5-8(d). Similar DSC and SEM image results were observed for PLGA (50:50).
Figure 5-7. DSC traces of polymer blends composed of (a) 5 wt% or 2.5 wt% of 5 with 8 and PLGA (85:15) and (b) 5 wt% or 2.5 wt% of 6 with 9 and PLGA (85:15).
5.3.4 Hydrolytic Degradation of Block Copolymers

Hydrolytic degradation of the miscible blends was studied. The solid samples were hydrolyzed over a period of 6 weeks with the pH and mass loss monitored at each time point. Hydrolysis of the blended samples with PLAGA (50:50) resulted in pH values of 3 while 20% to 35% of the original mass remained, as shown in Figure 5-9. These low pH values are a direct result of the hydrolysis of PLAGA (50:50), which was also evident by the low molecular mass that remained. On the other hand, blends with PLAGA (85:15) had pH values that were near neutral with only 10% to 30% mass loss. The slower hydrolysis is attributed to the increased
amount of lactic acid in PLAGA (85:15). The degradation of PLAGA (50:50) and PLAGA (85:15) were affected by the poly(amino acid ester phosphazene) that was used to fabricate the blends. The rate of hydrolysis decreased with increases in the steric hindrance of the amino acid ester substituted along the polyphosphazene backbone, which resulted in reduced mass loss and higher pH values. The rate of hydrolysis followed the trend of alanine ethyl ester > valine ethyl ester > phenylalanine ethyl ester. This resulted in blends between 7 and PLGA characterized by greater mass loss and lower pHs when compared to blends between 9 and PLGA.

**Figure 5-9.** Hydrolysis of PLAGA blended with poly(amino ethyl ester phosphazene) using corresponding PLA-block-PPhos (Ala = alanine ethyl ester, Val = valine ethyl ester, and Phe = Phenylalanine ethyl ester substituted polyphosphazenes) blend compatibilizers: (a) pH of hydrolysis media and (b) percent mass remaining with PLAGA (50:50) and (c) pH of hydrolysis media and (d) percent mass remaining with PLAGA (85:15).
5.4 Conclusions

The synthesis of poly(lactic acid)-co-poly(bis-alanine ethyl ester phosphazene), poly(lactic acid)-co-poly(bis-valine ethyl ester phosphazene), and poly(lactic acid)-co-poly(bis-phenylalanine ethyl ester phosphazene) have been completed using the living cationic route. This is the first reported synthesis of completely hydrolysable polyphosphazene block co-polymers. The addition of 5 wt% to 7.5 wt% of these block co-polymers to mixtures of corresponding poly (amino acid ester phosphazenes) and PLAGA (50:50) or PLAGA (85:15), produced completely miscible blends. Thus, these block copolymers allowed the formation of miscible blends hitherto immiscible systems. These compatibilized blends ensure that mechanical stability is retained within the fabricated composites. This is especially important for load-bearing applications like hard tissue engineering scaffolds.

5.5 Acknowledgment

This work was supported by NIH RO1 EB004051.
5.6 References


Chapter 6

Biodegradable Dipeptide-based Polyphosphazene Blends for Regenerative Medicine: Analysis, Characterization, and Implications of A Unique Polymer Matrix Erosion on Morphology and In Situ Porous Structure

6.1 Introduction

Tissue engineering aims to repair, restore, and regenerate lost or damaged tissues by using biomaterials, cells, and factors alone or in combination.¹ Biomaterials including synthetic biodegradable polymers and composites play vital roles in a tissue engineering approach.² A significant interest has focused on the synthesis of synthetic biodegradable materials and fabrication of biomaterials into appropriate constructs that mimic the architecture of native tissues. In a regeneration strategy for musculoskeletal tissue, biomaterials should be able to provide mechanical support during the tissue in-growth, gradually degrade to biocompatible products, present an open porous structure of adequate porosity to allow cell infiltration, and promote matrix synthesis. Synthetic biodegradable polymers that have currently been investigated for orthopedic tissue engineering applications include polyesters such as poly(lactide), poly(glycolide), poly(lactide-co-glycolide) (PLAGA), polyanhydrides, polycarbonates, and polyphosphazenes.² Unlike biostable materials, all these polymers undergo hydrolytic degradation and remove the need for a second surgery to retrieve the implant. Although the degradation rate varies with the chemical composition, the mechanism of the polymer hydrolytic degradation can be broadly classified into two types: bulk erosion
and surface erosion. For example, synthetic biodegradable polymers such as polyesters are known to undergo bulk erosion, whereas polyanhydrides degrade through surface erosion.

In bulk erosion, the polymer undergoes degradation with significant decrease in molecular weight and the corresponding material properties (such as mechanical properties) as a function of degradation time. As illustrated in Figure 6-1a, the matrix dimension remains constant until the structure fails catastrophically during hydrolytic degradation. Bulk eroding polymers have been widely used in orthopedic applications. Unfortunately, these polymers do not generate an interconnected porous structure during the degradation process, which necessitates the prefabrication of the materials into three-dimensional (3D) structures with interconnected pores. Many of the prefabricated structures possess significantly poorer mechanical properties compared to the bulk form. These structures may also deform and collapse, thus compromising the 3D porous structure and their ability to function, particularly as bone graft substitutes. They also suffer from limitations in nutrient and oxygen diffusion through the porous structures, which are crucial for complete cell infiltration and tissue formation. Furthermore, these prefabricated structures lack the control in scaffold properties post-implantation, resulting in a rate mismatch between scaffold degradation and tissue in-growth. In the case of surface erosion, the polymer undergoes degradation from the surface with insignificant decrease in the molecular weight of the bulk material. The matrix becomes smaller but maintains its original geometric shape as a function of degradation time until the structure is completely eroded as indicated in Figure 6-1b. Thus, the surface-eroding polymers do
not generate an interconnected porous structure during the degradation process and also necessitate the prefabrication of the material into 3D structures.

Figure 6-1. Schematic illustration of different types of polymer erosion a) bulk erosion; b) surface erosion; c) a unique polymer erosion through which the polymer changes from a solid coherent film to an assemblage of microspheres with an interconnected porous structure.

Our studies presented here demonstrate for the first time a unique degradation process for biodegradable biomaterials. As indicated in Figure 6-1c, the polymer system exhibits an erosion mechanism by which the polymer changes from a solid coherent film to an assemblage of microspheres with interconnected porous structures. This could have significant implications toward developing biodegradable materials for tissue engineering. The polymer system is made of polyphosphazene-polyester blends where the degradation products of the polyphosphazene neutralize the acidic degradation of the polyester. The blend approach is attractive since it imparts the ability to tailor the
polymer properties by simply changing the blend composition. In addition, biodegradable polyphosphazene-PLAGA blends show several distinct advantages. PLAGA is a Food and Drug Administration-approved polymer that has been widely investigated for a variety of biomedical applications. The synthetic flexibility of polyphosphazene allows us to design specific side group chemistry that enables both intramolecular and intermolecular interactions such as hydrogen bonding. The degradation mode of the polymer can also be tuned efficiently by incorporating different side groups. For example, amino acid ester side groups confer hydrolytic instability into the polymer, while addition of phenylphenoxy side groups increases its hydrophobicity. Inspired by the strong hydrogen bonding in the dipeptide polymer systems, combined with the ability of phenylphenoxy side groups to retard the degradation of polyphosphazene and maintain structural integrity, we have employed a mixed-substituent polyphosphazene co-substituted with glycylglycine dipeptide and phenylphenoxy side groups in this work. We hypothesized that the combination of glycylglycine dipeptide with the hydrophobic phenylphenoxy group would lead to a suitable degradation time frame of 12-24 weeks. Further, we hypothesized that the low molecular weight polyester with relatively high molecular weight polyphosphazene in the blend would favor the 3D porous structure formation and multi-phase degradation kinetics. Finally, the degradation pattern could be controlled via blend compositional changes.
6.2 Experimental

6.2.1 Fabrication and Characterization of Blend Matrices

Blend matrices were prepared using a mutual solvent method. Briefly, the polymers with two weight ratios of PPHOS to PLAGA (25:75 and 50:50) were dissolved in a mutual solvent (chloroform) to obtain a homogeneous solution. Samples of the polymer solution were subsequently poured into petri dishes lined with Bytac paper and the solvent was allowed to evaporate slowly at 4°C for 48 hours followed by freeze drying. Finally, circular disks of 10 mm diameter were bored from the films.

The surface morphology of the composite matrices was examined using a JEOL 6700F scanning electron microscope (SEM) (JEOL, Boston, MA, USA) after being coated with Au/Pd using a Hummer V sputtering system (Technics Inc., Baltimore, MD). The glass transition temperatures ($T_g$) were determined by differential scanning calorimetry (DSC) using a TA DSC Q1000 instrument with Thermal Analysis software. Polymer samples were heated from -40°C to 100°C at a heating rate of 3°C/minute under a nitrogen atmosphere. The $T_g$ was determined from the half height point of the heat capacity change in the thermogram. The Fourier transform infrared (FTIR) spectra of thin films of polymers and composite matrices were recorded using a Brucker Vector 22 FTIR spectrophotometer at a resolution of 4 cm$^{-1}$ and with an average of 50 scans. The composite matrices were cast on KBr plates and were sufficiently thin to be within an absorbance range where the Beer-Lambert law was obeyed.
6.2.2 High-resolution solid-state NMR Section

The water-in-air contact angle was measured at room temperature using a Digital Contact Angle Measurement System equipped with a CCD camera (CAM 100 series, KSV instruments, USA). Temporal images of the water droplet on slabs of polymer matrices fixed on glass slides were recorded and the contact angle values were calculated using ramé-hart DROPinimage Advanced software.

6.2.3 In Vitro Degradation

Blend disk matrices with dimensions of 0.5 mm × 10 mm (T × D) and weighing ~95 mg each were incubated in 10 mL of distilled water at pH 7.4 up to 12 weeks at 37°C. The vials were maintained at 37°C in a shaker water bath for 12 weeks at 250 rpm. At specific time points (2, 4, 7, 10, and 12 weeks), the matrices were removed from distilled water and were dried under vacuum for 2 weeks. The results were reported as percentage mass loss versus time as calculated from the equation:

\[
\text{Percentage mass loss} = \frac{W_0 - W_t}{W_0} \times 100\%
\]

where \( w_t \) is the dry weight of the matrix at predetermined time points, and \( w_0 \) is the initial matrix dry weight. The molecular weight of the degraded matrices was also determined using GPC and reported as percentage molecular weight remaining: Percentage molecular weight remaining = \( \frac{M_t}{M_0} \) \times 100% where \( M_t \) is the molecular weight of polymer component at predetermined time points, and \( M_0 \) is the initial molecular weight of polymer. Samples were visualized by SEM. Each week, the media were also collected and the pH values were recorded by a pH meter.
6.2.4 Rat Subcutaneous Implantation Model

Blend disk matrices with dimensions of 0.5 mm × 10 mm (T × D) were implanted subcutaneously in 45 male retired breeder Sprague-Dawley rats weighing ~450 grams (Charles River Laboratories, Wilmington, MA) after being exposed to UV for 30 min on each side. The animals were cared for according to the procedures approved by the Animal Care and Use Committee at the University of Virginia, and following the guidelines established by the U.S. National Institutes of Health. Two incisions (10 mm apart) of about 10 mm were made laterally on the dorsum using a No. 10 surgical blade. A subcutaneous pouch on opposite sides of the incision was created using blunt dissection technique and a disk implant was inserted into each pouch. Each rat was implanted with two matrix disks. The skin was closed using a sterile stapler. At specific time points (2, 4, 7, 10, and 12 weeks), the implanted matrices were harvested and extraneous tissue was removed. The recovered matrices were rinsed with phosphate buffer saline solution, washed with deionized water, and dried under vacuum for 1 week. The samples were analyzed for surface morphology, degradation kinetics, and in situ porosity/pore volume.

6.2.5 Scanning electron microscopy

The surface morphology of the blend matrices was examined by SEM using a Hitachi TM-1000 tabletop microscope after being coated with Au/Pd using a Hummer V sputtering system (Technics Inc., Baltimore, MD).
6.2.6 Characterization of Degradation Kinetics

The degradation of the polymer matrix \textit{in vivo} was followed by measuring the mass, molecular weight of the polymer matrix before implantation, and those at different time points.

6.2.7 Micro-CT analysis

Micro-CT was used to quantify total pore volume within the scaffolds. Scaffolds were scanned using a micro-CT 40 imaging system (SCANCO Medical), and linear attenuation data were reconstructed to generate 3D images. To quantify pore volume, samples were scanned at 12 mm resolution with an integration time of 300 ms. Images of full size samples were generated from scans with 12 mm resolution. The 2D data are the average of 10 slices for each sample. The area is a square that measures $0.103 \text{ cm}^2$.

6.2.8 Histological Staining

At specific time points (2, 4, 7, 10, and 12 weeks), the implants and the surrounding tissues were excised and fixed in a 10% formalin solution (Surgipath, USA) for 7 days. The samples were embedded in paraffin and sectioned using a microtome to about 4-5 μm thickness followed by staining with hematoxylin and eosin (H&E) and Masson’s trichrome stain (TRI). The thickness of the inflammatory zone (H&E) and collagen deposition (TRI) was characterized according to a reported protocol.\textsuperscript{19}
6.2.9 Statistical Analysis

All the experiments were run in triplicate per sample. Quantitative data were reported as mean ± standard deviation (SD). Statistical analysis was performed using a one-way analysis of variance (one-way ANOVA). Comparison between means was determined using the Tukey post-hoc test with a minimum confidence level of p< 0.05 for statistical significance.

6.3 Results

6.3.1 Design and Fabrication of Blend Matrix

The blend matrices consist of a mixed-substituent polyphosphazene and commercially available PLAGA polymer with a 50:50 lactide to glycolide ratio (M_w=34 kDa). The mixed-substituent polyphosphazene was co-substituted with the ethyl ester of glycylglycine dipeptide and with the phenylphenoxy group in a 50:50 ratio namely poly[(glycine ethyl glycinate)_(1)(phenylphenoxy)_(1)phosphazene] (PPHOS). The polymer was synthesized according to a standard two-step polymerization / substitution route (Figure 6-2). The two side groups for the PPHOS were selected based on the following rationale: (i) the dipeptide was incorporated to provide multiple hydrogen bonding sites (two proton donors per monomer unit) for coordination with PLAGA while the phenylphenoxy group was beneficial for maintaining mechanical function and hydrophobicity; (ii) the glycylglycine dipeptide ester is known to be biocompatible in vivo and can be hydrolyze to glycine units, one of the common amino acids in the body; (iii) the combination of the two side groups in a 50:50 ratio will result in a complete degradation time of 12-24 weeks. Selection of this specific PLAGA was based on a criterion
of having less steric hindrance from the α-CH3 groups in order to achieve a higher probability of hydrogen bonding interactions between the two polymers (Figure 6-2). In addition, this specific PLAGA has a relatively fast degradation rate among the PLAGA family, which is advantageous to induce morphology change and pore formation. The thermal ring opening polymerization of (NPCl2)3, followed by nucleophilic substitution reactions of the resultant poly(dichlorophosphazene) produced the yellow polymers of PPHOS (Figure 6-2). The structure and side-group ratios of PPHOS were confirmed by 1H NMR and 31P NMR spectroscopies (Table S1). The detailed characterization and physical properties of PPHOS polymer (Mw=767 kDa) are described elsewhere. Blend matrices were fabricated using a mutual solvent method. Two different weight ratios of PPHOS and PLAGA, namely 25:75 and 50:50, yielded Matrix1 (light yellow) and Matrix2 (yellow), respectively. Therefore, by taking advantage of the difference in degradation rates of the two parent polymers, PLAGA was used as a “dynamic porogen” in the 3D void space formation and evolved into the pores during the blend degradation.
6.3.2 Blend Matrix Characterization

The miscibility of the blend matrices has been confirmed by Differential Scanning Calorimetry (DSC), Fourier Transform Infrared Spectroscopy (FTIR), Scanning electron microscopy (SEM), and high-resolution solid-state $^{13}$C NMR. For example, the blend matrices showed a single glass transition temperature ($T_g$) intermediate between two parent polymers. The FTIR spectra also showed that the C=O stretching vibrations of
PPHOS and PLAGA occurred at 1739 and 1750 cm\(^{-1}\), respectively. In addition, for Matrix1 and Matrix2, a second band developed at 1673 cm\(^{-1}\), which indicated hydrogen bonded carbonyl groups. A smooth and uniform surface morphology was found for the blend matrices. Furthermore, \(^{13}\)C NMR has also been widely used to study the intermolecular hydrogen-bonding interactions. It is known that the \(^{13}\)C nuclei show some downfield shifts when they are involved in hydrogen bonding.\(^{17-18}\) As shown in Figure 6-2, a 5 ppm downfield shift of the carbonyl carbon resonance was detected in the high-resolution solid-state \(^{13}\)C NMR spectra of blends. Thus, all the evidence supported the formation of miscible blends via hydrogen bonding between amide and amine protons in the dipeptide units of PPHOS and the carbonyl groups in PLAGA. Furthermore, the water-in-air contact angle of Matrix2 (80.3 ± 0.1º) was significantly higher than Matrix1 (70.9 ± 0.2º) (p<0.05), indicating the presence of the hydrophobic phenylphenoxy groups on the surface.

**6.3.3 In Vitro Degradation Study**

The most unique phenomenon about the polymer erosion of the dipeptide-based polyphosphazene-PLAGA blend system lies in the morphology change of the matrix as a function of time. Fig. 2 shows the representative SEM images of blend matrices 0.5 mm × 10 mm (T × D) during *in vitro* hydrolysis in aqueous media as a function of time. At week zero, both the matrices appeared to be smooth and uniform (Figure 6-3a, b). After 4 weeks of hydrolysis, the PLAGA component had experienced significant molecular weight loss and retained only 7.87 ± 0.38% and 9.39 ± 0.08% of its original molecular
weight, respectively (Figure 6-4a). Such a rapid degradation of the PLAGA phase resulted in spherical structures on the surface of Matrix1 and Matrix2 (Figure 6-3c, d). Following 7 weeks of continued rapid bulk hydrolysis of the PLAGA, more spherical structures were formed on the surface as evidenced by SEM imaging, and only a small amount of the PLAGA component was found on the surface as indicated in Figure 6-3e and f. During the first 7 weeks, the percentage of mass loss for Matrix1 and Matrix2 was 77.05 ± 1.07% and 56.58 ± 1.01%, respectively (Figure 6-4b). However, after the rapid PLAGA hydrolysis phase, the degradation rate of the blend matrices significantly decreased beyond 7 weeks. During the remaining 5 weeks of the degradation study only ~8% and ~18% mass loss was observed. Thus Matrix1 and Matrix2 experienced a total mass loss of 85.26 ± 0.27% and 74.21 ± 0.49% after 12 weeks of degradation, respectively (Figure 6-4b). Such multi-phase degradation profiles of blend matrices would allow accommodating gradual tissue in-growth during the different stages of bone healing. By 12 weeks, completely open 3D continuous architecture with macropores (10-100 µm) between spheres in combination with micro/nanopores on the sphere surfaces formed with well packed polymer spheres (Figure 6-3g, h). This unique in situ-created porous structure has significant potential for cell in-growth and tissue regeneration. Furthermore, the 3D porous structure can be modulated by altering the composition of the matrix to enable the potential for dynamic control of the matrix in situ. For instance, a higher PLAGA content in Matrix1 resulted in faster degradation as well as smaller spheres than in Matrix2. Such uniform porous structure formation was found throughout the matrix as evidenced by the SEM images of matrix cross-sections (data not shown). The spherical phase of the degraded blend was confirmed to be primarily
polyphosphazenes through the use of energy dispersive x-ray spectroscopy and solid-state $^{31}$P NMR (data not shown). Furthermore, the molecular weight loss of the polyphosphazene component followed a similar trend to that of PLAGA during the matrix degradation. The blend matrices degraded much slower than pristine PLAGA over 12 weeks (Figure 6-4b). It was also observed that an increase in the ratio of polyphosphazene in the blend resulted in a slower degradation rate (Figure 6-4b,c). In summary, SEM images of the blend matrices during degradation in media revealed the formation of in situ 3D porous structure that is suitable for cell infiltration and tissue ingrowth (Figure 6-3). The SEM images also showed that the spheres were imbued with micropores and nanopores.
Figure 6-3. Surface morphologies of the blend matrices incubated in aqueous media at 37°C over 12 weeks as a function of time. Top row: SEM images showing surface morphologies of Matrix1 following 0, 4, 7, and 12 weeks of *in vitro* degradation. The insets of (e) and (g) show the detailed 3D spherical structures. Bottom row: SEM images showing surface morphologies of Matrix2 following 0, 4, 7, and 12 weeks of *in vitro* degradation. The unique polymer erosion of the blend system resulted in the change of matrix morphology from a solid coherent film to an assemblage of microspheres with interconnected porous structures characterized by macropores (10-100 µm) between polyphosphazene spheres as well as micro/nanopores on the sphere surface.
Figure 6-4. *In vitro* degradation profiles of the blend matrices in pH 7.4 aqueous media at 37°C over 12 weeks: a) Percentage of PLAGA molecular weight remaining in the pristine PLAGA and blend matrices over 12 weeks; b) Percentage of mass remaining; c) Percentage of polyphosphazene molecular weight remaining in the blend matrices; d) pH change of degradation media with the pristine PLAGA and blend matrices. The blend matrices showed slower degradation rate than pristine PLAGA. The changes of molecular weight for both the PLAGA and polyphosphazene components in the matrix further confirmed that the two components degraded in a similar pattern.

One of the major advantages of the blend matrices resides in their self-neutralizing ability where the buffering degradation products of the polyphosphazene neutralize the acidic PLAGA hydrolysis products. The self-neutralizing ability of the blend matrices was assessed by recording the pH of the degradation media throughout the study. In general, the pH of the media with blend matrices was higher than that of pristine
PLAGA (Figure 6-4d). For example, the pH decreased dramatically to ~3.1 after 4 weeks for pristine PLAGA, which was due to the faster degradation of PLAGA to lactic acid and glycolic acid. On the other hand, the pH of degradation media was 14% and 22% higher with Matrix1 and Matrix2 than with PLAGA after 4 weeks of degradation, respectively. The increase in the pH of the degradation media with blends was maintained until the end of the hydrolysis study. This suggests that the acidity, which originated from the bulk degradation of PLAGA, was buffered by the phosphates and ammonia produced from the PPHOS backbone. This buffering effect further reduced the auto-catalyzed PLAGA hydrolysis. In anatomical defect sites where there is less body fluid, such as articular cartilage, the resultant low pH environment from the degradation of the polyester materials is detrimental to implant functions. With the use of blend materials, the resulting pH environment can be well tuned for better tissue regeneration by simply adjusting the blend composition. In addition, the changes of molecular weight for both the PLAGA and polyphosphazene components in the matrix during degradation further confirmed that the two components degraded in a similar pattern (Figure 6-4a, c). Initially, the acidic hydrolysis products from PLAGA accelerated the degradation of itself as well as PPHOS. As a consequence, the degradation of PPHOS neutralized the acidity and retarded the degradation of PLAGA.

6.3.4 Rat Subcutaneous Implantation Model

To examine their performance under physiological conditions, blend matrices with dimensions of 0.5 mm × 10 mm (T × D) were implanted in Sprague-Dawley rats
subcutaneously. PLAGA was used as the control due to its recognized biocompatibility. The morphology and porosity of the matrix was characterized and analyzed using a variety of techniques including SEM, microcomputed tomography (micro-CT), and histology staining. The hydrolytic degradation was quantified by measuring matrix mass loss and molecular weight change. The molecular weight for both the PLAGA and polyphosphazene components in the blend system was analyzed using GPC. PLAGA was completely degraded whereas the blend matrices were so infiltrated with collagen tissues that it is inappropriate to characterize the mass loss after 7 weeks of implantation. Both the blend matrices were well tolerated throughout the duration of the 12-week of subcutaneous implantation period. No acute inflammation, tissue necrosis, or abscess formation was observed around either polymer matrices.

Blend matrices were obtained at 2, 4, 7, 10, and 12 weeks for characterizing morphological changes by SEM. As shown in Figure 6-5, the blend system also resulted in the formation of 3D porous structures in vivo during polymer degradation. Specifically at 2 weeks post-implantation, spherical structures appeared on the surface of Matrix1 and Matrix2 (Figure 6-5a, b). Following 7 weeks of continued hydrolysis, more spherical structures were formed on the surface as evidenced in Figure 6-5c and d. At 12 weeks post-implantation, completely open 3D porous structures formed with well packed polymer spheres (Figure 6-5e, f). These in situ polymer spheres were less than 100 µm in diameter, which potentially generates high surface area for cell-material interactions. Furthermore, the size of in situ formed spheres increased significantly with the increase of polyphosphazene ratio in the blend system (Figure 6-5). In addition, SEM images
revealed the intricate pore structure with great surface area and 3D space within the polymer sphere (Figure 6-6).

![Figure 6-5](image)

**Figure 6-5.** Surface morphologies of blend matrices as a function of subcutaneous implantation time. Top row: SEM images showing surface morphologies of Matrix1 following 2, 7, and 12 weeks of implantation. Bottom row: SEM images showing surface morphologies of Matrix2 following 2, 7, and 12 weeks of implantation. The polymer spheres were less than 100 μm in diameter. Furthermore, the size of spheres increased significantly with the increase of polyphosphazene ratio in the blend system.
Figure 6-6. Representative SEM image of sphere cross-sections showing the intricate pore structure within the formed polymer spheres of Matrix2 after 10-week implantation. Such continuous porous structure within the polymer spheres provides additional surface area and space for promoting cell-material interactions.

The in vivo hydrolysis rate of the blend matrix was also examined by measuring the mass loss of polymer matrices. No residual PLAGA was found after 7 weeks of implantation, whereas Matrix1 and Matrix2 showed 75.10 ± 0.64% and 55.17 ± 4.14% mass loss, respectively (Figure 6-7a). Both the PLAGA and polyphosphazene components in the blend experienced a similar degradation pattern as characterized by the molecular weight loss (Figure 6-7b, c). The number and weight average molecular weights ($M_n$ and $M_w$) of both the PLAGA and polyphosphazene components decreased exponentially with degradation time throughout the implantation period. Furthermore, the apparent hydrolysis rate, $K$, can be obtained according to the following exponential
relationship between molecular weight and degradation time: \( \lg M = \lg M_0 - Kt \). For example, the apparent hydrolysis rates based on \( M_w \) were calculated to be 0.1014 and 0.0859 week\(^{-1} \) for the PPHOS component in Matrix1 and Matrix2, respectively. This indicates that PPHOS degraded faster in Matrix1 due to higher PLAGA composition. A similar trend in the apparent degradation rates was found for the PLAGA component in pristine PLAGA, Matrix1, and Matrix2. This suggests that the PLAGA degraded the slowest in Matrix2 due to the higher PPHOS composition. These results agree well with our \textit{in vitro} observations that the hydrolysis products from PPHOS retard the hydrolysis of PLAGA, whereas the PLAGA hydrolysis accelerated the PPHOS degradation.
Figure 6-7. *In vivo* degradation profiles of blend matrices and pristine PLAGA over 12 weeks: (a) Percentage of mass remaining; (b) Percentage of PLAGA molecular weight remaining in the pristine PLAGA and blend matrices during 12 weeks of implantation; (c) Percentage of polyphosphazene molecular weight remaining in the blend matrices during 12 weeks of implantation. The mass loss profiles during the implantation suggested the degradation rate to be PLAGA > Matrix1 > Matrix2. The molecular weight for both PLAGA and polyphosphazene components in the blend decreased in a similar trend throughout the 12-week implantation period.
Representative 2D micro-CT images in Figure 6-8a-h revealed the progression of morphological and structural changes within the blend system. Quantitative measurements of \textit{in-situ} porosity (\((\text{total volume-polymer volume})/\text{total volume}\)) of the matrix were performed based on the 3D micro-CT reconstructions during the polymer degradation (Figure 6-8i). During the first two weeks of implantation, only a small percentage of mass loss in the blends occurred and resulted in a porosity of \(\sim 14\%\) and \(12\%\) for Matrix1 and Matrix2, respectively (Figure 6-7a and 6-8a, b, i). At weeks 2 through 10, dramatic mass losses from fast degradation of both components led to a significant porosity increase to \(\sim 86\%\) and \(\sim 82\%\) for Matrix1 and Matrix2, respectively (Figure 6-7 and 6-8e, f, i). At weeks 10 through 12, the hydrolysis rate of the blends decreased dramatically and the porosity was maintained at \(\sim 87\%\) and \(82\%\) (Figure 6-7 and 6-8g, h, i). Since the PLAGA component served as a “dynamic” porogen, the interconnectivity of the resultant porous structures was \(100\%\) as indicated in the 2D micro-CT images. In addition, it was shown that the blend composition had no significant effect on the porosity of the blend matrices. Both blend matrices had a porosity of \(82-87\%\) after 12 weeks of implantation, which is very important in providing sufficient space for cell infiltration and tissue in-growth.
Figure 6-8. Micro-CT analysis for the blend matrices during in vivo implantation where a-h) Representative 2D micro-CT images illustrating the progression of morphological and structural changes within the blend system; i) In-situ porosity ((total volume-polymer volume)/total volume) of the matrix based on the 3D micro-CT reconstructions during the polymer degradation. Since the PLAGA component served as a “dynamic” porogen, the interconnectivity of the resultant porous structures was 100% as indicated in the 2D micro-CT images. In addition, it was shown that the blend composition had no significant effect on the porosity of the blend matrices. Both the blend matrices had a porosity of 82-87% after 12 weeks of implantation.
As seen from both hematoxylin and eosin (H&E) and Masson’s trichrome stain (TRI), implantation of the blend matrices also resulted in the formation of 3D porous structures in vivo (Figure 6-9). As indicated by the arrows, the porous structure formed during matrix degradation demonstrated a strong potential for directing and hosting tissue in-growth in vivo. A polymer sphere based porous structure was formed after 7 weeks of implantation (Figure 6-9a, b). These observations are in line with in vitro data discussed previously. Cellular infiltrates were found to fill in the void space between spheres as indicated by the arrows. Thereafter, the 3D porous structure continued to evolve to promote robust tissue in-growth. By 12 weeks, collagen tissues were guided to surround the polymeric microspheres and filled in the pore space between spheres (Figure 6-9c, d), which is consistent with the in vivo findings using sintered microsphere scaffolds for tissue repair.20-12
Figure 6-9. Histology images illustrating the formation of polymer spheres with pore system that is capable of accommodating cell infiltration and tissue in-growth within the blend matrices. (a,b, H&E): The arrows indicate the polymer sphere formation within Matrix1 and Matrix2 after 7 weeks of implantation, respectively; (c,d, H&E): The arrows indicate the polymer sphere formation within Matrix1 and Matrix2 after 12 weeks of implantation, respectively. The insets of c) and d) with TRI show robust collagen tissue infiltration within the matrix through the in situ formed pores after 12 weeks of implantation. It demonstrated that the in situ formed 3D interconnected porous structure enabled accommodation of cell infiltration and collagen tissue in-growth.

Matrix tissue compatibility was characterized by the extent of inflammation and fibrous capsule formation.\textsuperscript{19} Both the blend matrices were found to elicit minimal inflammation with improved biocompatibility than PLAGA during 12 weeks of implantation. A minimal inflammatory response indicated an apparent lack of local
toxicity, which could be due to the non-toxic and near-neutral pH hydrolysis products. It was also observed that an increase of polyphosphazene content in the blend resulted in both a reduced inflammatory response and fibrous capsule formation. Reduction in the fibrous capsule thickness is highly advantageous to improve mass transfer between the implants and surrounding tissues.

6.4 Discussion

One of the limitations for the use of biodegradable polymers as temporary substrates is the predictability of the degradation time to provide an appropriate physical, chemical, and biological function for a specific application. For a polymer blend, miscibility is required in generating a binary system with predictable degradability. For example, blending two polymers with different degradation kinetics result in a predictable degradation pattern only when they constitute a miscible system. In general, the miscibility of any two polymers is a result of strong intermolecular interactions such as hydrogen bonding, dipole-dipole interactions, and/or van der Waals forces. In a polyphosphazene-polyester blend, hydrogen bonding between the amino groups of polyphosphazenes and the ester groups of PLAGA is predominant over dipole-dipole and van der Waals forces of attraction. High-resolution solid-state $^{13}$C NMR confirmed the formation of miscible blends through the hydrogen bonding between the amide and amine protons in the dipeptide units of PPHOS and the carbonyl groups in PLAGA, which is in line with our observations through SEM, DSC, and FTIR. Furthermore, blending of PLAGA with PPHOS resulted in a single $T_g$ for Matrix1 and Matrix2
~35.9°C and 40.3°C, respectively. For a miscible blend system, the $T_g$ of the blend can be predicted by the Wood equation:\[T_g = w_1 T_{g1} + w_2 T_{g2}\] where $w_i$ and $T_{gi}$ are the weight fraction and the $T_g$ of polymer i (1 and 2 designate PPHOS and PLAGA, respectively). The glass transition temperatures estimated using the Wood equation for Matrix1 and Matrix2 are 34.1°C and 40.4°C, respectively, which are very close to the experimental values from DSC thermograms.

Polymer erosion is highly desirable in regenerative medicine because it eliminates the need for the surgical removal of implants after healing is complete. Polymer erosion results from the polymer hydrolysis. The process involved in the erosion of a degradable polymer generally include several phases: 1) hydration with or without swelling, 2) oligomers and monomers generation by water intrusion, 3) progressive degradation, and 4) oligomers and monomers release leading to mass loss. There are several important factors that affect the polymer degradation rate such as type of chemical bond, molecular weight, hydrophobicity, environmental conditions (pH, \textit{in vitro}, and \textit{in vivo}, etc.), and copolymer composition. PLAGA is known to degrade by simple hydrolysis of the ester bonds into lactic and glycolic acids.

In this study, the degradation of the PLAGA-PPHOS blends was investigated both in aqueous media (pH=7.4) as well as under physiological conditions via a rat subcutaneous implantation over 12 weeks. The rationales for using aqueous media over phosphate buffered saline were: 1) phosphate containing buffer solutions would interfere with the $^{31}$P NMR spectrum of the polyphosphazene hydrolysis products; 2) whether the PPHOS hydrolysis can neutralize the acidic pH resulted from the hydrolysis of PLAGA. For example, it was found that the pH of the degradation media decreased from 7.4 to 3.1
after 4 weeks of degradation due to the rapid hydrolysis of the PLAGA alone, whereas the pH of the degradation media with Matrix2 was 3.8. The decrease in pH also accelerated the polyester hydrolysis into carboxylic acids through catalysis. The polyphosphazene component in the blend also showed a similar molecular weight decline, which indicates that the polyphosphazene hydrolyzed to buffer the medium to some extent. The pH of the aqueous media with blends decreased dramatically during the first four weeks which is a consequence of rapid acidic hydrolysis of the PLAGA component (Figure 6-4a, d). It further declined slightly through week 7, by which the majority of PLAGA had completely degraded. Thereafter, the pH was maintained until the study was concluded at week 12. The acid neutralization of degradation media differs from our previous degradation studies with polyphosphazene-PLAGA blends in which a significant buffering effect was detected. As evidenced from Figure 6-4, the polyphosphazene component underwent a ~93% decrease in original molecular weight during 12 weeks of degradation. The number of polymer chains degraded was much less than that for PLAGA component due to the 23-fold difference in original molecular weight. Furthermore, there are still ~15% and 26% of mass remaining for Matrix1 and Matrix2 which were primarily composed of polyphosphazenes as confirmed by energy dispersive x-ray spectroscopy and solid-state $^{31}$P NMR. Therefore, the degradation byproducts of PPHOS were not enough to compensate the acidic hydrolysis of PLAGA. The slower degradation of PPHOS is also partially due to the steric effects from the bulky phenylphenoxy group that hinders water intrusion.
The erosion pattern of a polymer matrix in vivo is an important measure for new materials to be used in tissue engineering applications due to the additional external factors (such as enzymes, phagocytes, lymphocytes and fibroblasts) in the physiological environment. A rat subcutaneous implantation model was used in the present study in order to characterize the degradation kinetics of the blend matrix under physiological conditions. The mass loss profiles during the implantation suggested the degradation rate to be PLAGA > Matrix1 > Matrix2. The molecular weight for both PLAGA and polyphosphazene components in the blend decreased in a similar trend throughout the 12-week implantation period, which is in line with in vitro findings. These observations are consistent with literature reports for degradation of PLAGA and PLAGA-polyphosphazene blends in vivo. In addition, a minimal inflammatory response characterized by the presence of a few neutrophils, erythrocytes, and lymphocytes was found for both blend matrices. This suggests an apparent absence of local toxicity that demonstrates the non-toxic nature of blend hydrolysis products. Furthermore, the fibrous capsules surrounding the blend matrices were much thinner than with PLAGA and other biocompatible polymers. For example, the thickness of the fibrous capsules surrounding the widely used polyesters has been reported to be more than a few hundred microns. In addition, Matrix2 produced less inflammatory reactions with thinner fibrous capsules than Matrix1, indicating the tunability in blend biocompatibility by changing the blend composition. Most interestingly, both blend matrices were robustly infiltrated with collagen tissue after 12 weeks of implantation. This demonstrates that the in situ formed porous structures are capable of accommodating cell infiltration and tissue in-growth.
A widely used method to tune the polymer hydrolysis rate is to introduce a second monomer into the polymer chain. For example, the degradation rate of poly(lactide-co-glycolide) depends on the copolymer composition. The molecular weight loss during hydrolysis is accelerated with an increase in glycolide content. This is attributed to greater absorption of water into the polymer matrix. Therefore, the significant difference in degradation rates between Matrix1 and Matrix2 suggests that the degradation rate of the blend matrix can be effectively controlled by changing blend composition. For example, slower degradation rate can be obtained both in vitro and in vivo by increasing the polyphosphazene composition. In addition, the increase of hydrophobicity indicated by the significant increase of contact angle of the polymer system was also attributed to the slower degradation rate.

The absence of an interconnected porous structure during the bulk or surface erosion of other polymers often necessitates prefabrication of the polymers into three-dimensional (3D) structures with interconnected pores for tissue engineering applications. A variety of matrix fabrication techniques including particulate leaching, gas foaming, phase separation, and sphere sintering that have been developed to produce 3D porous matrices for skeletal tissue regeneration during the past two decades. It has been demonstrated that a porosity of ~90% is highly desirable for an ideal scaffold by providing sufficient space for extracellular matrix (ECM) synthesis, a high surface area for cell–material interactions, and minimal diffusion constraints. There have also been quite a few reports on the diffusion and cell migration limitations of the closed pore system resulting from the prefabrication processes. Therefore, porosity and interconnectivity are key factors in the success of a matrix for promoting tissue in-growth
and integration [Hulbert et al., 1970]. In addition, the optimal pore diameters for neoangiogenesis, osteoid, and mineralized bone formation have been reported to be in the range of 5-350 µm.\textsuperscript{23} Interestingly, the hydrolysis of the dipeptide-based polyphosphazene-PLGA blends resulted in the formation of 3D porous structures with 100\% interconnectivity and with pore sizes less than a hundred microns. The morphology of such \textit{in situ} formed porous structures resembled our sintered microsphere matrix, which is a biomimetic 3D pore system resulted from fused polymer microspheres.\textsuperscript{24} In particular, a sintered microsphere matrix from PLAGA has attracted significant interest as a load-bearing scaffold for orthopedic tissue engineering. The structure of a sintered microsphere matrix has been shown to be able to support the attachment and proliferation of human osteoblasts \textit{in vitro}.\textsuperscript{25} However, for large porous structures the use of a bioreactor might be essential for complete cell infiltration, because the preformed structures present nutrient and oxygen diffusion limitations. \textit{In vivo} studies in New Zealand White rabbits further demonstrated that the sintered matrix structure showed formation of bone at the implant-bone interface with penetration of new bone into the matrix by 6 weeks.\textsuperscript{20} A 12-week of blend degradation \textit{in vivo} resulted in a porosity of 82-87\%, which is structurally similar to cancellous bone. Furthermore, the sphere size increases with the increase of polyphosphazene composition in the blend, whereas no significant changes to porosity were observed. This offers an advantage for maintaining a highly porous structure while fine-tuning the pore system for facilitating cell infiltration and tissue in-growth.\textsuperscript{26} For example, it has been verified by image analysis that the average pore size increases with the sphere diameter. This is consistent with our earlier observations for the sintered microsphere matrix.\textsuperscript{24} In addition, the intricate porous
structure within the polymer spheres provides additional surface area and space for promoting cell-material interactions. In the blend matrix, the fast degradation of PLAGA facilitated pore formation while the polyphosphazene microspheres maintained the matrix structural integrity during the tissue in-growth. Therefore, this unique in situ porous structure has significant potential for cell in-growth and tissue regeneration. By contrast, the porosity and structural integrity of prefabricated porous matrices are often compromised during the course of polymer degradation which limits cell infiltration and tissue in-growth.

The underlying mechanism for the unique erosion process of such a polymer system into spheres and interconnected porous structure is still under investigation. Several possible mechanisms have been proposed including phase separation, hydrogen bonding, and hydrophobic interactions. Phase separation that occurs during blend hydrolysis may be the result of combined water ingress and hydrolysis of PLAGA, which disrupts hydrogen bonding between the two polymers. Microspheres of the polyphosphazene would be formed from the relatively intact polyphosphazene molecules due to the coalescence of hydrophobic side groups. Whether the microspheres are formed by random aggregation of the polyphosphazene molecules or from nanospherical nucleating sites in the original blend is not yet established. Nevertheless, this represents a novel hydrolysis sequence when hydrogen bonding and hydrophobic entities are present. We believe that the dynamic pore formation process accompanying the matrix erosion will significantly enhance cell infiltration and tissue in-growth compared to the prefabricated matrices. In contrast to the compromised porosity and structural integrity observed for 3D prefabricated matrices during polymer degradation, the blend gradually
evolved into a 3D porous structure with interconnected pores during degradation which enables accommodation of the gradual cell infiltration and tissue in-growth. In addition, the blend matrices have the ability to self-neutralize to achieve a near neutral-pH environment with a multi-phase degradation pattern. The blend properties such as physico-chemical properties and *in vitro* and *in vivo* biocompatibility can also be well tuned by simply varying the blend composition (data not shown). The results presented in this study suggest that the blend matrices hold the promise of creating a new paradigm in scaffold-based tissue regeneration.

### 6.5 Conclusions

We have synthesized a unique polyphosphazene polymer with strong hydrogen bonding ability and slow degradation rate by co-substituting the polymer backbone with both glycylglycine dipeptide ester and hydrophobic phenylphenoxy groups. The co-polymer formed a completely miscible blend system with the polyester. Most importantly, the blends showed a unique polymer erosion process *in situ* in which the solid blend film degraded to form a 3D porous structure with interconnected pores. Specifically, a 12-week of degradation *in vivo* resulted in a 3D porous structure with 82-87% porosity and with 100% interconnectivity. It was observed that this *in situ* formed 3D interconnected porous structure enabled accommodation of cell infiltration and collagen tissue in-growth. This is the first time a biodegradable and biocompatible synthetic material has been developed which has the unique property of forming a macro-porous spherical structure during degradation. It will help us to develop solid matrices
with very high initial mechanical properties that will allow tissue in growth and integration as the matrix degrades. Further study is currently underway to further fine-tune the properties of dipeptide-based polymers with other side groups that could further enhance the mechanical properties of the polymers and evaluate the efficacy of these polymers as a drug delivery vehicle and tissue engineering scaffold for orthopedic applications. Attempts are also ongoing to apply this phenomenon to other polymer blends.

6.6 Acknowledgements

This work was supported by NIH RO1 EB004051 and NSF EFRI-0736002. Dr. Laurencin was the recipient of Presidential Faculty Fellow Award from the National Science Foundation. The authors thank Justin L. Brown from Department of Biomedical Engineering at the University of Virginia and Yusuf M. Khan from Department of Orthopedic Surgery at the University of Connecticut for valuable discussions in preparation of the manuscript. Supporting Information is available online from Wiley InterScience or from the author.
6.7 References


Chapter 7

Phase Changes of Poly(alkoxyphosphazenes), and their Behavior in the Presence of Oligoisobutylene

7.1 Introduction

Polyphosphazenes are hybrid inorganic-organic macromolecules in which the properties are determined by both the inorganic phosphorus-nitrogen backbone and the organic side groups. The backbone imparts properties such as fire resistance, thermo-oxidative stability, macromolecular flexibility, and low glass transition temperatures, while the side groups control solubility and many other properties including an influence on overall molecular flexibility. Several hundred different polyphosphazenes are now known, each with a combination of different properties and solubilities. The blend-type interactions of polyphosphazenes with classical polymers that provide opportunities for polymer-polymer coordination such as polyesters and polyethers have been studied as part of efforts to integrate the sometimes unique properties of polyphosphazenes with those of classical organic polymers. However, relatively little has been reported about the interactions of polyphosphazenes with low- or medium molecular weight hydrocarbon polymers or oligomers that are widely used in the field of lubricants and sealants.

Oligo- and poly-isobutylene (1) are important liquids, gums, and elastomers in technology. They also have a number of structural and physical properties that are similar to several alkoxy-substituted linear polyphosphazenes (2) (Figure 7-1). For example, they are all amorphous macromolecules with liquid-, gum-, or elastomer-like morphology, and with low glass transition temperatures. They are characterized by unusual high chain flexibility due to the low barrier to torsion of the backbone bonds. In the case of alkoxy- and alkyl ether-substituted
polyphosphazenes, this chain flexibility is accentuated by the high torsional mobility of short chain alkoxy or alkyl ether side chains. The solubility of one polymer in another or of a polymer in an oligomeric liquid is favored by enthalpy-lowering interactions between the structural components of the two macromolecules. However, given the well-known entropy-driven disincentives for mixing that exist when two high polymers are brought together, the overall properties of multi-component systems depend on the ability of polymers with similar structures and similar characteristics to form homogeneous mixtures.

![Polymer Structures](image)

**Figure 7-1.** Structural similarity between poly(isobutylene) and polyphosphazenes.

Polyolefins such as polyisobutylene, polypropylene, polybutylene, polyethylene, and polybutadiene form blends with related classical organic polymers. This blending effectiveness has been studied using techniques such as NMR, differential scanning calorimetry (DSC), small angle neutron scattering (SANS), wide angle x-ray scattering (WAXS), melt rheological measurements, and dynamic mechanical thermal analysis. These techniques are used to determine the extent to which polymer mixtures form miscible, compatible, or immiscible polymer systems. In the case of DSC experiments, the following criteria are employed to determine the character of a polymer mixture: 1) miscible blends are characterized by a single glass transition ($T_g$), and are the result of the two polymers interacting at
the microscale level; 2) compatible blends cause shifts of both parent Tg’s as a result of weak polymer-polymer interactions; and 3) immiscible blends are identified by no shift in the Tg’s of the parent polymers. Most polymer blends are favored by low molecular weights that reduce the energy required to overcome entropy with the mixing of two macromolecules. Low molecular weight polymers or oligomers can act as a solvent for another polymer to form miscible blends and may also be characterized using the above criteria. For example, low molecular weight polyisobutylene is a good solvent for polyethylene.

With these facts in mind, it was of interest to examine the ability of low or medium molecular weight oligoisobutylene to serve as a solvent for and a blend component with polyphosphazenes. Interest in such systems arises from the fire-resistance of some polyphosphazenes, and the low Tg and cation-coordination ability of others, quite apart from the fundamental scientific issues involved.

In this work, polyphosphazenes were synthesized with side groups such as propoxy, pentoxy, hexoxy, octoxy, isostearylxy, and 2-(2-methoxyethoxy)ethoxy (MEE). The lengths of the alkyl side chains were varied to determine the influence of this feature on the solubility and miscibility of the polyphosphazenes in low molecular weight polyisobutylene. Concurrently, these same alkoxy side groups were combined with co-substituent MEE side groups to form mixed-substituent polyphosphazenes. The solubilities of the polyphosphazenes in oligoisobutylene were determined at room temperature (25 °C) and at elevated temperature (80 °C). After cooling, these solutions/blends were then examined by differential scanning calorimetry (DSC) to monitor any changes in Tg. The polyphosphazenes were also studied by means of thermo-gravimetric analysis (TGA) to estimate their potential thermal stability in mixed polymer/oligomer systems.
7.2 Experimental

7.2.1 Reagents and Equipment

Polymer synthesis reactions were carried out under a dry argon atmosphere using standard Schlenk line techniques. Tetrahydrofuran (EMD) was dried using solvent purification columns. Propanol, pentanol, hexanol, octanol (Sigma-Aldrich), and 2-(2-methoxyethoxy)ethanol (Acros) were dried over calcium hydride, distilled, and stored over molecular sieves. Isostearyl alcohol, oligoisobutylene (Mₓ=880g/mol, PDI=1.02) (Penreco), and a 60% sodium hydride dispersion in mineral oil (Aldrich) were used as received. Poly(dichlorophosphazene) was prepared via the thermal ring-opening polymerization of recrystallized and sublimed hexachlorocyclotriphosphazene (Fushimi Chemical Co., Japan) in evacuated Pyrex tubes at 250°C. This was then used as a reactive macromolecular intermediate for reactions with sodium alkoxides. ³¹P and ¹H NMR spectra were obtained with a Bruker 360 WM instrument operated at 145 MHz 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 (GPC) 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 calibrated with polystyrene standards. Thermal degradation estimates were obtained using a Perkin Elmer TGA7 Thermogravametric Analyzer with Perkin Elmer TAC 7/DX Thermalanalysis Controller with a heating rate of 10°C/min.
7.2.2 Synthesis of poly(alkoxyphosphazenes) (Polymers 3-6)

Polymers 3-6 were synthesized in a similar manner following previous synthetic procedures\textsuperscript{13-14}. The resulting polymers were characterized by \textsuperscript{31}P and \textsuperscript{1}H NMR spectroscopy to confirm full replacement of the chlorine atoms. The final yields were 80-90\% based on the reactive intermediate poly(dichlorophosphazene).

7.2.3 Synthesis of poly(diisostearyloxyphosphazene) (Polymer 7)

Polymer 7 was synthesized using the following procedure. Poly(dichlorophosphazene) (2.00g, 17.3mmol) was dissolved in dry THF (200mL). Isostearyl alcohol (18.67g, 69.0mmol) was treated with 60\% NaH (2.08g, 51.9mmol) suspended in THF (50mL). The alkoxide solution was then added to the poly(dichlorophosphazene) solution, and the mixture was stirred under reflux for 48hours. It was then concentrated via rotary evaporation and was precipitated into methanol. The product was further purified by precipitation twice from THF into ethanol and water. Final structural characterization was by \textsuperscript{31}P and \textsuperscript{1}H NMR spectroscopy. The overall yield was 85\%.

7.2.4 Synthesis of poly[bis(2-(2-methoxyethoxy)ethoxy)phosphazene] (Polymer 8) (MEEP)

Polymer 8 was prepared by a method described previously\textsuperscript{14-16}.
7.2.5 Synthesis of poly((alkoxy)$_1$(2-(methoxyethoxy)ethoxy)$_1$phosphazenes) (Polymers 9-13)

Polymers 9-13 were synthesized by similar procedures. The synthesis of polymer 9 is described as an example. Poly(dichlorophosphazene) (5.00 g, 43.1 mmol) was dissolved in dry THF (500 mL). 2-(methoxyethoxy)ethanol (6.21 g, 51.7 mmol) was treated with 60% NaH (1.72 g, 43.1 mmol) suspended in THF (150 mL). This solution was added drop wise to a dilute solution of poly(dichlorophosphazene) over 1 hour. Propanol (3.62 g, 60.3 mmol) was added to 60% NaH (2.07 g, 51.7 mmol) suspended in THF (150 mL). The sodium propoxide solution was added to the polymer mixture, which was stirred for 24 hours under reflux. The final polymer solution was concentrated via rotary evaporation and was precipitated into methanol. The product was further purified by precipitation twice from THF into ethanol and then from THF into water. The final product purity was confirmed by $^{31}$P and $^1$H NMR spectroscopy. The overall yields were 75-80%.

7.2.6 Solubility testing

The solubility of the polyphosphazenes in oligoisobutylene was determined using the following method. Oligoisobutylene (25g) was placed in a mechanical mixer at 25 °C. The polyphosphazene (1 wt%) was added and the mixture was stirred. Following complete dissolution another 1 wt% polyphosphazene was added until the solubility limit was reached at room temperature. After the limit of room temperature solubility was reached, the polymer solution was then heated to 80 °C and solubility testing resumed until the solubility limit was reached at 80 °C.
7.3 Results and Discussion

7.3.1 Synthesis of polyphosphazenes

Two variants of the polyphosphazene side group structural arrangement were examined. First, polymers 3-7 are single-substituent species with increasing lengths of the hydrocarbon component in the alkoxy side chains, culminating with the isostearyloxy derivative 7. Polymers 3-6 have been described in previous reports \(^{13}\). Polymer 7 is new. Table 7-1 shows the \(^{31}\)P and \(^{1}\)H NMR shifts and thermal transitions of these polymers. Polymer 7 is a pale yellow elastomer (\(T_g \approx 73^\circ C\)) that is soluble in THF.

Second, a series of mixed-substituent polyphosphazenes, 9-13, were prepared, all of which contained the methoxyethoxyethoxy (MEE) side group together with the same alkoxy groups utilized in the single-substituent macromolecules. The syntheses of 9-13 are illustrated in Figure 7-2. All five mixed-substituent polymers were obtained by the reactions of poly(dichlorophosphazene) with the appropriate alkoxide and etheric nucleophiles. Random substituent distributions were favored by the initial addition of 2-(2-methoxyethoxy)ethoxide drop-wise to a dilute, stirred solution of poly(dichlorophosphazene) over a 1 hour period, followed by the addition of the second alkoxide to the polymer solution. The molecular structures were confirmed by \(^{31}\)P and \(^{1}\)H NMR spectroscopy. All these mixed-substituent polymers are pale yellow gums that are soluble in THF.
Table 7-1. Characterization Data for Polymers 3-13, OIB and PIB.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>$^{31}$P NMR (ppm)</th>
<th>$^1$H NMR (ppm)</th>
<th>$T_g$ ($^\circ$C)</th>
<th>Molecular Weighta (g/mol)</th>
<th>PDIb</th>
<th>Solubility in OIB at 80$^\circ$C (wt/wt)%</th>
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</thead>
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<tr>
<td>1 (OIB)</td>
<td>-----</td>
<td>0.9(6H), 1.1(2H)</td>
<td>-87</td>
<td>880</td>
<td>1.02</td>
<td>------------</td>
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<tr>
<td>2 (PIB)</td>
<td>-----</td>
<td>0.9(6H), 1.1(2H)</td>
<td>-65</td>
<td>890000</td>
<td>1.33</td>
<td>------------</td>
</tr>
<tr>
<td>3</td>
<td>-7.3</td>
<td>0.9 (6H), 1.6(4H), 3.9(4H)</td>
<td>-99</td>
<td>710000</td>
<td>2.40</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>-5.4</td>
<td>0.9(10H), 1.2(4H), 1.6(4H), 3.9(4H)</td>
<td>-102</td>
<td>530000</td>
<td>1.75</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>-5.3</td>
<td>0.9(6H), 1.3(12H), 1.6(4H), 3.9(4H)</td>
<td>-104</td>
<td>1347000</td>
<td>1.83</td>
<td>9</td>
</tr>
<tr>
<td>6</td>
<td>-7.0</td>
<td>1.1(6H), 1.5(20H), 1.9(4H), 4.1(4H)</td>
<td>-104</td>
<td>2030000</td>
<td>1.95</td>
<td>11</td>
</tr>
<tr>
<td>7</td>
<td>-11</td>
<td>3.2(5H), 3.4(3H), 3.5(4H), 3.6(2H), 4.0(2H), 4.2(2H)</td>
<td>-67</td>
<td>2970000</td>
<td>1.86</td>
<td>3</td>
</tr>
<tr>
<td>8 (MEEP)</td>
<td>-7.8</td>
<td>3.3(3H), 3.5(2H), 3.62(2H), 3.66(2H), 4.0(2H)</td>
<td>-80</td>
<td>1132000</td>
<td>1.98</td>
<td>------------</td>
</tr>
<tr>
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<td>-5.7</td>
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<td>-74</td>
<td>131000</td>
<td>1.83</td>
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<tr>
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<td>-65</td>
<td>217000</td>
<td>1.82</td>
<td>5</td>
</tr>
<tr>
<td>13</td>
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<td>1.1(9H), 1.6(24H), 1.9(3H), 3.5(4H), 3.8(4H), 4.1(2H)</td>
<td>-66 / -80</td>
<td>1779000</td>
<td>1.93</td>
<td>21 (16 @ RT)</td>
</tr>
</tbody>
</table>

*aThe molecular weights approximate to $M_w$. *PDI was determined by $M_w / M_n$. 
Figure 7-2. General synthesis of polydichlorophosphazene and subsequent substitution with alkoxide (OR). Chemical structures of polymers 3-13.
7.3.2 Glass transition temperatures

DSC analysis was used to identify second order transitions of all ten polyphosphazenes, oligoisobutylene, and polyisobutylene. The values are shown in Table 7-1. All the polymers except 13 had a single glass transition temperature (T<sub>g</sub>) in the range of -65 to -104 °C, and only one 31P NMR signal that indicated random distribution of the different side groups in the mixed-substituent polymers. By any standard these indicate a high order of molecular flexibility, similar in many ways to poly(dimethylsiloxane) or polyisobutylene. However, polymer 13 had two T<sub>g</sub>'s, at -66 °C and -80 °C, even though it gave only a single 31P NMR signal. Possible reasons for this dual glass transition temperature are discussed below.

The thermal transition behavior of polymer 13 is unusual for polyphosphazenes. Polymer 13 in a solution of deuterated tetrahydrofuran gives a single 31P NMR signal at -5.3 ppm, which suggests that nearly every phosphorus atom bears one isostearyloxy and one 2-(2-methoxyethoxy)ethoxy side group. In other words, there were no 31P signals detected that would be consistent with the presence of two side groups of the same type on a given phosphorus atom or of a blocky structure. Because 31P NMR spectroscopy is sensitive only down to 5% concentrations of a given species, this suggests that at least 95% of the repeating units have a randomized side group disposition. This structure should generate a polymer that has a single glass transition (T<sub>g</sub>) in a DSC experiment. However, this is not the case as seen in Figure 7-3. Polymer 13 has two second order transition temperatures, at -66 °C and -80 °C. Two separate transitions suggest the existence of a blocky structure with a different T<sub>g</sub> for each block, the existence of a two-domain morphology, or the onset of two different types of coordinated side group motions. Polymer 13 is not a block copolymer as indicated by the single 31P NMR signal.
The possibility of micro- or nano-scale domain segregation cannot be discounted. However, the marked differences in hydrophobicity of the two different types of side groups suggest that the side groups, though randomized, are organized in an unusual manner. Thus, isostearyloxy groups are associated with their counterparts, as are the alkyl ether side groups, and the cross combination interactions are minimized. A possible orientation is illustrated in Figure 7-4. Each individual domain would affect the backbone mobility in a different way, thus causing two second order transitions to appear in the DSC trace (Figure 7-3).

**Figure 7-3.** DSC traces of poly(di(2-(2-methoxyethoxy)ethoxy)phosphazene), polymer 12, and polymer 13.
Figure 7-4. One possible orientation of the side group structure in polymer 13.
7.3.3 Solubility and Miscibility of Polymers 3-13 in Oligoisobutylene (OIB)

The solubilities of polymers 3-13 in oligoisobutylene depend on the side groups linked to the polyphosphazene chains. The presence of alkoxy groups with the longer hydrocarbon chains allows solvation type interactions with oligoisobutylene due to the similar polarities of the different constituents. It also leads to chain entanglements between OIB and the polyphosphazene alkoxy side groups. Conversely, MEEP (8) is insoluble in OIB probably due to the dissimilar polarities. However, when both long carbon chain alkoxy and 2-(2-methoxyethoxy)ethoxy side groups are present (9-13) the solubility increases.

The solubilities of the single-substituent poly(dialkoxyphosphazenes) in OIB are summarized in Table 1. The solubility is low, but it increases to a small extent as the length of the hydrocarbon side chain is increased. For example, the solubility of poly(dipropoxyphosphazene) (3) is only 1 (wt/wt)% in OIB at 80 °C, but poly(dipent oxyphosphazene) (4) has a solubility of 5 (wt/wt)%, and so on. However, at some point a solubility threshold is reached and further increases in alkoxy chain length cause a plateau or a decrease in solubility. This is demonstrated by the behavior of poly[bis(isostearyloxy)phosphazene] (7), which has a solubility of only 3 (wt/wt)% in OIB. It is likely that, beyond a certain side group length, the side groups on the polyphosphazene have a greater affinity for their counterparts along the same chain or on adjacent phosphazene chains than with the OIB molecules. Moreover, increases in side group length in the polyphosphazene cause increased intramolecular side group entanglements, thus making it sterically harder for solvation by oligoisobutylene to occur.

The solubility of the mixed-substituent poly[(alkoxy)\(_1\)(2-(2-methoxyethoxy)ethoxy)\(_1\) phosphazenes] (9-13) in OIB is also dependent on the length of the alkoxy cosubstituent group. Thus, cosubstituent polyphosphazenes with short alkoxy cosubstituents such as propoxy, pentoxy,
or hexoxy, together with the methoxyethoxyethoxy component, are completely insoluble in OIB, as shown in Table 7-1. Moreover, poly[bis(2-(2-methoxyethoxy)ethoxy)phosphazene] (MEEP) (8) is also completely insoluble in OIB.

However, the influence of the 2-(2-methoxyethoxy)ethoxy side groups on the insolubility of the co-substituted polymers in OIB is counteracted as the length of the alkoxy cosubstituent side group is increased. Thus, a slightly enhanced solubility in OIB (5 wt/wt % solubility at 80 °C) is achieved for the mixed-substituent polymers once the alkoxy cosubstituent side chain reaches the length of the octoxy group (Polymer 6).

By contrast, the solubility of the co-substituted polymer with both isostearyloxy and 2-(2-methoxyethoxy)ethoxy side units (Polymer 13) is much higher than that of any of the other macromolecules. Polymer 13 has a solubility in OIB of 16 (wt/wt) % at room temperature and 21 wt/wt% at 80 °C. The 2-(2-methoxyethoxy)ethoxy side groups may affect solubility in two ways: first, by reducing the intramolecular interactions between the isostearyl side chains, which are probably responsible for the insolubility of 7 in OIB; and second, by increasing the free volume in 9, which enhances the opportunities for intermolecular interactions between the isostearyl units and OIB.

The solubility studies were accompanied by differential scanning calorimetry experiments of the mixtures to examine the interactions between the different polyphosphazenes and OIB. Polyphosphazenes mixed with OIB rely on London dispersion forces and chain entanglements to form miscible systems. DSC studies were carried out with solutions of polymer 6 in OIB as a representative of the poly(dialkoxyphosphazene) series and of polymer 13 in OIB representing the poly[(alkoxy)₁(2-(2-methoxyethoxy)ethoxy)₁phosphazene] group. The DSC characterization of polymer 6 initially in 5wt/wt% OIB is shown in Figure 7-5. The separate glass transition temperatures (T_g) of both 6 and OIB were detected at -104 °C and -86 °C respectively. This indicates that, in spite of the solubility at room temperature or at 80 °C, the
system underwent phase segregation by the time the temperature had been reduced to -80 or -100 °C. This is consistent with the trend of the solubility being reduced as the temperature is lowered. It also reflects the low molecular weight of the oligoisobutylene since a high polymeric isobutylene would presumably have a lower tendency to phase separate, at least over a short time scale.

![Graph showing DSC traces](image)

**Figure 7-5.** DSC traces of polymer 6, oligoisobutylene, and a blend of polymer 6 with OIB.

The presence of 2-(2-(methoxyethoxy)ethoxy) side groups might be expected to yield immiscible blends with OIB due to the hydrophilic nature of the etheric side group. However, the DSC characterization of mixed-substituent polymer 13 dissolved in OIB (15 wt/wt%) suggests that the system does not completely phase-segregate at low temperatures. Figure 7-6 shows a shift of the two parent peaks of polymer 13 from -66 °C and -80 °C to one peak at -86 °C in the
mixture. This is accompanied by a negligible shift in the peak of pure OIB from -87 °C to -86 °C (Figure 7-6). These results suggest that, although the overall solubility of this polymer in OIB is reduced at the lowest temperatures, some interactions between the two components remain even at -90 °C. If this interpretation is correct, the 2-(2-(methoxyethoxy)ethoxy side group is probably responsible for the remaining interactions between the two components.

**Figure 7-6.** DSC traces of polymer 13, oligoisobutylene, and a blend of 13 with OIB.

Repulsion between the two types of side groups in 13 is believed to be the phenomenon that affects the solubility of 13 in OIB. As shown in Figure 7-7, polymer 13 may be enthalpy-driven to form segregated oriented domains. Thus the isostearloxy groups prefer to interact with other hydrophobic units rather than with 2-(2-methoxyethoxy)ethoxy units. The introduction of OIB may induce an entropy-driven rearrangement in molecular conformation. This might favor
the partial dissolution of 13 in OIB due to the increased interactions between the isostearyl units and OIB and, at the same time, cause the change in the second order transition temperatures detected experimentally. For comparison, a blend of 15 wt% mixed-substituent polymer 13 with high molecular weight polyisobutylene (PIB) 2 cast from THF resulted in an immiscible blend. Therefore, the interaction and dissolution phenomenon only occur when polymer 13 is dissolved by oligoisobutylene.

Figure 7-7. Possible interactions between 13 and oligoisobutylene that leads to increased solubility compared to 12.

7.3.4 Thermal Decomposition of Polymers 3-13 and OIB

Thermo-gravimetric analysis (TGA) was used to monitor the thermal behavior of polymers 3-13, OIB, and PIB. As shown in Figure 7-8 (a) and (b), the onset of 10wt% weight loss of poly(dialkoxyphosphazenes) and poly((alkoxy)₁(2-(2-methoxyethoxy)ethoxy)₁phosphazenes) occurs at least 100 °C higher than that for OIB and at the
same temperature as PIB. Polymer 13 has the highest 10 wt% loss (248°C), compared to the 10% weight loss of OIB at 123 °C and 10% weight loss of PIB at 269 °C. Mass spectrometric analysis of the products liberated from OIB at 123 °C or higher indicates that the principal volatile products are butylene (major) and propylene (minor). This suggests that OIB and PIB undergoes depolymerization, unlike the polyphosphazenes studied here which decompose semi-randomly at high temperatures. If these weight loss data truly reflect relative thermal stabilities, the results suggest that polymers 3-13 may be useful as additives to OIB for a number of applications such as lubricants or cooling oils for low to medium temperature applications.
Figure 7-8. Thermal characteristics of (a) poly(dialkoxyphosphazenes) polymers 3-6 and 12; (b) poly((alkoxy)\(_3\)(2-(2-methoxyethoxy)ethoxy)\(_2\)phosphazene) polymers 8-11 and 13. The thermal characteristics of oligoisobutylene is included in both (a) and (b).
7.4 Conclusions

One of the unusual characteristics of most of the polyphosphazenes described here is their low solubility in oligoisobutylene. Even the single-substituent polymer with octoxy side groups, a species with a highly aliphatic hydrocarbon-like side chain structure, has a solubility of only 11% at 80 °C. Moreover, an extension of the side chains to isostearyloxy actually reduces the solubility to less than 2%. The solubility of polymers with both hydrophilic methoxyethoxyethoxy and linear alkoxy side groups, as in polymer 10, increases with the length of the (hydrophobic) alkoxy side chain to give a maximum solubility of 21% in OIB, even though the presence of alkyl ether groups alone results in total insolubility. These results suggest that a great deal of the behavior of these polymers may be explained by the interactions within the side group system on each phosphazene chain rather than on their interactions with a second component such as oligoisobutylene. This possibility is reinforced by the appearance of two separate second order low temperature transitions for the polyphosphazene that bears randomly substituted methoxyethoxyethoxy and isostearyloxy side groups (13), a phenomenon that is ascribed to phase segregation brought about by the two different types of side groups undergoing preferential association with their counterparts rather than with the cosubstituents that have a different character.

7.5 Acknowledgement

This work was supported by Penreco Inc.
7.6 References


VITA

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