SYNTHESIS AND CHARACTERIZATION OF POLYPHOSPHAZENES FOR SURFACE AND BIOMEDICAL APPLICATIONS

A Thesis in
Chemistry
by
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ABSTRACT

The work presented in this thesis is based on the modification of surface and bulk properties of polyphosphazenes to form polymers with new and/or improved properties that are useful in advanced applications. Chapter 1 provides an introduction to this field and sketches the history and purpose of research in this area. Chapter 2 reviews the field of hydrophobic polyphosphazenes and their potential applications. Hydrophobic polymers play a crucial role in many biomedical and commercial applications. Hydrophobic polyphosphazenes offer opportunities for the tuning of surface properties that are not found for many conventional hydrophobic materials. Chapter 3 describes a study involving surface modification of a hydrophobic, fluorinated polyphosphazene to form a superhydrophobic surface. Superhydrophobic surfaces, with contact angle to water as high as 159°, were created by electrospinning polymer films of poly[bis(trifluoroethoxy)phosphazene]. The extremely high hydrophobicity of these films was a combined result of a highly fluorinated surface and the inherent surface roughness of an electrospun mat. Surface properties were analyzed by water contact angle measurements, X-ray photoelectron spectroscopy, atomic force microscopy and scanning electron microscopy. The development of these superhydrophobic surfaces constitutes a significant advancement for fluorinated polyphosphazenes. It not only offers great potential as biomaterials and membranes for separation purposes but also widens the scope of applicability of these polymers in fields like self-cleaning surfaces and protective clothing applications.
Chapter 4 discusses the development of biodegradable polyphosphazenes for bone tissue engineering application. This chapter reports on the design, synthesis, characterization and biological evaluation of L-alanine co-substituted polyphosphazenes. Polymer properties, such as, glass transition temperature, hydrolytic degradation, surface wettability, tensile strength and modulus of elasticity varied over a wide range following changes to the type of co-substituents on the polymer backbone, thus demonstrating the tunability of biodegradable polyphosphazenes. Chapter 5 deals with the processing of nanofiber and nanofiber composite scaffolds of poly[bis(ethyl alanato)phosphazene] by the process of electrospinning. The nanofiber scaffolds were characterized by scanning electron microscopy, profilometry and hydrolysis studies. These degradable nanofiber scaffolds are useful in biomedical applications such as tissue engineering and drug delivery.

Chapter 6 reports on the synthesis and characterization of tyrosine-functionalized polyphosphazenes. The physical and chemical properties of the polymers varied with the type of linkage between the tyrosine unit and phosphazene backbone. Poly[(ethyl glycinato) (ethyltyrosinato)phosphazenes] (linkage via the amino group of tyrosine) were found to be hydrolytically erodible. Poly[(n-propoxy) (tyrosinato)phosphazene] (linkage via the hydroxyl group of tyrosine) were hydrolytically stable, showed a pH-dependant solubility behavior and formed ionotropic gels. Thus, the tyrosine functionalized polyphosphazene system offers the opportunity to incorporate properties such as bioerosion or pH sensitive behavior into one material by structural variations at the molecular level and are useful in applications such as tissue engineering and controlled drug delivery.
Appendix a describes the development of low temperature setting polyphosphazene/hydroxyapatite composites, potentially useful as bone tissue engineering materials. These composites were characterized by various techniques such as XRD, SEM, solution chemistry and mechanical property evaluation. The in vivo biological response of the composites was tested in a unicortical rabbit model.

Appendix b reports on novel blends of hydrophobic, biodegradable polyphosphazene, poly[bis(ethyl alanato) phosphazene] and poly(lactic-co-glycolic)acid (LA: GA; 85:15), developed as candidates for bone tissue engineering applications. Blending of biodegradable polyphosphazenes with PLAGA was attempted in order to combine the beneficial features of PLAGA such as recognized biocompatibility and widespread applicability with the osteoconductivity, well tuned degradability as well as the buffering capacity of the degradation products of polyphosphazenes.
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PREFACE

Portions of this thesis have been adapted for publication. Chapter 2 was adapted for publication in *Polymer International* and was coauthored by H.R. Allcock and L. Steely. Chapter 3 has been adapted for publication in *Langmuir* and was coauthored by H.R. Allcock and L. Steely. Chapter 4 has been adapted for publication in *Biomacromolecules* and *Journal of Biomedical Materials Research* and was co-authored by H.R. Allcock, N Krogman, C. Laurencin, P. Brown, J. Sturgeon, S. Sethuramanan, L Nair, S El-Amin, R Ferrar and M. N. Nyugen. Chapter 6 has been adapted for publication in *Biomacromolecules* and was coauthored by H.R. Allcock, A. Ambrosio and W. Laredo. Portions of Appendix a have been adapted for publication in *Orthopedic Research Society 2006 proceedings* and submission to *Advanced Materials* and *Journal of Biomedical Materials Research* and is coauthored by H R Allcock, C. Laurencin, P. Brown, J. Sturgeon, S. Sethuramanan, L Nair, S El-Amin, Y Khan and M Kwon. Appendix b was adapted for publication in *Materials Research Society Proceedings, 2005* and was coauthored by H R Allcock, C. Laurencin, P. Brown, J Bender, S. Sethuramanan and L Nair.
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Chapter 1

Introduction

1.1 General Introduction to Polymers

The last century has seen a rapid growth in the field of polymers. Polymers are defined as macromolecules derived from many small molecules (known as monomers) linked together. Thus, polymers are high molecular weight materials often with hundreds to thousands of monomers linked together in one polymer chain. Natural polymers such as rubber and silk are the earliest examples of polymers used by mankind. However, even though polymers have been used for several hundred years, their real potential has been realized only in the last 60 to 70 years. The foundation for modern day polymer chemistry was laid out in 1920’s, when a German chemist, Hermann Staudinger, suggested for the first time that natural compounds such as rubber and cellulose were macromolecules made up of several thousand atoms [1]. Initially his observations were met with criticism in the scientific world. But through the works of several chemists, including Carothers, the macromolecular hypothesis was accepted by the 1930’s [2]. Since then, hundreds of synthetic polymers have been developed for a variety of applications. Even today, researchers are continuously working on developing new and improved polymers or finding new applications for the existing polymers.

Polymers have several useful properties and this had led to their use in many different applications. These materials are inherently light weight, cost effective and can
be easily processed into various shapes or fibers. Properties such as flexibility, high strength, chemical resistance, thermal resistance, thermal and electrical insulation etc. can be readily found in these materials. Figure 1-1 illustrates the structures of some common synthetic polymers. Polyethylene is a commodity polymer with a wide range of desirable properties including high strength, flexibility, good impact resistance, good resistance to solvents and chemicals and electrical insulation [3]. It is widely used for packaging purposes, construction applications, containers and as insulation for electrical wires. Polystyrene is a rigid plastic with good strength, electrical insulation, optical clarity, resistance to acids and bases and easy processability [3]. It is widely used in making disposable containers, toys, lighting and decoration applications, and sterilized medical items such as petri dishes and pipettes. Poly(methyl methacrylate) has excellent dimensional stability, high strength, very good weatherability and exceptional optical clarity [3]. These properties make the polymer useful as lighting covers, signs, skylights, architectural structures, optical fibers, eyeglass lenses and contact lenses. Poly(ethylene terephthalate) has good mechanical strength and fatigue resistance at high temperatures and good resistance to solvents and chemicals [3]. It is widely used as a container for soft drinks, beers etc., automobile parts, domestic or office appliances and as fibers in various textile applications. Examples of other common synthetic polymers include polypropylene, poly(vinyl chloride), poly(acrylonitrile), polytetrafluoroethylene, nylon 6/6, polybutadiene and phenolic polymers.
Figure 1-1: Common synthetic polymers

Polyethylene

Poly(styrene)

Poly(methyl methacrylate)

Poly(ethylene terephthalate)
The polymers discussed so far are organic in nature with a carbon-based backbone structure. Most of these polymers are synthesized from monomers derived from the petrochemical industry. The low cost of production and ease of processability is one of the reasons for the commercial success of carbon based macromolecules. However, despite their overwhelming success, there are some drawbacks associated with these polymers. Most of the carbon based polymers are flammable and have low thermal and oxidative stability. This mainly arises due to their tendency towards free radical breakdown. Many of these polymers have low chemical stability towards hot organic liquids or lubricating oil. Also, organic polymer based elastomers cannot be used at very low or high temperatures as they lose their flexibility. These reasons have prompted researchers to look for alternative materials with improved properties.

Among the different materials that have been developed, inorganic polymers have shown the desired properties and are thus the focus of current research in this field. The inorganic component in the polymer adds thermal, chemical and oxidative stability and more backbone flexibility, thus overcoming the shortcomings of many organic polymers [4, 5, 6, 7]. Some of the more common examples of inorganic polymers are polysiloxanes (silicones), polysilanes, polycarbosilanes, polyphosphazenes, poly(sulfur nitride) and ferrocene containing polymers (Figure 1-2). These materials can combine the attributes of both organic and inorganic components or be completely inorganic in nature. For example, polysiloxanes, which are hybrid organic-inorganic polymers, have a backbone structure of alternating silicon and oxygen atoms with organic substituents attached to every silicon atom. On the other hand, poly(sulfur nitride), which is a completely inorganic polymer, consists of a backbone structure of alternating nitrogen and sulfur
atoms. Inorganic polymers offer a unique combination of properties not attainable by their organic counterparts. Some of their useful properties include high temperature flexibility, low temperature elastomeric properties, superconducting properties and biocompatibility.
Figure 1-2: Common inorganic polymers

Polysiloxanes

Polysilanes

Polycarbosilanes

Polyphosphazenes

Poly(sulfur nitride)

Polyferrocenylsilane
1.2 Polyphosphazenes

Polyphosphazenes are hybrid organic-inorganic polymers. These polymers are high molecular weight species with a backbone of alternating phosphorus and nitrogen atoms. Each phosphorus atom bears two substituents. A wide variety of side groups are available for property optimization. The general structure of polyphosphazenes is shown in Figure 1-2 where R can be alkoxy, aryloxy, or amino groups, or other units. The type of side group attached to the phosphazene backbone has a profound effect on the chemical and physical properties of the polymer such that a polymer can be tailored to generate a specific set of properties. Different types of polyphosphazenes have been developed as high performance commercial elastomers, electro-optical glasses, ion transport membranes, and biomedical materials [8].

1.2.1 History

The history of polyphosphazenes is over 170 years old. It began in 1834, when Liebig and Rose reported the formation of a white crystalline product from reacting phosphorus pentachloride with ammonia [8, 9, 10]. In the next 30 years, studies by Gerhardt, Gladstone, Holmes and Wichelhaus, helped to establish the empirical formula of this compound as NPCl$_2$ with a trimer structure [11, 12, 13]. In the 1890’s Stokes in the USA proposed a cyclic structure with the formula (NPCl$_2$)$_3$. Also, in 1897, Stokes provided the first report on polymeric phosphazenes where he studied the effect of heating (NPCl$_2$)$_3$ to high temperatures [14]. He observed that when (NPCl$_2$)$_3$ was heated above 250ºC it formed an insoluble elastomeric material which could be broken down
into smaller compounds at temperatures above 350°C. This material, also known as “inorganic rubber” was completely insoluble in organic solvents and thus could not be further developed. The next 78 years saw very little interest in chlorophosphazenes until the 1960’s, when Allcock, Kugel and Valan reported on the first successful synthesis of soluble polyphosphazenes [15, 16, 17]. The study showed that, with careful control of conditions such as temperature and time, soluble, uncrosslinked poly(dichlorophosphazene) can be obtained from the ring opening polymerization of \( \text{(NPCl}_2 \text{)}_3 \). Furthermore, the chlorine groups in poly(dichlorophosphazene) can be replaced by organic nucleophiles to form completely organo-substituted polymers. This work has laid the foundation of current day phosphazene chemistry and is still followed in various laboratories and industries, world wide.

1.2.2 Synthesis of Polyphosphazenes

The most widely accepted synthesis of polyphosphazenes is a two step reaction scheme. The first step involves the synthesis of a macromolecular intermediate, poly(dichlorophosphazene), and the second step involves replacing the chlorine atoms of poly(dichlorophosphazene) with organic nucleophiles. Poly(dichlorophosphazene) can be prepared through several routes, the most common of which is thermal ring opening polymerization of hexachlorocyclotriphosphazene. The main advantage of this route is that polymer can be produced in large quantities. Other routes include, living cationic polymerization, and Lewis acid catalyzed solution polymerization.
1.2.2.1 Synthesis of Chlorophosphazenes

1.2.2.1.1 Thermal Ring Opening Polymerization

Ring opening polymerization of hexachlorocyclotriphosphazene (also known as phosphazene trimer) is achieved by heating the molten compound at 250 °C, in an evacuated glass tube (Figure 1-3). The extent of reaction is followed by visual inspection of the viscosity of the molten material and the polymerization is terminated just before the viscous flow stops. The polymerization is terminated by cooling the reaction vessel to room temperature. This is usually done at about 70-75 % conversion, because crosslinking can occur rapidly beyond this point [8]. The unreacted trimer is removed from the polymer by vacuum sublimation. Important parameters which affect the efficiency of this process are time, temperature and purity of the trimer. If the reaction is allowed to proceed beyond the point when the viscous flow of the molten material stops, an insoluble, crosslinked polymer is obtained. Increasing the reaction temperature beyond 250 °C increases the rate of polymerization. However, since the ceiling temperature of poly(dichlorophosphazene) is around 350 °C, the reaction temperature should not exceed 300 °C. Also, the reaction temperature can be lowered to 210 °C by using lewis acid catalysts such as BCl$_3$.OP(OPh)$_3$ or AlCl$_3$ [8, 18]. The purity of the trimer is also an important factor as impure trimer can retard the polymerization rate or lead to crosslinked material.

The mechanism of thermal ring opening polymerization of the trimer is not well understood. The most widely accepted theory is based on a cationic chain growth propagation in which initiation occurs via ionization of P-Cl bond [19] (Figure 1-4).
Thermal ionization of P-Cl bond leads to the formation of a phosphazenium cation which can interact with a neighboring trimer molecule inducing ring cleavage and eventually leading to high molecular weight polymer. This theory is supported by several facts. Since free radical-producing compounds do not affect the rate of polymerization and electron spin resonance studies have shown the absence of free radical species in the system, the mechanism is not a free radical process. The ionic conductivity of molten trimer increases with temperature suggesting the presence of ionic species. The polymerization is catalyzed by Lewis acids such as BCl$_3$ and AlCl$_3$ which can assist in the ionization of P-Cl bond. And lastly, replacement of the chlorine atoms of trimer by organic groups has an effect on the polymerization conditions, presumably through its effect on the formation of the initiating species. Thus, increasing the strength of the phosphorus–halogen bond increases the temperature of polymerization and replacing the halogens with organic groups inhibits polymerization [8].

Even though the ring-opening polymerization route is currently the most widely accepted route, it suffers from some disadvantages. The polymerization conditions give poor control over molecular weight with polydispersity indices greater than 1.5. The reaction temperatures are high. It is impossible to obtain 100% conversion in an uncatalyzed reaction due to the occurrence of crosslinking reactions. And only linear or branched polymer architectures can be synthesized. To overcome these disadvantages several other polymerization techniques have been examined, the most promising of which is the ambient temperature solution polymerization.
Figure 1-3: Thermal ring opening polymerization of hexachlorocyclophosphazene
Figure 1-4: Mechanism of thermal ring opening polymerization of hexachlorocyclophosphazene [8]
1.2.2.1.2 Solution Polymerization of Phosphoranimines

Poly(dichlorophosphazene) can be prepared at ambient temperatures by the living cationic polymerization of a phosphoranimine [20, 21] (Figure 1-5). The reaction is catalyzed by PCl$_5$ and is usually carried out in an organic solvent such as methylene chloride. One molecule of phosphoranimine is initiated by 2 molecules of PCl$_5$ followed by cationic chain propagation until all the monomer in the system is consumed. There are several advantages for synthesizing poly(dichlorophosphazene) by this route compared to other available techniques. Polymerization can be carried out at room temperature. Since this is a living polymerization, a narrow polydispersity index (Mw/Mn close to 1) is obtained. The method allows good control over the polymer molecular weight. Block copolymers and architectures such as star, branched etc. are readily available through this route [22, 23, 24].
$\text{PCl}_5 + \text{Cl}_3\text{P}═\text{NSiMe}_3 \xrightarrow{\text{CH}_2\text{Cl}_2, 25^0\text{C}} \left[ \text{Cl}_3\text{P}═\text{N}−\text{PCl}_3 \right]^+ [\text{PCl}_6 ]^-$

$n \text{Cl}_3\text{P}═\text{N}−\text{SiMe}_3 \xrightarrow{\text{CH}_2\text{Cl}_2, 25^0\text{C}} \left[ \text{Cl}−\left(\text{P}═\text{N}\right)\text{PCl}_3 \right]_{n+1}^+ [\text{PCl}_6 ]^-$

Figure 1-5: Solution polymerization of phosphoranimines
1.2.2.2 Macromolecular Substitution of Poly(dichlorophosphazene)

The second step in the synthesis of polyphosphazenes is the macromolecular substitution of poly(dichlorophosphazene) (Figure 1-6). The P-Cl bonds in this polymer are highly reactive and can be replaced by a variety of nucleophiles, presumably through an $S_N2$ type mechanism [8, 15]. Substitution is carried out in solution and the reaction time and temperature can vary with the type of nucleophile used. The high reactivity of the P-Cl bonds ensures complete replacement of the backbone chlorine atoms with the nucleophile units. This can be confirmed by $^{31}$P NMR spectroscopy. Nucleophiles such as alkoxides, aryloxides, amines and organometallic reagents can be used in these reactions.

The macromolecular substitution approach towards the synthesis of polyphosphazenes has several advantages. The reaction temperatures almost never exceed 100 °C and thus polymers can be produced without side reactions such as decomposition or depolymerization. Model reactions can be carried out on a small molecule, hexachlorocyclotriphosphazene [(NPCl$_2$)$_3$], and the products are easier to characterize than a polymer. Another advantage is that single-substituent or mixed-substituent polymers can be readily synthesized. But, by far, the most attractive feature of macromolecular substitution is the synthetic versatility and tailoribility of this approach. A large number of polymers can be synthesized from this route due to a wide variety of alcohols, amines and organometallic reagents that can be used. Over 250 nucleophilic reagents have been used to form more than 700 different types of polyphosphazenes [8]. This type of synthetic versatility is not found in any other known polymer system.
Figure 1-6: Macromolecular substitution of poly(dichlorophosphazenes)
1.2.3 Structure-Property Relationships in Polyphosphazenes

A comprehensive list of structure-property relationships can be derived from the large number of different polyphosphazenes that have been synthesized so far. In general, the backbone structure and the type of side group determine the overall polymer properties. The inorganic –P=N- backbone structure confers properties such as flexibility, elasticity and stability to high temperatures and high energy radiation. The flexibility of the –P=N- backbone arises due to its low barrier to torsion which is less than 1 Kcal/mol [8, 25]. This gives rise to some of the lowest glass transition temperatures recorded for polymers, typically in the range of -90 °C to -100 °C. The stability of the backbone toward high temperatures and high energy radiation (gamma and X rays) arises due to a high bond energy (approximately 70 Kcal/mol) and resistance to homolytic cleavage to form free radicals [4, 26, 27]. This is a major advantage compared to organic polymers that have a tendency towards free-radical breakdown. Also, since the phosphorus atoms in the backbone are in their most stable oxidation state (pentavalent), this resists thermo-oxidative breakdown. The –P=N- backbone is transparent to radiation from the near infrared to the mid-UV at 220 nm, a property that can be used in a variety of optical applications [8]. Another interesting property that arises due to the –P=N- backbone is the hydrolytic instability of a few derivatives, which is a very useful property in applications such as biomedicine [31, 35]. With an appropriate choice of side groups, the polyphosphazene backbone is highly susceptible to hydrolytic breakdown, catalyzed by either acidic or basic media. The hydrolysis products of the backbone are phosphates and
ammonia which are non-toxic and form a near-neutral solution due to their buffering capacity [28].

In addition to the influence of the –P=N- backbone structure, the type of side group also has a tremendous affect on the overall polymer properties. This can be judged from the fact that over 250 different side groups have been used to form over 700 polymers, each with their own set of properties. The properties can range from elastomeric to highly rigid, infinitely stable to hydrolytically unstable, highly hydrophobic to highly hydrophilic, among other properties. The fluoroalkoxy and alkyloxy side groups form polymers with low glass transition temperature and elastomeric properties [15, 16, 29]. On the other hand, aryloxy side groups form polymers with high glass transition temperatures and possibly liquid crystalline properties [30]. Side groups such as fluoroalkoxy, alkyloxy and aryloxy form polymers with hydrolytic stability. On the other hand, side groups such as amino acid esters, glucosyl, glyceryl, glycolate, lactate and imidazole, sensitize the polymer backbone to hydrolysis [8, 31, 32, 33, 34, 35]. It is beyond the scope of this chapter to discuss the effect of each type of side group on the resulting polymer properties, but a few general examples are discussed in the next section with their potential applications. The reader is directed to a comprehensive book written on polyphosphazene chemistry to learn more about the effect of different side groups on polymer properties [8].
1.2.4 Applications of Polyphosphazenes

Figure 1-7 illustrates the structure of some representative polyphosphazenes. Polymer 1 is a fluorinated polyphosphazene, poly[bis(trifluoroethoxy)phosphazene]. The combination of fluorinated side groups with an inorganic backbone generates a number of interesting properties many of which are specific to fluorinated polyphosphazenes. For example, coherent films of 1 are highly hydrophobic, are resistant to many chemicals, are bioinert, have a high flame resistance, and have high radiation stability. They are also easy to fabricate into microfibers and films. In addition to this, mixed-substituent fluoroalkoxyphosphazene polymers are elastomers and have low temperature flexibility and a very wide range of physical properties [36]. Poly[bis(2,2,2-trifluoroethoxy)phosphazene] and its co-substituted analogues have been investigated in a number of potential applications such as membranes for gas transport, solvent pervaporation and ion separation; hemocompatible materials or substrates for enzyme immobilization; textile protective coatings; fire-resistant additives and various elastomeric applications [8, 37, 38].

Polymer 2 is an aryloxy polyphosphazene, poly[bis(phenoxy)phosphazene]. This polymer has a glass transition temperature of -8 °C and a melting transition at 390 °C [39]. Linkage of aryloxy units to the phosphazene backbone raises the glass transition temperature of the polymer as the aromatic groups restrict the conformational mobility of the –P=N- backbone. Polymer 2 has good fiber forming properties and high hydrophobicity. One of its main potential applications is as a fire-retardant. Halogenated
aryloxy polyphosphazene, such as polymer 3, have high refractive index and are thus important materials for optical applications [40].

Polymer 4 is an oligoethyleneoxy functionalized polyphosphazene, poly[bis(2-(2-methoxy ethoxy ethoxy)phosphazene]. It has a very low glass transition temperature of -83 °C, a completely amorphous structure and a high concentration of etheric oxygens, making it an ideal candidate for solid polymer electrolyte applications [41, 42, 43]. Polymer 4 has shown a Li-ion conductivity of $10^{-5}$ S/cm. This number is three orders of magnitude higher than poly(ethylene oxide) which is one of the most widely investigated polymers for this application. Acid functionalized polyphosphazenes have been investigated as proton transfer membranes. Crosslinked membranes of polymer 5, a sulfonamide functionalized polyphosphazene, have shown proton conductivity as high as $10^{-2}$ S/cm, which is comparable with Nafion membranes, the current standard in fuel cell technology [44, 45].

Polymer 5 is a water-soluble polyphosphazene, poly[bis(sodium carboxylatophenoxy)phosphazene]. Microspheres of this polymer have been used to encapsulate cells, protein or drugs for tissue engineering and drug delivery applications. Langer et al. have reported on the successful encapsulation of proteins, liposomes and hybridoma cells within calcium crosslinked microspheres of polymer 5 [46, 47]. In addition, polymer 5 is known to have immunoadjuvant property for a range of bacterial and viral vaccine antigens and is currently under clinical trials [48]. Besides drug delivery applications, polyphosphazenes have been investigated in various other biomedical applications. The next section will discuss the advantages of using polyphosphazenes in biomedical applications with emphasis on their biodegradability.
Figure 1-7: Structures of representative polyphosphazenes
1.2.5 Polyphosphazenes for Biomedical Applications

The use of polymers in biomedicine is a rapidly growing field with researchers on a constant look out for materials that can satisfy the stringent requirements of a biomaterial such as appropriate physical and mechanical properties and biocompatibility. Biocompatibility is defined as ‘the ability of a material to perform with an appropriate response in a specific application’ such as resistance to blood clotting or lack of immunogenic response [49]. Polyphosphazenes which have shown great promise as biomaterials offer a number of advantages. First, the synthetic versatility of polyphosphazene allows fine-tuning of polymer properties to meet a certain set of requirements. Second, biodegradable polymers with good control over degradation characteristics are accessible through the phosphazene platform. The hydrolysis products are non-toxic and can be metabolized or excreted by the body. Also, since the phosphazene backbone degrades into phosphates and ammonia, a pH buffered solution is formed. Third, polymers that are water soluble or form hydrogels can be synthesized readily. These types of polymers are especially suited for applications such as drug delivery. Fourth, surface modifications can be readily carried out on these polymers to optimize surface properties. And fifth, studies by several researchers have shown the in vitro and in vivo biocompatibility of these polymers, making them ideal candidates for biomedical applications [50, 51, 52].

Polyphosphazenes have been investigated in several potential biomedical applications [8]. Hydrolytically stable polyphosphazenes have been investigated in cardiovascular, ophthalmologic and dental applications. Water soluble polyphosphazenes
have been investigated as polymeric drug carriers and in control targeted drug delivery. Bioerodible polyphosphazenes have been investigated in tissue engineering and drug delivery applications. Polyphosphazenes have also been investigated as biomedical membranes and as surfaces for immobilizing bioactive agents.

Among the listed properties of polyphosphazenes, biodegradability is often prized for a number of applications. Specific side groups such as amino acid esters, glucosyl, glycercyl, glycolate, lactate and imidazole, sensitize the polymer backbone to hydrolysis. On the other hand, hydrophobic side groups such as aryloxy, fluoroalkoxy and C$_4$ and higher alkoxy units protect the polymer backbone against hydrolytic breakdown. Therefore, a co-substituted polymer, with both hydrolysis-sensitizing and hydrolysis-retarding groups offers considerable opportunities for controlling the rate of degradation through changes in the ratio of the two side groups. It is believed that the hydrolysis of polyphosphazenes proceeds through the formation of P-OH units along the backbone (Figure 1-8). Hydrolytic attack displaces the side group attached to the phosphorus atom to form $-\text{P-OH}$ units. Proton migration to a neighboring nitrogen atom forms a phosphazane unit. This can be followed either by a hydrolytic cleavage of the nitrogen phosphorus bond or removal of the second side group from the phosphorus atom followed by proton transfer to a neighboring nitrogen atom. Irrespective of the pathway chosen, the end products left are ammonia and phosphates from the backbone and the small molecule(s) from the side group unit. Side groups that can protect the phosphazene backbone against this hydrolytic attack form inherently stable materials and side groups that do not offer this protection or rather enhance the interaction between the backbone and water molecules form hydrolytically erodible polymers. For example, several
possible mechanisms have been proposed by which random chain scission can be initiated in poly[(amino acid ester) phosphazenes] [8]. In one, water hydrolyzes the ester units of the side groups to form the corresponding polymer-bound amino acid with a deprotected carboxylic acid unit. The phosphorus atoms in the backbone are then susceptible to attack by the carboxylic acid units. In a second mechanism, it has been suggested that water displaces the amino acid esters from the phosphorus atoms to form the hydroxyphosphazene species, which then undergoes chain cleavage to phosphates and ammonia. In both the proposed mechanisms, it is the formation of hydroxyphosphazene species that is responsible for the hydrolytic instability of the polymer. If access to this intermediate is blocked, for example by hydrophobic or very bulky side groups, then hydrolysis is retarded.

The hydrolytic instability of amino acid ester functionalized polyphosphazenes has been exploited in several tissue engineering applications. For example, Laurencin et al. studied glycine ethyl ester and its co-substituted polyphosphazenes as scaffolds for skeletal tissue regeneration [53]. These scaffolds supported osteoblast cell growth and showed controllable degradation rates. Langone et. al. have investigated the potential of alanine ethyl ester and its co-substituted polyphosphazenes in peripheral nerve repair as nerve guide conduits [54]. The nerve guide conduits were degradable into nontoxic products. Nussdorfer et.al. have reported on the successful growth of neuromicrovascular endothelial cells on poly[bis(ethyl alanato)phosphazene] scaffolds making them useful in blood vessel engineering [55].
Figure 1-8: Mechanism for hydrolytic degradation of polyphosphazenes [8]
1.3 Research Objectives

The work presented in this thesis is based on the modification of surface and bulk properties of polyphosphazenes to form polymers with new and/or improved properties, useful in advanced applications.

Hydrophobic polymers play a crucial role in many biomedical and commercial applications. Hydrophobic polyphosphazenes offer opportunities for the tuning of surface properties that are not found for many conventional hydrophobic materials. Chapter 2 reviews the field of hydrophobic polyphosphazenes and their potential applications. Chapter 3 reports on the processing of superhydrophobic surface from the polyphosphazene platform. Superhydrophobic surfaces, with high water repellency (water contact angle above 150º) and self-cleaning properties (low sliding angle) have attracted considerable interest over the past few years for potential uses in applications such as self-cleaning windows, coatings and biomaterials. There are two distinct advantages of creating a superhydrophobic surface using a polyphosphazene. First, the phosphazene platform allows access to some of the most hydrophobic materials known coupled with ease of processability. Second, it offers the opportunity to combine different properties along with superhydrophobicity such as UV and thermal stability useful in outdoor coating applications or biocompatibility and biodegradability useful in biomedical applications.

Chapters 4 and 5 discuss the development of biodegradable polyphosphazenes as candidate materials for bone tissue engineering. Polyphosphazenes are attractive candidates for tissue engineering applications due to their synthetic versatility,
biodegradation, non-toxic and neutral degradation products and biocompatibility. **Chapter 4** reports on the design, synthesis, characterization and biological evaluation of L-alanine co-substituted polyphosphazenes. The design of these novel polymers was based on improving the mechanical properties of amino acid ester substituted polyphosphazenes. **Chapter 5** reports on processing nanofiber scaffolds and nanofiber composite mats of degradable polyphosphazenes by electrospinning. Several studies in the literature have shown that nanofiber matrixes that closely resemble the fibrous structure of natural extra cellular matrix (ECM) can lead to a better organization of cells and can also reduce chances of adverse tissue reaction after implantation and are thus desirable as tissue engineering scaffolds.

**Chapter 6** discusses the synthesis and characterization of novel tyrosine-functionalized polyphosphazenes. The development of tyrosine-functionalized polyphosphazenes is of special interest because, unlike the previously studied poly[(amino acid ester)phosphazenes], the tyrosine unit can be linked to the phosphazene backbone via the amino or the phenolic group. Poly[(ethyl glycinato) (ethyltyrosinato)phosphazenes] (linkage via the amino group of tyrosine) were found to be hydrolytically erodable. Poly[(n-propoxy) (tyrosinato)phosphazene] (linkage via the hydroxyl group of tyrosine) were hydrolytically stable, showed a pH-dependant solubility behavior and formed ampholytic or ionotropic hydrogels.

**Appendix a** reports on the development of polyphosphazene/hydroxyapatite composites that can serve as a practical alternative to current bone repair materials. The composites were characterized by physico-chemical analysis and biological testing.
Appendix b deals with novel blends of hydrophobic, biodegradable polyphosphazene, poly[bis(ethyl alanato) phosphazene] and poly(lactic-co-glycolic)acid (LA: GA; 85:15), developed as candidates for bone tissue engineering applications. Blending of biodegradable polyphosphazenes with PLAGA was attempted in order to combine the beneficial features of PLAGA such as recognized biocompatibility and widespread applicability with the osteoconductivity, well tuned degradability as well as the buffering capacity of the degradation products of polyphosphazenes.

1.4 References


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Chapter 2
Hydrophobic and Superhydrophobic Surfaces from Polyphosphazenes

2.1 Introduction

In simple terms, a hydrophobic [Greek, hydro = water and phobos = fear] surface shows poor affinity to water whereas a hydrophilic [Greek, hydro = water and philia = friendship] surface shows a strong affinity to water. Hydrophobicity of a polymer surface is an important property that underlies applications that range from waterproof fabrics to cardiovascular implants. Block copolymers that contain a hydrophobic block linked to a hydrophilic block can form micelles in water that can serve as vehicles for the delivery of hydrophobic drugs. Hydrophobicity is also a crucial requirement for many types of electrical insulation and for surface coatings that are exposed to the outdoors. The “non-stick” character of many hydrophobic surfaces and their lubricity are additional properties that are utilized in technology.

Classical hydrophobic polymers include silicones [poly(dimethylsiloxane)] and a variety of fluorinated organic polymers such as poly(tetrafluoroethylene) (Teflon®), Viton®, and Kalrez®. However, an entirely different class of hydrophobic polymers is emerging, based on the polyphosphazene platform. These are polymers with a backbone of alternating phosphorus and nitrogen atoms and two organic side groups attached to each phosphorus atom. Polyphosphazenes with fluorinated organic or organosilicon side groups comprise some of the most hydrophobic materials known.
2.2 Synthesis of Polyphosphazenes

The development of hydrophobic properties in polyphosphazenes is facilitated by the special methods of synthesis that are employed. Fluorinated organic polymers are generally produced by the polymerization of fluorinated monomers. This limits the number of different side groups that can be incorporated, because different polymerization conditions may be needed for different monomers. By contrast, most polyphosphazenes are synthesized by a macromolecular substitution process in which the side groups of a reactive polymer intermediate (3) are replaced by selected organic groups. The overall process is illustrated in Figure 2-1.

The reactive macromolecular intermediate is poly(dichlorophosphazene) (3), itself produced either by the thermal ring-opening polymerization of the corresponding cyclic trimer (2) or via a room temperature living cationic condensation process from a phosphoranimine monomer (4) [1, 2]. This synthesis protocol allows hydrophobic fluorinated aliphatic side groups, organosilicon, or aryloxy groups to be linked to the phosphazene chain. Examples are shown as structures 5-14 in Figure 2-2 [3, 4, 5, 6, 7, 8]. Polyphosphazenes that bear only one type of side group can be crystalline. Those that bear two or more different types of side groups are amorphous, and several of those with fluoroalkoxy side groups (such as 6) are hydrophobic, high performance elastomers.
Figure 2-1: Synthesis and functionalization of polyphosphazenes
Figure 2-2: Hydrophobic polyphosphazenes [3, 4, 5, 6, 7, 8]
2.3 Enhanced Hydophobicity via Surface Modification

The synthesis protocol just described gives access to hydrophobic polymers through changes at the molecular level. However, an additional approach is to select a polyphosphazene that is optimized for its bulk properties and then introduce different side groups by surface reactions [9]. This is possible because side groups that are already present in these polymers can often be replaced by simple metathetical nucleophilic exchange reactions (Figure 2-3). Trifluoroethoxy side groups are especially suited for replacement reactions because of the electron-withdrawing character of these units and their relatively small size. In another approach, surface properties of hydrophobic polyphosphazenes have been modified by grafting organic polymers such as polystyrene and poly(ethylene oxide) by photochemical, thermal or \( \gamma \)-radiation techniques [10].

The surface modification method to replace trifluoroethoxy by longer chain fluoroalkoxy units has been used to make the fluoroalkoxyphosphazene elastomer 6 more resistant to hydrocarbons and other fluids [11]. Many of the surface exchange reactions we have reported in the literature involve the replacement of trifluoroethoxy groups by hydrophilic or functional units [12, 13]. Surface reactions to introduce hydrophilic units allow one side of a polymer film to become adhesive, while the opposite side remains hydrophobic and resistant to adhesion. It is also possible to alter the hydrophobic or hydrophilic properties of aryloxyphosphazene polymers by surface chemistry [3, 14, 15]. For example, surface hydrosilation chemistry has been used to link organosilicon units to aryloxyphosphazenes to give surface structures of type 14 [16]. These enhance the properties of an already hydrophobic polymer.
Figure 2-3: Surface modification by the introduction of new polymer side groups at an interface.
2.4 Origins of hydrophobicity

Although the phosphazene backbone is hydrophilic, mainly due to the presence of the nitrogen lone pair electrons, polyphosphazenes can be made hydrophobic by an appropriate choice of side groups. Side groups such as –OCH₂CF₃, -O(CH₂)ₓCH₃, –OC₆H₅, or –Si(CH₃)₃, which are both hydrophobic and large enough to shield the skeleton, generate strong water repellency. The origins of water repellency from C-F, Si-CH₃, and aromatic or aliphatic hydrocarbon groups have been debated for many years and are still not fully understood. The bond polarizability and bond lengths of C-F and C-H bonds are different. However, the surface area of the peripheral units is cited as a key factor in determining the hydrophobic properties of a molecule. Two dominant mathematical models for determining the surface area of a molecule are solvent accessible surface area (SASA) and molecular surface area (MSA) [17, 18]. Experimentally, terminal CF₃ and CF₂H units differ in hydrophobic behavior but, after correcting for the difference in their hydrophobic surface area with the SASA or the MSA, their hydrophobic character should be almost the same [19].

Within the polyphosphazene series, hydrophobic effects are associated with fluorinated or organosilicon side groups or with block or graft copolymers that combine fluoroalkoxyphosphazene units with poly(organosiloxane) components. These may be compared with block copolymers that have both fluoro-organic and poly(dimethylsiloxane) blocks [20].

Hydrophobicity is frequently measured by surface contact angles to water. Water droplets spread out on a hydrophilic surface forming contact angles lower than 40°.
the other hand, water droplets retract to a semi-spherical shape on a smooth hydrophobic surface with contact angles in the range of $90^\circ < \Theta < 120^\circ$. The wetting of a flat solid surface with water is described by the Young’s equation which correlates the water contact angle with the interfacial tensions between the solid, liquid and gas phase, as shown by the equation below [26].

$$\cos \Theta = \frac{(\gamma_{sv} - \gamma_{sl})}{\gamma_{lv}}$$

where $\Theta$ = water contact angle, $\gamma_{sv}$ = solid/vapor interfacial tension, $\gamma_{sl}$ = solid/liquid interfacial tension, $\gamma_{lv}$ = liquid/vapor interfacial tension

### 2.5 Specific Examples of Hydrophobic Polyphosphazenes

Some of the hydrophobic polyphosphazenes bear fluorinated side groups such as the trifluoroethoxy groups shown in polymer 5. Other examples include polymers 6, 7, 8, and 12 shown in Figure 2-2, with the contact angles to water given beneath each structure. The results indicate that side units with terminal -CF$_2$H groups are less hydrophobic than those with terminal –CF$_3$ groups. Moreover, polymers with –OCH$_2$CF$_2$OCF$_2$CF$_2$OCF$_2$CF$_3$ side groups are more hydrophobic than those with –OCH$_2$CH$_2$OCH$_2$CH$_2$OCH$_2$CF$_3$ units. Clearly, the density of C-F bonds in the side group system is an important factor that determines the overall surface wetting behavior. Moreover, a low density of C-H bonds and relatively few exposed oxygen atoms that can hydrogen bond to the water are critical factors for high hydrophobicity in these systems.

The inherent flexibility of the polyphosphazene backbone also plays an important role by allowing the hydrophobic side groups to orient toward the surface and dominate
the interfacial properties of the polymer [21, 23]. That flexibility must be taken into account when designing new low surface energy materials.

Organosilicon side groups also play a role in forming hydrophobic polyphosphazenes. Examples are shown as structures 7, 8, 9, 13, and 14 in Figure 2-2. These polymers can be synthesized in three ways: (1) through the polymerization of small-molecule cyclophosphazenes that bear organosilicon side groups; (2) by the reactions of organosilicon nucleophiles with polymer 3 to give species such as 8, 9, or 13, or (3) by surface reactions that replace one type of side group at the interface by another (structure 14). A further variation of structure is via the formation of block copolymers that contain both fluoroalkoxyphosphazene and poly(dimethylsiloxane) blocks (structure 7).

Hydrophobicity at a milder level is also generated by the use of aryloxy side groups, as illustrated by structures 10 and 11. Fluorinated aryloxy groups or those with fluoroalkyl substituents generate a more impressive hydrophobicity. In all of these polymers the steric size of the side groups prevents hydrogen bonding between water and the backbone nitrogen atoms. Given the dimensions of most of these side units it is perhaps surprising that the relatively small trifluoroethoxy side group is so effective at shielding the backbone.

Several different polyphosphazene skeletal architecture types in addition to linear macromolecules have been utilized to generate hydrophobic materials. These include star, block and graft copolymers [11]. High polymers are not the only hydrophobic phosphazene systems known. Low molecular weight oils and waxes can be produced with any of the above side groups by using the living cationic polymerization of 4 to
control the molecular weight of the phosphazene or by using the cyclic trimer (2) as a reaction substrate.

### 2.6 Superhydrophobic Nanofibers

Superhydrophobic surfaces are characterized by a high water contact angle (> 150°) and a low sliding angle (the angle to which a surface must be tilted to cause mobility of a droplet of water). These types of surfaces, with their high water repellency and self-cleaning properties have attracted considerable interest over the past few years for their potential uses in applications such as stain and dust resistant fabrics, self-cleaning windows, microfluidic devices, and as biomaterials [24, 25, 26, 27]. Superhydrophobicity is generated either by increasing the surface roughness of a hydrophobic material or by applying a hydrophobic material to an inherently rough surface. Thus, both surface chemistry and surface roughness play an important role [26, 28, 29]. Techniques such as plasma etching, lithography, and controlled crystallization have been used to create inherently rough surfaces [30, 31, 32]. Recently, electrospinning has emerged as a convenient alternative to generate highly porous polymer mats with high surface roughness [27, 33, 34]. Electrospinning is a rather simple technique that can produce submicron size fibers of polymers from an electrically charged polymer solution [35]. Variations in the electrospinning conditions yield fiber or fiber-bead morphology (Figure 2-4), which drastically alters the surface wetting properties.

Recently we have electrospun superhydrophobic surfaces from poly[bis(2,2,2-trifluoroethoxy)phosphazene] (5) [6]. The maximum contact angle observed for spun-cast
films of this polymer was 104°. However, electrospun mats (with the appearance of tissue paper) show contact angles as high as 159°. This 55° increase is a dramatic illustration of the role played by surface morphology. Poly[bis(2,2,2-trifluoroethoxy)phosphazene] nanofibers can be spun readily from common organic solvents like tetrahydrofuran, methyl ethyl ketone, or acetone (Figure 2-4). A decrease in fiber diameter increases the hydrophobicity of these mats to give contact angles in the range of 135°-159°. Superhydrophobic properties were most obvious for polymer mats that had predominantly fiber-bead morphology (Figure 2-5). Polymer 11 can also be electrospun, and a range of other hydrophobic polyphosphazenes are also being electrospun in our laboratories.
Figure 2-4: SEM micrograph of electrospun poly[bis(2,2,2 trifluoroethoxy)phosphazene] nanofibers from THF at a concentration of (a) 5% (wt/v) of the polymer and (b) 0.5% (wt/v) of the polymer.
Figure 2-5: Water droplet on electrospun poly[bis(2,2,2 trifluoroethoxy)phosphazene] film.
2.7 Applications of Hydrophobic Polyphosphazenes

Hydrophobic materials are used in a variety of high performance applications. Hydrophobic elastomers, especially if they are also oil-resistant, are employed as seals in aircraft and marine applications, oil drilling, gas pipelines, fabrics, and surface coatings [36]. The mixed-substituent fluoroalkoxy phosphazene polymers such as 6 described earlier have been developed extensively as seals and gaskets for military applications [37, 38]. Superhydrophobic surfaces, with water contact angles greater than 150°, are of special interest as self-cleaning windows, biomedical implants, or protective coatings for masonry or metals [6, 39]. One hydrophobic small-molecule cyclic phosphazene \( \text{N}_3\text{P}_3(\text{OPhF})_2(\text{OPhCF}_3)_4 \) is used as a component in hard drive lubricants [40, 41]. The p-fluoroaryloxy groups provide coordination to an aluminum surface and the m-trifluoromethylaryloxy groups provide a hydrophobic protective coating for the hydrophilic aluminum substrate. Other oligomeric hydrophobic polyphosphazenes have been patented as lubricants and hydraulic fluids. Textile fibers can also be coated with hydrophobic polyphosphazenes to produce water repellent fabrics [37]. Micelles with hydrophobic cores are under development for the controlled release of hydrophobic drugs and have been considered for the encapsulation of hydrophobic dyes in water based inkjet media [42]. Poly(diphenoxyphosphazene) (10) has been employed as a hydrophobic liquid separation membrane to remove methylene chloride from water [43].
2.8 Conclusions

Classical hydrophobic organic fluoropolymers are some of the most useful macromolecules known. Their utility stems from their general inertness to aqueous media, their radiation resistance, and especially their hydrophobicity. Polyphosphazenes with fluorinated organic side groups possess these same properties, supplemented by ease of fabrication due to their solubility in some organic media and, in certain cases, their elasticity at low, normal, and high temperatures. It is also possible to modify the surfaces of fluorinated polyphosphazenes in ways that are not possible for fluorocarbon polymers. Hydrophobic properties in these polymers can also be generated by the presence of aryloxy or organosilicon units, and by the electrospinning of nanofibers to yield superhydrophobic surfaces. These developments widen the opportunities for producing interfaces with a broad range of uses in science and technology.

2.9 References


Chapter 3

Poly[bis(2,2,2-trifluoroethoxy)phosphazene] superhydrophobic nanofibers

3.1 Introduction

Electrospinning has emerged as a versatile tool for producing submicron size fibers of polymers [1, 2, 3]. In a typical electrospinning process, the surface of a polymer droplet, suspended at the tip of a needle, is charged by application of an electric field. As mutual charge repulsion on the drop surface overcomes surface tension, a charged polymer jet is ejected, travels a certain distance in air, and is collected as a fiber mat on a grounded collector screen. Evaporation of solvent and electrostatic repulsion between the surface charges causes continuous stretching of the polymer jet which results in the formation of submicron size fibers. In cases where the surface tension of the solution exceeds the surface charges, beads or fiber bead morphology is obtained. Non-woven textiles composed of electrospun fibers have a large specific surface area and high porosity, making them excellent candidates for filters, membranes, and protective fabric applications [2, 4]. Other potential uses include tissue engineering scaffolds, drug delivery matrices and other biomedical applications [2, 5, 6] as well as nanoelectronics [2, 7, 8].

This chapter reports on the electrospinning of a highly fluorinated polymer, poly[bis(2,2,2-trifluoroethoxy)phosphazene] (3), to form non-woven mats with high surface hydrophobicity. Nanofibers of polymer 3 were readily produced by
electrospinning solutions in tetrahydrofuran, methylethyl ketone or acetone. The degree of hydrophobicity was tuned by fiber diameter and surface morphology, with contact angles to water being in the range of 135º - 159º. Hydrophobicity of a material is a key property that depends on both surface chemistry and surface roughness [9, 10, 11]. Hydrophobic polymers (with water contact angle above 90º) are useful in many applications such as biomaterials, environmentally resistant coatings and low-friction devices, while superhydrophobic materials (with water contact angles above 150º) are of special interest as self-cleaning surfaces and stain-resistant textiles [12, 13]. Actay et al reported superhydrophobic surfaces by electrospinning low molecular weight poly(acrylonitrile-co-α,α-dimethyl-m-isopropenylbenzyl isocyanate) with a perfluorinated linear diol [14]. A contact angle of 167º was observed for a polymer film with predominantly bead morphology. Jiang et al report a contact angle of 160.4º for an electrospun composite film of polystyrene and porous polystyrene microspheres [15].

The advantage of our system over the previously studied approaches is its simplicity. We have taken a highly hydrophobic fluorinated organic-soluble polymer and used electrospinning to further enhance its surface hydrophobicity. The high hydrophobicity of fluorinated polymers is due to the unique surface activity of fluorine-containing groups, which tend to concentrate at the polymer surface and minimize the surface free energy [16, 17, 18]. The advantage of using a fluorinated phosphazene, rather than, for example, poly(tetrafluoroethylene), is its solubility in common organic solvents such as tetrahydrofuran, acetone, or methylethyl ketone. The basic structure of this polymer consists of trifluoroethoxy side groups linked to a backbone of alternating phosphorous and nitrogen atoms (3). The combination of fluorinated side groups with an
inorganic backbone generates a number of interesting properties many of which are specific to fluorinated polyphosphazenes. For example, coherent films of 3 are highly hydrophobic, are resistant to many chemicals, are bioinert, and have a high fire resistance, and radiation stability. They are also easy to fabricate into microfibers and films. The unique properties of poly[bis(2,2,2-trifluoroethoxy)phosphazene] and related polymers are the reason for their use in membrane research, biomedicine, surface coatings and elastomers [19, 20, 21]. However, this is the first report of superhydrophobicity generated by these polymers.

The significance of this approach is that it yields polymer mats with either highly hydrophobic or superhydrophobic surfaces. Moreover, this work is one of the few examples in which nanofibers of a highly fluorinated polymer have been produced because most hydrophobic fluoropolymers are too insoluble to allow electrospinning to be used.

### 3.2 Materials and Method

#### 3.2.1 Reagent and Equipment

Polymer synthesis was carried out under an atmosphere of dry argon using standard Schlenk line techniques. Hexachlorocyclotriphosphazene (1) (Ethyl Corp. and PCS) was obtained from a trimer-tetramer mixture by sublimation (30°C / 0.2 mm Hg). 2,2,2-Trifluoroethanol 99.8% (Acros) and sodium hydride 60% (Acros) were used as received. Tetrahydrofuran and acetone were purchased from EM Sciences and were
degassed and dried with an alumina bed. Methylethyl ketone was obtained from Aldrich and was used without further purification. Proton and phosphorus NMR characterization was obtained using a Bruker AMX-360 instrument. Molecular weights were determined using a HP 1090 liquid chromatograph equipped with Phenomenex columns calibrated against polystyrene standards. Glass transition temperatures were determined from a TA Instruments Q10 differential scanning calorimetry (DSC) apparatus with a heating rate of 10°C/min under an inert atmosphere. Electrospinning was accomplished with the setup described previously [2]. Parameters that were kept constant during spinning were, working distance at 20 cm, flow rate of polymer solution at 1ml/hr and the applied potential at 15 kV. The variable parameters were type of solvent and concentration of the polymer solution. SEM was conducted using a FEI-Philips XL-20. XPS data were obtained with use of a Kratos Analytical Axis Ultra instrument and the take off angle for the measurements was 0°. Water contact angle measurements were obtained using a Rame-Hart contact angle goniometer. Water was dispensed from a needle attached to a Gilmont microliter syringe filled with ultrapure water (Millipore system, 18 MΩ cm). Water droplets, 12 µl in size, were placed on the surface and images of the drop silhouette were taken with a video camera and stored for analysis on a computer. For advancing contact angle measurements, 10µl of water was brought in contact with the surface followed by addition of 2 µl of water. For receding contact angle measurements, 2 µl of water was withdrawn from the surface. The reported values are average for five measurements.
3.2.2 Synthesis of Poly[bis(2,2,2-trifluoroethoxy)phosphazene]

Synthesis of polymer 3 was carried out with the use of poly(dichlorophosphazene) prepared by the ring-opening polymerization of hexachlorocyclotriphosphazene [23]. Poly[dichlorophosphazene] (2) (20 g, 0.173 mol) was dissolved in THF (2000 ml). The sodium salt of trifluoroethanol (86.32 g, 0.862 mol) was added and the reaction mixture was stirred for 48 hours. A white fibrous polymer was isolated by precipitation of a concentrated solution of the reaction mixture into acidic water. The polymer was further purified by repeated precipitations from THF into water and hexanes. The synthetic route is summarized in Figure 3-1. $^1$H NMR (d8-THF), ppm: $\delta$ 4.54 (singlet); $^{31}$P (d8-THF), ppm: $\delta$ -5.17 (singlet). Mn 656,000, Mw 1,560,000, PDI 2.38; Tg -62 ºC, T (1) 64 ºC, Tm 241ºC.
Figure 3-1: Synthesis of poly[bis(2,2,2-trifluoroethoxy)phosphazene]
3.3 Results and Discussion

In the electrospinning of polymers, nature of the solvent plays a significant role because solution properties such as dielectric constant, boiling point, viscosity, and surface tension affect the morphology and diameter of the resulting fibers [24]. Thus electrospinning of poly[bis(2,2,2-trifluoroethoxy)phosphazene] was attempted from three solvents: methylethyl ketone (MEK), acetone, and tetrahydrofuran (THF) (Figure 3-2). The average diameter of fibers produced from different solvents was: MEK, 256 ±102 nm; acetone, 397 ± 80 nm; and THF, 498 ± 51 nm. Electrospinning from THF gave the most regular fibers with a narrow size distribution.

By electrospinning from THF solution, the average fiber diameter could be varied from 80 nm to 1.4 µm by variations in the concentration of the polymer solution. The fiber diameter decreased with decreases in solution concentration. However, as the concentration decreased, both fibers and beads strung along the fiber morphology were formed. Several studies in the literature have reported the effect of solution concentration on fiber morphology during electrospinning [4, 25]. Low concentration solutions, due to a reduced solution viscosity, tend to form beaded fibers due to surface tension effects. Figure 3-3 illustrates the effect of solution concentration on fiber morphology. At higher concentrations, ≥ 15 wt%, a broad distribution of fiber size was obtained, with the fiber diameter in the range of ±555 nm. However, below this concentration, a drastic improvement in size distribution was achieved with fiber diameters in the range of ±52 nm (Figure 3-4).
Figure 3-2: SEM micrograph of electrospun nanofibers from 10 % (wt/v) of polymer 3 from (a) methylethyl ketone (b) acetone (c) tetrahydrofuran.
Figure 3-3: SEM micrograph of electrospun nanofibers from THF at a polymer concentration of (a) 25% (wt/v) (b) 5% (c) 0.5%.
Figure 3-4: Effect of solution concentration on fiber diameter and static water contact angle on electrospun polymer 3 films.
The surface properties of electrospun mats were analyzed by XPS, AFM and static water contact angle (WCA) measurements. XPS analysis showed no change in fluorine content on an electrospun surface when compared to a spun cast film (Table 3-1). However, WCA measurements showed a marked increase in hydrophobicity of the electrospun mats. The WCA on a spun cast film was 104°. However, electrospun fiber mats showed WCA values in the range of 135°-159° (Figure 3-4). The contact angle increased with a decrease in fiber diameter and reached a ‘superhydrophobic state’ as both beads and fibers were formed on the surface of the spun mats (Figure 3-5).

The surface topography and surface roughness of polymer 3 films were analyzed by tapping mode AFM. Figure 3-6 illustrates a more porous surface structure for electrospun film compared with solution cast film. Also, the surface roughness of the electrospun film was 10 times higher compared to solution cast film. However, it should be mentioned that quantitative analysis of the electrospun film with AFM was difficult due to the rough nature of the sample. During measurements, the scanning tip tended to stick and slip, despite adjustments made to the applied force, tip velocity, and feedback loop. Several factors that influence the numerical value of the measured surface roughness include, tip and surface geometry, specific region analyzed on the sample surface, flattening and leveling algorithms to remove scanner offsets and drifts, and the skipping and dragging of the tip as it tracks the surface. Since all these factors affected measurements for the electrospun film, the roughness value recorded are approximate and semi-quantitative. $R_q$ is the RMS roughness, and the $R_a$ is the average roughness.
Figure 3-5: Water contact angle on spun cast and electrospun poly[bis(2,2,2-trifluoroethoxy)phosphazene] films.
Table 3-1: XPS data of spun cast and electrospun poly[bis(2,2,2-trifluoroethoxy)phosphazene] films

<table>
<thead>
<tr>
<th>Sample</th>
<th>F%</th>
<th>O%</th>
<th>N%</th>
<th>C%</th>
<th>P%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spun Cast Film</td>
<td>43.2</td>
<td>14.3</td>
<td>7.1</td>
<td>28.2</td>
<td>7.1</td>
</tr>
<tr>
<td>Electrospun Film</td>
<td>43.3</td>
<td>14.2</td>
<td>7.3</td>
<td>28.0</td>
<td>7.3</td>
</tr>
</tbody>
</table>
Figure 3-6: Surface topography by tapping mode AFM for (a) spun cast PTFEP film and (b) Electrospun PTFEP film (0.5 wt% solution concentration)
Superhydrophobic surfaces, with high water repellency and self-cleaning properties have attracted considerable interest over the past few years. These surfaces have a contact angle to water above 150° and a low sliding angle [9, 10, 11]. In most cases, hydrophobic materials with high surface roughness show superhydrophobic properties. Two different models, Wenzel and Cassie, have been proposed for explaining the wetting behavior of rough surfaces [10]. The Wenzel model hypothesizes that an increase in surface roughness causes an increase in surface area which leads to enhanced hydrophobicity. Since the liquid fills up the spaces on the rough surface leading to a better pinning, these types of surfaces show a high hysteresis. The Cassie model suggests that a rough surface will lead to the creation of grooves with trapped air. Liquid droplets remain suspended on these air trapped grooves and thus are not pinned to the surface leading to a low hysteresis.

Poly[bis(2,2,2-trifluoroethoxy)phosphazene] mats electrospun from 1 wt% and 0.5 wt% THF solution showed a contact angle of 152° ± 2.6° and 155° ± 2.6°, respectively. The advancing and receding contact angles were 149° and 145°, respectively, for a mat spun from 1 wt% solution and 150° and 147°, respectively, for a mat spun from 0.5 wt% solution. An electrospun mat has a higher degree of surface roughness, compared to a spun cast film [14, 24]. This roughness is further enhanced by the formation of micron size beads on the surface [14]. Thus poly[bis(2,2,2-trifluoroethoxy)phosphazene] mats electrospun from 1 wt% and 0.5 wt% THF solutions showed ‘superhydrophobic properties’ as the WCA on these films was over 150° and a low value for contact angle hysteresis (< 4°) was recorded.
In addition to poly[bis(2,2,2-trifluoroethoxy)phosphazene], electrospinning of three other hydrophobic polyphosphazenes was attempted. These polymers were poly[bis(phenoxy)phosphazene] (4), poly[bis(p-fluorophenoxy)phosphazene] (5) and poly[bis(m-fluorocresoxy)phosphazene] (6). Polymer 4 formed regular fibers in chloroform or dioxane solution. Polymer 5 formed fibers in THF solution. However, electrospinning conditions could not be optimized to obtain bead morphology with these polymers. On the other hand, polymer 6 formed both fiber and beads strung along fiber morphology from methyl ethyl ketone or THF solution (Figure 3-7). Static water contact angle measured by the sessile drop method showed these surfaces to be highly hydrophobic, with WCA in the range of 133°-148°. Table 3-2 lists the maximum WCA observed on these polymers and the optimized electrospinning conditions to form fiber or bead morphology.
Figure 3-7: SEM micrographs of electrospun nanofibers of (1) poly[bis(phenoxyp)phosphazene] (2) poly[bis(p-fluorophenoxy)phosphazene] (3) poly[bis(m-fluorocresoxy)phosphazene]
Table 3-2: Properties of electrospun fluorinated polyphosphazene fibers.

<table>
<thead>
<tr>
<th>Polymer Sample</th>
<th>Electrospinning Conditions</th>
<th>Fiber Morphology</th>
<th>Maximum Static WCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>poly[bis(phenoxy)phosphazene]</td>
<td>Solvent: CHCl₃</td>
<td>Fibers (average diameter: 2.18 µm)</td>
<td>139°</td>
</tr>
<tr>
<td></td>
<td>Concentration: 5 wt%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Voltage: 25 kV</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fibers (average diameter: 1.16 µm)</td>
<td>133°</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Beads (average diameter: 2.69 µm)</td>
<td></td>
</tr>
<tr>
<td>poly[bis(p-fluorophenoxy)phosphazene]</td>
<td>Solvent: THF/DMF</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Concentration: 5 wt%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Voltage: 15 kV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>poly[bis(m-fluorocresoxy)phosphazene]</td>
<td>Solvent: MEK</td>
<td>Fibers (average diameter: 146 nm)</td>
<td>148°</td>
</tr>
<tr>
<td></td>
<td>Concentration: 0.5 wt%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Voltage: 25 kV</td>
<td>Beads (average diameter: 2.69 µm)</td>
<td></td>
</tr>
</tbody>
</table>
3.4 Conclusions

Highly hydrophobic, nanostructured mats of poly[bis(2,2,2-trifluoroethoxy)phosphazene] were produced via electrospinning. Fibers with average diameters in the range of 80 nm to 1.4 µm were fabricated. The hydrophobicity of the electrospun mats varied with fiber diameter and surface morphology, with contact angles to water being in the range of 135º -159º. The extremely high hydrophobicity of these surfaces is a combined result of surface enrichment with fluorinated units together with the inherent surface roughness associated with an electrospun mat. The development of electrospun mats from polymer 3 constitutes a significant advancement for fluorinated phosphazenes that may eventually be utilized in membranes and filters as well as in fabric technology.

3.5 References

2. Huang, Z.M.; Zhang Y.Z.; Kotaki, M.; Ramakrishna, S. Composite Science Technology 2003, 63, 2223-2253.


Chapter 4

Synthesis, Characterization and Biological Testing of Biodegradable Polyphosphazenes for Bone Tissue Engineering

4.1 Introduction

Treatment of bone defects is a major clinical problem in the US and around the world. As decreed by the World Health Organization and United Nations, ‘2000-2010 is the Bone and Joint decade’ where researchers continue to look for improved therapies. Autografts, which are bone grafts obtained from the patient, have been the gold standard in orthopedic treatments due to their relatively high success rate. However, they suffer from disadvantages such as limited availability and donor site morbidity [1]. Allografts, which are donor bones from another source (such as cadaver) can potentially transmit diseases or elicit an unfavorable immunogenic response [1]. Bone graft substitutes, such as metals and ceramics, help to overcome some of the problems with traditional graft materials. However, they also suffer from a number of disadvantages. For example, metals do not integrate with the surrounding tissue, can cause stress-shielding and the implant may eventually fail due to infection or fatigue loading. Ceramics, such as calcium phosphates, are brittle and have poor mechanical properties [1]. Thus, at present, there is a great need to develop materials that can be used in the treatment of bone defects.

Bone tissue engineering, an emerging area in orthopedic research, aims at overcoming the disadvantages of traditional bone graft and bone graft substitute materials
It involves the development of biodegradable material scaffolds, which in combination with cells, can initiate the repair and regeneration of damaged bone. In this respect, synthetic biodegradable polymers have shown promise because of their ease of synthesis, unlimited availability, and the potential of coupling polymer degradation and removal with simultaneous tissue regeneration [4, 5]. Polyesters such as poly(lactic-co-glycolic) acid (PLAGA) have been widely used in bone repair studies as they are commercially available and also have Food and Drug Administration (FDA) approval for some biomedical applications. However, PLAGA is known to degrade by a bulk-erosion mechanism which leads to a loss of over 50% of its mechanical strength in less than two months [6, 7]. Also, due to acidic degradation products, the use of PLAGA can cause some biocompatibility concerns [6, 7]. Thus, at present, there is a need to examine other polymer systems which can be used in bone tissue engineering. Polypophosphazenes is one such class of polymers that can potentially overcome the problems associated with current materials due to their synthetic versatility, controllable degradation rate, and nontoxic, neutral degradation products [8].

Polypophosphazenes are hybrid polymers with a backbone of alternating phosphorus and nitrogen atoms and with two organic side groups attached to each phosphorus atom. These polymers are synthesized by the reactions of alkoxides, aryloxides or amines with a highly reactive macromolecular intermediate, poly(dichlorophosphazene) [8]. Because a large number of different side groups can be introduced in these reactions, a wide range of properties may be generated with this polymer system. Specific side groups such as amino acid esters, glucosyl, glyceryl, glycolate, lactate and imidazole, sensitize the polymer backbone to hydrolysis [8, 9, 10, 11, 12, 13]. Among the various classes of
Degradable polyphosphazenes, poly[(amino acid ester) phosphazenes] have met with most success in terms of potential biomedical applications. These polymers are synthesized by the attachment of an ester derivative of naturally occurring amino acids to the phosphazene backbone via the amino terminus. Hydrolysis of these polymers gives biologically benign products which include the amino acid group, an alcohol from the ester, and a pH buffered system of phosphate and ammonia [10, 14]. These polymers have been investigated for various potential applications including drug delivery vehicles and tissue engineering scaffolds [8].

The aim of the study was to develop novel polymers based on amino acid ester functionalized polyphosphazenes for bone tissue engineering applications. The first part of the study involved evaluating the effect of side group chemistry on the degradation and mechanical properties of poly[(amino acid ester)phosphazenes]. The second part of the study involved determining the in vitro and in vivo biological response to the synthesized polymers. The base polymer selected for structural and property comparisons was poly[bis(ethyl alanato)phosphazene] (1) because this polymer can be readily synthesized as a single substituent or a co-substituent polymer. Veronese and coworkers reported the use of polymer 1 as a membrane for tissue regeneration in the treatment of periodontal disease [16]. Polymer 1 has also been studied as a successful nerve guide conduit for the regeneration of severed nerves [17]. Laurencin and co-workers demonstrated the osteocompatibility of the same polymer as a tissue engineering scaffold [18, 19].

This chapter describes the synthesis, characterization and properties of poly[bis(ethyl alanato)phosphazene] and its co-substituted analogues (Figure 4-1): poly[(ethyl alanato)1 (ethyl glycinato)1 phosphazene] (2), poly[(ethyl alanato)1 (p-methyl
phenoxy)₁ phosphazene] (3) and poly[(ethyl alanato)₁ (p-phenylphenoxy)₁ phosphazene] (4). These polymers were synthesized by the macromolecular substitution route. Hydrolytic degradation of the polymers was studied by following molecular weight decline and mass loss in phosphate buffer saline solution, over a period of 7 weeks. Mechanical properties of the polymers were measured by micro-tensile testing. The in-vitro osteocompatibility of polymers was evaluated by studying the cellular response and gene expression of primary rat osteoblast cells on the surface of polymer films. The in-vivo biocompatibility of the polymers was evaluated in a subcutaneous rat model where the tissue responses to the implanted polymer films were determined by histology.
Figure 4-1: Polymer structures of L-alanine co-substituted polyphosphazenes. 1: poly[bis(ethyl alanato)phosphazene]; 2: poly[(ethyl alanato)₁ (ethyl glycinato)₁ phosphazene] ; 3: poly[(ethyl alanato)₁ (p-methyl phenoxy)₁ phosphazene] ; 4: poly[(ethyl alanato)₁ (p-phenyl phenoxy)₁ phosphazene] .
4.2 Experimental

Polymer design, synthesis and characterization was carried out in Dr Allcock’s laboratory.

In-Vitro and in-vivo biological testing of the polymers was carried out in Dr Laurencin’s laboratory.

4.2.1 Reagent and Equipment

Synthesis reactions were carried out under an atmosphere of dry argon using standard Schlenk line techniques. Hexachlorocyclotriphosphazene (Ethyl Corp. and PCS) was obtained from a trimer-tetramer mixture by recrystallization from heptane followed by sublimation (30°C / 0.2 mm Hg). Poly(dichlorophosphazene) was prepared by the ring-opening polymerization of hexachlorocyclotriphosphazene in a sealed evacuated Pyrex tube at 250°C. The same batch of poly(dichlorophosphazene) was used in the synthesis of polymers 1-4. Ultra pure, anhydrous tetrahydrofuran (THF), toluene and triethylamine were obtained from solvent dispensing system designed by J C Meyer. L-Alanine ethyl ester hydrochloride (Chem Impex International Inc), L-glycine ethyl ester hydrochloride, 4-methylphenol, 4-phenylphenol (all from Aldrich), and sodium hydride (60% dispersion in mineral oil, Aldrich) were used as received. Spectra/Por regenerated cellulose dialysis membranes with a molecular weight cut-off of 12,000-14,000 were used for purification of the polymers. $^{31}$P NMR (145 MHz) and $^1$H NMR (360 MHz) data were obtained with use of a Bruker 360 MHz spectrometer. $^{31}$P NMR chemical shifts are reported in ppm relative to 85% H$_3$PO$_4$ at 0 ppm. Gel permeation chromatography
(GPC) was carried out with use of a Hewlett-Packard HP-1090 liquid chromatograph fitted with an HP-1047A refractive index detector and two phenogel 10-µm linear columns (Phenomenex, CA), calibrated with polystyrene standards (Polysciences, PA). The samples were eluted at 40°C with a 0.1 wt% solution of tetra-n-butyl ammonium nitrate (Aldrich, WI) in THF (EM Science, NJ). Glass transition temperatures were determined from a TA Instruments Q10 differential scanning calorimetry (DSC) apparatus with a heating rate of 10°C/min. Water contact angle measurements were obtained using a Rame´-Hart contact angle goniometer. A conventional dual-stage scanning electron microscope (SEM) (FEI-Philips XL 20) was used to study the surface morphology of the degrading films. The samples were gold coated and viewed under a SEM at a working distance of 8 mm, with an accelerating voltage of 20 kV. Tensile tests were carried out using an Instron 5866 instrument equipped with a 100 N load cell and operated at a crosshead speed of 5.08 mm/min at room temperature (20-25°C). The reported results are mean values of three measurements for each sample.

4.2.2 Synthesis

**Synthesis of Polymer 1.** L-Alanine ethyl ester was prepared by treatment of alanine ethyl ester hydrochloride (106.04 g, 0.690 mol) in refluxing THF (500 mL) with triethylamine (288 mL, 2.071 mol). After the solution had been stirred for 24 hours, the reaction mixture was filtered and the filtrate was added to a stirred solution of poly(dichlorophosphazene) (20.00 g, 0.173 mmol) in THF (2000 mL). The reaction mixture was then stirred at room temperature for 48 hours. The insoluble salts were
removed by filtration and a white fibrous polymer was obtained by precipitation of the viscous polymer solution into hexanes. Purification of the polymer was accomplished by repeated precipitations from THF into hexanes (3X), followed by dialysis against a THF/methanol (50/50) mixture for 3 days.

**Synthesis of Polymer 2.** The mixed-substituent polymers were synthesized by sequential addition of the two side groups. The bulky substituent was added first in stoichiometric amounts, followed by an excess of the second reagent. For polymer 2, a stoichiometric amount of L-alanine ethyl ester (14.58 g, 0.095 mol) was added to a solution of poly(dichlorophosphazene) (10 g, 0.086 mol) in THF (1000 mL). The reaction mixture was stirred for 24 hours and partial substitution of the phosphazene backbone was confirmed by $^{31}$P NMR. Excess amounts of L-glycine ethyl ester (48.18 g, 0.345 mol), in the presence of excess triethylamine, were then added to the reaction mixture to complete the substitution. The mixture was stirred for 48 hours. The insoluble salts were removed by filtration and a yellow, adhesive polymer was obtained by precipitation of the viscous polymer solution into hexanes. Purification of the polymer was accomplished by repeated precipitations from THF into hexanes (3X), followed by dialysis against methanol for 5 days.

**Synthesis of Polymer 3.** Poly(dichlorophosphazene) (20.0 g, 0.173 mol) was dissolved in THF (2000 ml). In a separate reaction vessel, p-cresol (20.53 g, 0.190 mol) was added to a suspension of sodium hydride (4.36 g, 0.173 mol) in THF (250 mL) and the reaction was allowed to proceed for 24 hours. Sodium p-methylphenoxide solution was then added slowly to the polymer solution via an addition funnel. The reaction was allowed to proceed at room temperature for 24 hours. L-Alanine ethyl ester (79.54 g, 0.518 mol) in
THF (700 mL) was then added to the reaction mixture that contained the partially substituted polymer. The reaction solution was then heated at reflux for 48 hours. The polymer was purified by repeated precipitations from THF into hexanes (3X) and methanol (2X).

**Synthesis of Polymer 4.** The synthesis of polymer 4 was accomplished in a similar manner to polymer 3. A stoichiometric amount of the more bulky side group, sodium salt of p-phenylphenol (32.31 g, 0.173 mol), was added to poly(dichlorophosphazene) solution (20 g, 0.173 mol) followed by the addition of excess amounts of L-alanine ethyl ester (116.64 g, 0.759 mol). The polymer was purified by repeated precipitations from THF into hexanes (3X) and methanol (2X).

### 4.2.3 Hydrolysis of Polymers 1-4

Rectangular shaped polymer films (0.5cm x 0.5cm x 0.1cm) cast from concentrated THF solutions were used for these experiments. Three samples of each polymer, immersed in phosphate buffer solution (pH 7.4), were placed in a constant shaker bath, maintained at 37°C. After 1, 3, 5 and 7 weeks, the samples were removed from the buffer solution and dried under vacuum. The dried samples were weighed and then dissolved in THF for molecular weight analysis. A small piece of each sample was set aside for analysis of surface morphology by SEM.

**Detection of Hydrolysis Products of Polymers 1-4.**
Aliquots from the solutions (distilled water and phosphate buffer solution) that contained the polymer samples were analyzed for hydrolysis products. The presence of amino acids and ammonia were detected qualitatively with the use of ninhydrin. A 1.0 M solution of ninhydrin in ethanol was added to the experiment media. Formation of an intense violet coloration within minutes was evidence for the presence of ammonia or amino acid. For the detection of phosphates, aliquots were taken from aqueous media containing the polymer films. Addition of silver nitrate yielded a yellow precipitate of silver phosphate. $^1$H NMR spectroscopy was used for the detection of alcohols.

4.2.4 In-vitro Biological Evaluation: Cell Viability and Proliferation Studies

Cell proliferation, differentiation, and mineralization on the surface of polymers were evaluated to assess the osteocompatibility for bone regeneration. Circular matrices, 10mm in diameter, were made from dichloromethane solution. Each side of the polymer disk was exposed to ultraviolet light for 10 minutes to minimize bacterial contamination. The disks were washed with Ham’s F-12 media and 50,000 primary rat osteoblast cells were seeded on each scaffold to study the cell proliferation and alkaline phosphatase activity. To determine the gene expression of cells on the polymer surface 100,000 cells were seeded on each polymer matrix. Cell adhesion and proliferation on the polymer scaffolds were evaluated quantitatively after 1, 3, and 7 days post seeding. The cells were washed with phosphate buffer saline solution and lysed with 1% triton X-100 [Biorad, USA]. The DNA concentration in cell lysate at each time point was determined using a Picogreen ds-DNA assay (Molecular Probes, OR, USA). The DNA concentration
was measured as fluorescence using Tecan [Spectro Flour Plus, F129005, USA] at an emission and excitation wavelength of 485nm and 535nm respectively. The fluorescence was converted into cell number using a standard curve. The phenotypic marker of bone, alkaline phosphatase, was examined after 1, 3, and 7 days post seeding using an alkaline phosphatase substrate kit (Bio Rad, CA, USA). The cell lysate obtained from the DNA assay was used to evaluate the alkaline phosphatase activity. Briefly, 100µl of the cell lysate was added to 400µl of the substrate and solution was incubated at 37°C. The reaction was stopped by the addition of 0.4M sodium hydroxide solution. The absorbance was measured using a Tecan instrument [Spectro Flour Plus, F129005, USA] at 410nm. The absorbance was normalized based on the cell number. The effect of the polymer surface on type I collagen (T1C), alkaline phosphatase (ALP), osteocalcin (OCN), osteopontin (OPN), and bone sialoprotein (BSP) expression were evaluated. After 7 days in culture, the polymer matrices were washed with PBS solution and the total RNA from the cells was isolated using Trizol (Gibco BRL, USA) following the procedure described by the manufacturer (Qiagen, 74106, USA). The RNA extract was stabilized and centrifuged using a QIA Shredder Spin Column [Qiagen, USA] and dissolved in RNAse free water [Qiagen, USA]. The concentration of the RNA was measured using a spectrophotometer at 260nm. The gene expression was determined using a real time RT-PCR [Applied Biosystems, ABI Prism, 7900 HT Sequence Detector System, USA].
4.2.5 In-vivo Biological Evaluation: Implant Fabrication and Histology

Circular matrices, 7.5mm in diameter, of polymers 1, 3 and 4 were made from dichloromethane solution. Each side of the polymer disks was exposed to ultraviolet light for 10 minutes to minimize bacterial contamination. Sixty Sprague-Dawley rats weighing approximately 450 grams were acquired from Charles River Laboratories (Wilmington, MA). All procedures were approved by the University of Virginia, Animal Care and Use Committee, following the guidelines established by the National Institutes of Health. Anesthesia was administered to the animals by an intraperitoneal injection of ketamine (87 mg/kg body weight) and xylazine (13 mg/kg body weight). The dorsa of the animals were shaved and sterile prepped with betadine, and alcohol. Two incisions (10 mm apart) of about 10 mm were made laterally on the dorsum using a No. 10 surgical blade (Becton-Dickinson, Franklin Lakes, NJ). A subcutaneous pouch on opposite sides of the incision was created using blunt dissection technique and a polymer disk was inserted into each pouch. Each rat was implanted with two polymer disks. Following implantation the skin was closed using a sterile stapler (Ethicon Endo-Surgery Inc. USA). The animals were administered Buprenorphine (0.4 mg/kg) after surgery and were allowed to recover in the cage.

At specific time points (2, 4, and 12 weeks) the animals were euthanized by intraperitoneal injection of an overdose of pentobarbital (75 mg/kg), followed by carbon dioxide asphyxiation. The implants and the surrounding tissues were excised. The polymer and the surrounding tissues were fixed in 10% formalin solution (Surgipath, USA) for 7 days. The samples were embedded in paraffin (Tissue-Tek Vacuum
Infiltration Processor, Miles Scientific, Mishawaka, IN), sectioned using a microtome (Autocrit Microtome 040, Reichert-Jung) to about 4-5µm thickness, and stained with hematoxylin and eosin. Samples were viewed using a light microscope. The presence of neutrophils, lymphocytes, macrophages, and giant cells were used as evidence of tissue response by an independent pathologist.

4.3 Results and Discussion

4.3.1 Synthesis and Characterization

Synthesis of the polymers was accomplished via a macromolecular substitution route which involved two steps: thermal ring opening polymerization of hexachlorocyclotriphosphazene at 250ºC to form poly(dichlorophosphazene), followed by sequential substitution of the labile chlorine atoms of poly(dichlorophosphazene) by the sodium salt of the corresponding alcohol or by an ester protected amino acid. This synthetic route is summarized in Figure 4-2. For the synthesis of polymer 1, an excess of the amino acid ester was used to complete the chlorine substitution. For the mixed substituent polymers, the bulky side group was added first in stoichiometric amounts, and then an excess of the second side group was added. The extent of the substitution was determined by $^{31}$P NMR. For polymers 3 and 4 the reaction mixture was refluxed for 2 days to force complete chlorine replacement.

The synthesized polymers were characterized by NMR, GPC and DSC (Table 4-1). NMR spectroscopy was used to confirm the ratio of the two side groups and the
substitution pattern for the mixed substituents polymers. $^1$H NMR revealed a 1:1 ratio of the co-substituents for polymers 2, 3 and 4. The $^{31}$P NMR spectrum for polymer 2 showed a single peak at -2.4 ppm. Because the two side groups in this polymer are attached to backbone phosphorus through nitrogen atom, it is difficult to differentiate between geminal (same side group) and non-geminal (different side groups) substitution peaks. Thus the structure shown in Figure 4-1 and Figure 4-2 is an oversimplification since the repeating units can bear the same side group or two different side groups. Polymers 3 and 4 showed three different peaks in the $^{31}$P NMR spectra. The most prominent peak, corresponding to non-geminal substitution, was observed around -7 ppm. For polymer 3, this peak accounted for 82% of the total substitution and for polymer 4, 76% of the total substitution. Thus the sequential mode of substitution and the steric hindrance by the aryloxy groups resulted in predominantly nongeminal substitution.

The aryloxy/amoio acid ester substituted polymers (3, 4) had a higher molecular weight than the amino acid ester substituted polymers (1, 2). A possible explanation for this could be the occurrence of side reactions. Reactions of amino acid ester units with the chlorine atoms on the phosphazene backbone results in the formation of hydrogen chloride which normally reacts with excess triethylamine in solution to form a salt. However, the liberated HCl could also attack the phosphazene backbone and result in a decrease in molecular weight. In the case of the amino acid ester phosphazenes, the backbone is completely exposed for this type of side reaction to occur. In the case of aryloxy co-substituted polymers, the bulky aromatic groups can provide an effective shielding of the phosphazene backbone and thus prevent molecular weight decline.
Polymers 3 and 4 showed an increase in glass transition temperature in comparison to polymer 1. The co-substituents in case of polymer 3 and 4 were p-methyl phenol and p-phenylphenol groups, respectively. Polymer 1 has a Tg of -10°C which increased to -6°C for polymer 3 and 35°C for polymer 4. The glass transition temperature increased with an increase in the bulkiness of the side group. Bulky side groups restrict the conformational mobility of the phosphazene backbone and thus yield a more rigid polymer. We observed that the biphenyl units were more effective in raising the glass transition temperature of the polymer as compared to single phenyl units. This is attributed to a higher steric bulk and the possibility of π-π stacking of the biphenyl units [20].
Figure 4-2: General synthetic scheme for polymers 1-4.
Table 4-1: Characterization data for polymers 1-4

<table>
<thead>
<tr>
<th>Polymer</th>
<th>$^1$H NMR, ppm (^\text{a})</th>
<th>$^3$P NMR, ppm (^\text{a})</th>
<th>$M_N$ g/mol</th>
<th>$M_W$ g/mol</th>
<th>$T_g$, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.1-4.08 (br, 3.6H, -CH-, -CH(_2)-, -NH(_2)-), 1.4-1.27 (br, 3H, -CH(_3)-), 1.2-1.19 (t, 3H, -CH(_3)-)</td>
<td>-3.5</td>
<td>8.9 x 10(^{4})</td>
<td>1.96 x 10(^{5})</td>
<td>-10</td>
</tr>
<tr>
<td>2</td>
<td>4.2-3.6 (br, 7.5H, 3x –CH(_2)-, -CH-, -NH(_2)-), 1.4 (br, 3H, -CH(_3)-), 1.3 (br, 6H, 2-CH(_3)-)</td>
<td>-2.43</td>
<td>1.5 x 10(^{4})</td>
<td>3.7 x 10(^{4})</td>
<td>-9</td>
</tr>
<tr>
<td>3</td>
<td>7.7-6.4 (br, 4H, -C(_6)H(_4)-), 4.2-3.9 (br, 3.8H, -CH(_2)-, -CH-, -NH(_2)-), 2.2-1.9 (br, 3H, -C(_6)H(_4)-CH(_3)), 1.1-0.7 (br, 6H, 2x –CH(_3)-)</td>
<td>-5.8, -7.7, -18.1</td>
<td>3.3 x 10(^{5})</td>
<td>9.7 x 10(^{5})</td>
<td>-6</td>
</tr>
<tr>
<td>4</td>
<td>7.8-7.2 (br, 9H, -C(_6)H(_4)C(_6)H(_5)), 4.8-3.9 (br, 3.8 H, -CH-, -CH(_2)-, -NH(_2)-), 1.3-0.6 (br, 6H, 2x –CH(_3)-)</td>
<td>-5.2, -7.3, -17.97</td>
<td>1.02 x 10(^{6})</td>
<td>1.9 x 10(^{6})</td>
<td>35</td>
</tr>
</tbody>
</table>

\(^{a}\) NMR recorded in d\(_8\)-THF solution

* NH protons were difficult to quantify by $^1$H NMR
4.3.2 Hydrolytic Degradation

Hydrolytic degradation of these polymers was studied in phosphate buffer saline solution, at 37 ºC by monitoring the molecular weight decline as well as the mass loss over a period of 7 weeks. As shown in Figure 4-3, all the polymers showed a significant decline in molecular weight. The molecular weight loss decreased in the following order, \(2 > 1 > 3, 4\). This trend can be explained on the basis of the differences in the bulkiness and hydrophobicity of the side groups and also the differences in the initial molecular weight of the polymers. From the literature, it is known that poly[(amino acid ester) phosphazenes] degrade by a random chain scission of the backbone. Several possible mechanisms have been proposed by which this random chain scission can be initiated [8]. In one, water hydrolyzes the ester units of the side groups to form the corresponding polymer-bound amino acid with a deprotected carboxylic acid unit. The phosphorus atoms in the backbone are then susceptible to attack by the carboxylic acid units. In a second mechanism, it has been suggested that water displaces the amino acid esters from the phosphorus atoms to form a hydroxyphosphazene species, which then undergoes chain cleavage to phosphates and ammonia. In both the proposed mechanisms, it is the formation of hydroxyphosphazene species that is responsible for the hydrolytic instability of the polymer. If access to this intermediate is blocked, for example by hydrophobic or very bulky side groups, then hydrolysis is retarded. For polymer 2, co-substitution of alanine ethyl ester units with glycine ethyl ester units reduces the steric shield that can protect the polymer backbone against hydrolytic cleavage and leads to a faster loss in molecular weight. Another factor that might contribute to this fast degradation is the
molecular weight of the polymer. Because the initial molecular weight was relatively low, this allowed a greater degree of swelling and thus more water uptake. For polymers 3 and 4, the bulky aromatic groups increase the overall shielding of the polymer backbone and thus result in a lower molecular weight decline. Also the aromatic groups increase the overall surface hydrophobicity of the polymer (Table 4-2) which reduces the ingress of water to the phosphazene backbone.

Degradation in PBS solution was also monitored by recording mass loss over a period of 7 weeks. With the exception of polymer 2, none of the polymers showed a substantial decrease in mass (Figure 4-4). In the first week itself, polymer 2 lost 40% of its original mass. By week 7, 90% of the original mass was lost. The products of hydrolysis were identified as phosphates, ammonia, amino acid and the alcohol derived from the ester group on the amino acid unit (experimental section). The fast hydrolysis rate of polymer 2 is comparable to depsipeptide, imidazole or lactic acid ester substituted polyphosphazenes. In contrast, other polymers in the series did not show any significant decrease in mass, with mass loss for these polymers being only 4-5%. This difference in mass loss can be attributed to the differences in the molecular weight and hydrophobicity of the degrading units. For polymers 1, 3 and 4, both the hydrophobicity and molecular weight of the degrading units would be higher than for polymer 2 and thus these films did not record a significant mass loss.
Figure 4-3: Molecular weight decline for polymers 1, 2, 3, 4 in PBS solution at 37 °C. Molecular weight for polymer 2 could not be recorded beyond 3 weeks due to rapid hydrolysis.
Figure 4-4: Mass loss recorded for polymers 1, 2, 3, 4 in PBS solution at 37 ºC.
The surface wetting properties of polymers 1, 2, 3 and 4 was examined by static water contact angle measurements (Table 4-2). Co-substitution of alanine ethyl ester groups with glycine ethyl ester units increased the surface hydrophilicity and gave a contact angle for polymer 2 of 63°. Co-substitution with aryloxy units increased the surface hydrophobicity, with the highest contact angle recorded for polymer 4 at 107°.

The change in surface morphology following hydrolytic degradation was examined by SEM. Table 4-3 shows the surfaces of polymers 1 and 2. Prior to the polymer films being immersed in PBS solution, the surface of the films appeared smooth. After 7 weeks in the medium, the film for polymer 1 showed a rough surface with formation of small pores indicating surface erosion. Similar results were observed for polymer 3, and 4. SEM images for polymer 2 showed both small and large pores at 3 weeks indicating a simultaneous surface and bulk erosion. The degradation of this polymer was rather fast as most of the film material had dissolved by 3 weeks and the surface morphology could not be recorded beyond this time.

4.3.3 Mechanical Properties

Results from tensile testing for polymers 1, 2, 3 and 4 are shown in Figure 4-5. The modulus of elasticity and tensile strength were similar for polymers 1 and 2. However, these values increased with the introduction of aryloxy side groups. Polymer 4 showed a five-fold increase in modulus of elasticity and a two-fold increase in tensile strength, when compared to polymer 1. Because the mechanical properties of a polymer depends on factors like glass transition temperature and molecular weight, the aryloxy
co-substituted polymers showed higher strength. Thus, these results illustrate the positive
effect of co-substitution with bulky aromatic groups on the mechanical properties of
poly[(amino acid ester)phosphazenes].
Table 4-2: Static water contact angle measured by sessile drop method on polymer films 1 – 4.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water Contact Angle</td>
<td>73º ± 0.4º</td>
<td>63º ± 5º</td>
<td>101º ± 1º</td>
<td>107º ± 1.4º</td>
</tr>
</tbody>
</table>
Table 4-3: Scanning electron micrographs of polymer films in PBS, at 37 °C.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>0 Week</th>
<th>3 Weeks</th>
<th>7 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>2</td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td>--------</td>
</tr>
</tbody>
</table>
Figure 4-5: Tensile strength and modulus of elasticity at maximum load for polymers 1 (PNEA), 2 (PNEAEG), 3 (PNEAmPh), 4 (PNEAPhPh).
4.3.4 In vitro Biological Evaluation

The in vitro osteocompatibility of polymer films was evaluated by studying the adhesion and proliferation of primary rat osteoblast cells. Polymers 1, 3, and 4 supported osteoblast adhesion and proliferation whereas polymer 2 did not. A possible explanation for the poor osteocompatibility of polymer 2 could be a fast degrading surface which prevents cells from attaching to it. Cell adhesion and proliferation of primary rat osteoblast cells on the surface of polymers 1, 3 and 4 was evaluated using a ds-DNA quantification kit and the cell number on the surface of the polymers was calculated using a standard curve. The cell number on the polymer films were comparable after 3 days in culture (p<0.05) (Figure 4-6). At day 7, the cell number on the surface of polymer 4 was significantly higher than polymer 1 (p<0.05) (Figure 4-6).

The phenotypic marker of bone, alkaline phosphatase (ALP) activity of the cells on the surface of the polymers was evaluated. Figure 4-7 shows the ALP activity per cell on the surfaces of the matrices. Cells on the polymer surfaces expressed early ALP activity (at day 1) and further the cells expressed phenotypic activity throughout the entire period of study. Alkaline phosphatase (ALP) is one of the earliest phenotypic markers expressed by osteoblasts cells [21]. The cells on the surface of polymers 1, 3 and 4 expressed ALP throughout the study (Figure 4-7). The increased expression of ALP suggests that the PRO cells have shifted to a more differentiated stage.

The expression of type I collagen (TIC), alkaline phosphatase (ALP), osteocalcin (OCN), osteopontin (OPN), and bone sialoprotein (BSP) was evaluated using a real time RT-PCR after 7 days. In this study we observed that the PRO cells on the polymer
surfaces expressed all the genes that are characteristic of osteoblast phenotype, differentiation, maturation, and mineralization.

4.3.5 In vivo Biological Evaluation

Biocompatibility of polymers 1, 3 and 4 was evaluated in a subcutaneous rat model. Polymer disks of diameter 7.5 mm were prepared by a solvent evaporation technique and were implanted subcutaneously in rats. After 2, 4, and 12 weeks, the polymer along with the surrounding tissues were excised, prepared, and viewed by light microscopy to evaluate the tissue responses of the implanted polymers (Figure 4-8). The tissue responses were classified as minimal, mild or moderate based on a biocompatibility scheme developed in Dr Laurencin’s laboratory. Minimal inflammation was characterized by the presence of few neutrophils, erythrocytes, and lymphocytes; mild response was characterized by the predominant presence of macrophages, fibroblasts, or giant cells; and moderate inflammation was characterized by the abundance of macrophages, giant cells, and by the presence of tissue exudates.

Polymers 1 and 3 matrices elicited varying levels of tissue responses during the 12 weeks implantation period. At 2 weeks both polymers evoked a moderate response and by 12 weeks the response was found to be mild. However, polymer 4 elicited a mild response at the end of two weeks and demonstrated a further decreased inflammatory response after 12 weeks. The polymers were classified based on a biocompatibility scale to assess the tissue response to these novel polymers [22-25]. Materials such as titanium implants, and poly(hydroxyethyl methacrylate) which elicit very mild inflammatory
response are classified as level 1 biocompatible material [22]. Level 2 biocompatibility involves materials such as guttapercha, and zinc oxide – eugenol cements which initially provoke a mild to moderate response and subsides over time [23]. Materials that elicit a moderate to severe inflammatory response and the presence of giant cells, coagulation necrosis, and dystrophic calcification are classified as level 3 biocompatible materials [24]. Level 4 biocompatible materials include paraformaldehyde containing pastes which elicit a severe inflammatory response which does not decrease over time [25]. The results of this study demonstrated that the polymers 1, 3 and 4 showed varying levels of inflammatory response and could be classified as level 2 compatible biomaterials.
Figure 4-6: Number of primary rat osteoblast cells on films of polymers 1, 3 and 4 over 7 days. Statistical significance at p<0.05, n=4.
Figure 4-7: Alkaline phosphatase activity expressed by the cells on films of polymers 1, 3 and 4 over 7 days. Statistical significance at p<0.05, n=4.
Figure 4-8: Micrographs of rat subcutaneous tissue response to [(a) Polymer 1, (b) Polymer 3, (c) Polymer 4 after 12 weeks of implantation. P – Polymer, N – Neutrophils, F – Fibrous Tissue. (40X magnification).


4.4 Conclusions

Polyphosphazenes that contain ethyl alanato side groups together with other amino acid ester or aryloxy side groups can be readily synthesized by the macromolecular substitution route. The types of co-substituents on the polymers affect properties such as molecular weight, glass transition temperature and hydrophobicity which in turn affect properties such as degradation rate and tensile strength. Combination of alanine ethyl ester units with glycine ethyl ester side units leads to a drastic increase in the hydrolysis rate and also increases the surface hydrophilicity. Combinations of alanine ethyl ester side group with bulky aromatic groups leads to polymers with high molecular weight. These polymers show a substantial increase in glass transition temperature, hydrophobicity, and tensile properties. The properties can be tuned further by changes in the ratios of the two side groups.

The in vitro osteocompatibility results demonstrate that cells adhere, proliferate, and maintain their phenotype when seeded directly on the surface of polymers 1, 3 and 4. Moreover cells on the surface of the polymers express type I collagen, alkaline phosphatase, osteocalcin, osteopontin, and bone sialoprotein which are characteristic genes for osteoblast maturation, differentiation, and mineralization. The in vivo biocompatibility results show varying levels (minimal to moderate) of inflammatory responses at different time points for polymers 1, 3 and 4. Overall, the polymers present good biocompatibility, comparable to other commercially available biomaterials and thus form suitable materials for bone tissue engineering applications.
4.5 Acknowledgements

The authors acknowledge the financial support from NIH grant #AR 46560.

4.6 References


Chapter 5

Electrospinning degradable scaffolds of polyphosphazenes for biomedical applications

5.1 Introduction

Tissue engineering has been defined as “.....the application of principles and methods of engineering and life sciences toward fundamental understanding...and development of biological substitutes to restore, maintain and improve tissue functions” [1]. This technique involves combining cells with a biocompatible scaffold to form tissues [1]. The scaffold serves as a temporary ‘extra cellular matrix’ (ECM) until the host cells can repopulate and form natural matrix. Desirable properties of tissue engineering scaffolds include biocompatibility, biodegradability, high porosity and pore interconnectivity [2, 3, 4]. Fabrication techniques such as sintered microsphere fabrication [5] and phase separation [6] have been investigated to form porous scaffolds with interconnected pores. However, recently, researchers have shown that nanofiber matrixes that closely resemble the fibrous structure of natural ECM can lead to a better organization of cells and can also reduce the chances of adverse tissue reactions after implantation [7, 8, 9, 10]. Nanofiber matrixes can be processed by template synthesis [11], self-assembly [12] and electrospinning [13, 14]. Among these techniques, electrospinning has gained a lot of attention due to its simplicity and versatility.

With rapid advances being made in the field of nanoscience and nanotechnology, several techniques have been developed whereby materials can be processed with
dimensions in the nanometer range. Electrospinning is one such technique that allows processing submicron size fibers of polymers [13, 14, 15, 16]. A typical electrospinning setup consists of three main components: a high voltage power supply, a spinneret and a grounded collector screen (Figure 5-1). A polymer solution of sufficient viscosity is loaded into a syringe which is connected to a spinneret (a metallic needle). On application of high voltage, the surface of the polymer droplet suspended at the tip of the needle, also known as the Taylor cone, is charged. As mutual charge repulsion on the drop surface overcomes surface tension, a charged polymer jet is ejected, which travels a certain distance in air, and is deposited as a non-woven fiber mat on a grounded collector screen. Evaporation of solvent and electrostatic repulsion between the surface charges causes continuous stretching of the polymer jet which results in the formation of submicron size fibers. In cases where the surface tension of the solution exceeds the surface charges, beads or fiber bead morphology is obtained. Compared to other nanofiber producing techniques, electrospinning is a relatively low cost technique, with a high production rate. Non-woven textiles composed of electrospun fibers have a large specific surface area and high porosity, making them excellent candidates for filters, membranes, protective fabric applications and various biomedical applications including tissue engineering [14, 17]. An electrospun nanofiber scaffold of a biodegradable and biocompatible polymer will form an ideal matrix for tissue engineering application due to its high surface area and narrow fiber diameter which closely resembles the natural ECM [7, 8, 9, 10].
Figure 5-1: Experimental setup for electrospinning polymer nanofiber [18]
Nanofiber based scaffolds have been produced from natural polymers like gelatin and silk. Huang et al. have reported on electrospinning a gelatin nanofiber mesh from 2, 2, 2-trifluoroethanol as the solvent [19]. Rutledge et al. have electrospun B mori silk/poly(ethylene oxide) fibers with diameters in the nanometer range [20]. Even though natural polymers are considered to have improved biocompatibility compared with synthetic polymers, they suffer from disadvantages such as batch-to-batch variations and possibility of eliciting an unfavorable immunogenic response. Synthetic polymers such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(lactic-co-glycolic)acid (PLAGA) and poly(caprolactone) (PCL) have been electrospun to form biodegradable scaffolds for tissue engineering applications [21, 22]. Goldstein et al have studied the effect of PLA fiber diameter on osteoblast cell adhesion, proliferation and differentiation. In the presence of osteogenic factors, the MC3T3-E1 cell density on electrospun PLA samples was higher compared to spin-coated samples [9]. However, even though the polyesters have FDA approval for some biomedical applications and several studies have shown these polymers to be biocompatible, there are still concerns about the accumulation of acidic degradation products that can cause adverse tissue reactions. Thus, at present, there is a need to examine other polymer systems that can be used as scaffolds in tissue engineering. Polyphosphazenes is one such class of polymers that can potentially overcome the problems associated with current materials due to their synthetic versatility, controllable degradation rate, and non-toxic, neutral degradation products [23].

Polyphosphazenes are high molecular weight polymers with a backbone of alternating phosphorus and nitrogen atoms. Each phosphorus atom bears two substituents,
with a wide variety of side groups available for property optimization. The type of side
group attached to the phosphazene backbone has a profound effect on the chemical and
physical properties of the polymer such that a polymer can be tailored for a specific
application. Different types of polyphosphazenes have been developed as high
performance commercial elastomers, electro-optical glasses, ion transport membranes,
and biomedical materials [23].

Polyphosphazenes offer an ideal platform for the design and synthesis of novel
biodegradable polymers with efficient control over degradation rate and other material
properties. Side groups such as amino acid ester, imidazolyl, lactate, glycolate, glucosyl,
or glyceryl groups sensitize the polymer backbone to hydrolysis [23, 24, 25, 26, 27, 28].
On the other hand, hydrophobic side groups such as, aryloxy, fluoroalkoxy and C4 and
higher alkoxy units protect the polymer backbone against hydrolysis. Therefore, a co-
substituted polymer, with both hydrolysis-sensitizing group and hydrolysis-retarding
groups offers considerable opportunity for controlling the rate of degradation through
changes in the ratio of the two substituents. Another added advantage of
polyphosphazenes, is their nontoxic, neutral degradation products. The phosphazene
backbone breaks down into phosphates and ammonia, which forms a neutral solution,
thereby circumventing the problems associated with the acidic degradation products of
PLAGA [26, 29]. The unique properties of these polymers have sparked great interest in
their biomedical use.

In this study, degradable scaffolds of poly[bis(ethyl alanato)phosphazene] (1)
were developed by electrospinning. Polymer 1 belongs to the class of amino acid ester
functionalized phosphazenes. These types of polymers are synthesized by the attachment
of an ester derivative of naturally occurring amino acid to the phosphazene backbone via the amino terminus. Hydrolysis of these polymers gives biologically benign products which include the amino acid group, an alcohol from the ester, and a pH buffered system of phosphate and ammonia. These polymers have been investigated for various potential applications including drug delivery vehicles and tissue engineering scaffolds [23]. Polymer 1 has been investigated as a membrane for tissue regeneration in the treatment of periodontal disease [30], as a nerve guide conduit for the regeneration of severed nerves [31] and as a composite material for bone tissue engineering [32, 33].

In addition to native polymer scaffolds, composite fiber scaffolds of polymer 1 and hydroxyapatite were also fabricated by electrospinning. Hydroxyapatite is the main mineral component of bones and teeth and has excellent biocompatibility with natural tissue. Recent studies have shown that biomimetic nanocomposites of polymer and hydroxyapatite perform better as bone regenerative matrixes in comparison with only polymer matrixes [34, 35, 36].

5.2 Experimental Section

5.2.1 Reagents and Equipment

Synthesis reaction was carried out under an atmosphere of dry argon using standard Schlenk line techniques. Hexachlorocyclotriphosphazene (Ethyl Corp. and PCS) was obtained from a trimer-tetramer mixture by recrystallization from heptane followed by sublimation (30 °C / 0.2 mm Hg). Poly(dichlorophosphazene) was prepared by the
ring-opening polymerization of hexachlorocyclotriphosphazene in a sealed evacuated Pyrex tube at 250 °C. Ultra pure, anhydrous tetrahydrofuran (THF) and triethylamine were obtained from solvent dispensing system designed by J C Meyer. L-Alanine ethyl ester hydrochloride (Chem Impex International Inc) was used as received. Spectra/Por regenerated cellulose dialysis membranes with a molecular weight cut-off of 12,000-14,000 were used for purification of the polymers. $^{31}$P NMR (145 MHz) and $^1$H NMR (360 MHz) data were obtained with use of a Bruker 360 MHz spectrometer. $^{31}$P NMR chemical shifts are reported in ppm relative to 85% H$_3$PO$_4$ at 0 ppm. Gel permeation chromatography (GPC) was carried out with use of a Hewlett-Packard HP-1090 liquid chromatograph fitted with an HP-1047A refractive index detector and two phenogel 10-µm linear columns (Phenomenex, CA), calibrated with polystyrene standards (Polysciences, PA). The samples were eluted at 40°C with a 0.1 wt% solution of tetra-n-butyl ammonium nitrate (Aldrich, WI) in THF (EM Science, NJ). Glass transition temperatures were determined from a TA Instruments Q10 differential scanning calorimeter (DSC) apparatus with a heating rate of 10°C/min. Water contact angle measurements were obtained using a Rame-Hart contact angle goniometer. A conventional dual-stage scanning electron microscope (SEM) (FEI-Philips XL 20) was used to study the surface morphology of the degrading films. The samples were gold coated and viewed under a SEM at a working distance of 8 mm, with an accelerating voltage of 20 kV.
5.2.2 Synthesis of Polymer 1

L-Alanine ethyl ester was prepared by treatment of alanine ethyl ester hydrochloride (53.01 g, 0.345 mol) in refluxing THF (500 mL) with triethylamine (144 mL, 1.04 mol). After the solution had been stirred for 24 hours, the reaction mixture was filtered and the filtrate was added to a stirred solution of poly(dichlorophosphazene) (10.00 g, 0.0863 mmol) in THF (1000 mL). The reaction mixture was then stirred at room temperature for 48 hours. The insoluble salts were removed by filtration and a white fibrous polymer was obtained by precipitation of the viscous polymer solution into hexanes. Figure 5-2 summarizes the synthesis protocol. Purification of the polymer was accomplished by repeated precipitations from THF into hexanes (3X), followed by dialysis against a THF/methanol (50/50) mixture for 3 days. $^{31}\text{P NMR}$ (CDCl$_3$), ppm: $\delta$ -3.5; $^{1}\text{H NMR}$ (CDCl$_3$), ppm: $\delta$ 4.1-4.08 (3.6H), 1.4-1.27 (3H), 1.29-1.19 (3H). $M_n = 63249$ g/mol, $M_w = 182166$ g/mol, PDI = 2.9. $T_g = -12^\circ C$

5.2.3 Electrospinning of Polymer Nanofiber Scaffold

Figure 5-1 illustrates the electrospinning set-up that was used in this study. The apparatus consists of a 5 ml syringe fitted with a blunt-end needle, a ground electrode, and a high voltage source. The ground electrode consisted of a copper plate covered with aluminium foil. Parameters such as type of solvent, solution concentration, flow rate, applied voltage and working distance were varied to determine the optimum electrospinning conditions. Electrospinning was carried out at room temperature ($\sim 20^\circ C$)
C). The electrospun fiber scaffolds were dried in a vacuum oven for 48 hours to remove any trace amounts of solvent.

5.2.4 Degradation of Polymer Nanofibers

Hydrolysis of a polymer 1 nanofiber scaffold was studied in phosphate buffer saline solution, at 37 ºC for a period of 4 weeks. Solution cast films of polymer 1 were used as control. Rectangular shaped polymer films (0.5cm x 0.5cm x 0.01cm) were used for these experiments. Three samples, each of the electrospun and solution cast films, were immersed in PBS (pH 7.4) and placed in a constant shaker bath, maintained at 37 ºC. After 1, 2, 3 and 4 weeks, the samples were removed from the buffer solution and dried under vacuum. The dried samples were weighed and then dissolved in THF for molecular weight analysis. A small piece of each sample was set aside for analysis of surface morphology by SEM.

5.2.5 Synthesis of Hydroxyapatite Precursor

Tetracalcium phosphate (TetCP, Ca$_4$(PO$_4$)$_2$O) and dicalcium phosphate anhydrous (DCPA, CaHPO$_4$) were allowed to react to form calcium deficient hydroxyapatite precursor, CDSHAp. TetCP was made by ball milling CaCO$_3$ (Osram-Sylvania, PA) and monocalcium phosphate monohydrate (MCPM, Ca(H$_2$PO$_4$)$_2$.H$_2$O, FMC Corp., NY) at a 3:1 molar ratio for 16 hours in heptane (Alfa Aesar, Ward Hill, MA). After filtering and drying, the TetCP was fired in air at 1400 ºC for 1 hour and quenched rapidly. X-ray
diffraction was used to confirm phase pure TetCP. The TetCP was ground by hand, sieved, ball milled, and attrition milled to reduce particle size. TetCP and DCPA were then mixed in the desired Ca/P ratio and ball milled in heptane. Figure 5-3 summarizes the synthesis steps. After synthesis, the precursor powders were stored in a desiccator under vacuum to avoid hydration. The average particle size was 2.5um, as measured by SEM.
Figure 5-2: Synthetic scheme for polymers 1
Figure 5-3: Synthesis of calcium deficient hydroxyapatite
5.2.6 Electrospinning of Polymer/Hydroxyapatite Scaffold

Composite scaffolds were electrospun from a suspension of hydroxyapatite precursor, CDSHAp, in chloroform solution of polymer 1. Three different concentrations of CDSHAp, 31 wt/v, 63 wt/v and 94 wt/v, were used in the study. Electrospinning was carried out with the following optimized conditions: 20 kV applied voltage, 1 ml/hr flow rate and 20 cm distance between needle tip and collector screen.

Polymer 1/CDSHAp composite scaffolds were placed in a 0.5% phosphoric acid (Acros 201140010) solution and allowed to set for 24 hours at 37°C in a humidified atmosphere.

5.2.7 X–Ray Diffraction of Composite Scaffold

X-Ray diffraction studies of phase evolution were carried out on the dried polymer/hydroxyapatite composite scaffold using an automated diffractometer (Scintag Inc., Sunnyvale CA) with a step size of 0.02°, a scan rate of 2° per minute, and a scan range from 20° to 40° (2θ). Phases present in the pattern were compared to JCPDS cards 9-432, 9-80 and 25-1137, which correspond to HA, DCPA and TetCP, respectively.

5.2.8 Porosity of Electrospun Composite Scaffolds

The porosity of the composite scaffolds was evaluated using a mercury porosimeter. The pressure was varied from 0.1 to 50psi with an equilibration time of 60 seconds for each intermediate data point. The pore size, and porosity of the scaffolds
were determined from the amount of mercury that penetrated into the sample at different pressures.

5.3 Results and Discussion

5.3.1 Synthesis

Synthesis of polymer 1 was accomplished via a macromolecular substitution route which involved two steps: thermal ring opening polymerization of hexachlorotriphosphazene at 250°C to form poly(dichlorophosphazene), followed by sequential substitution of the labile chlorine atoms of poly(dichlorophosphazene) by an excess of L-alanine ethyl ester. This synthesis route is summarized in Figure 5-2 and the characterization data for the polymer is reported in the experimental section.

5.3.2 Electrospinning degradable nanofibers of poly[bis(ethyl alanato)phosphazene]

5.3.2.1 Optimization of Electrospinning Conditions

Formation of nanofibers by electrospinning is controlled by several parameters such as type of solvent, solution concentration, applied voltage, flow rate and distance between the needle tip and collection screen. The nature of the solvent plays a significant role because solution properties such as dielectric constant, boiling point, viscosity, and surface tension affect the morphology and diameter of the resulting fibers [37]. Solution concentration has a dominant effect on formation of beads over fibers during
Recently, Shenoy and coworkers have demonstrated that chain entanglements due to increased polymer concentration or high polymer molecular weight play an important role in fiber formation during electrospinning [38]. They propose that a critical number of chain entanglements are required for the formation of fibers. During electrospinning, these chain entanglements stabilize the ejected liquid jet until the solvent evaporates and fibers are formed. For a low concentration solution or low molecular weight polymer, the loss of chain entanglements due to elongational flow is faster than compared with a high concentration solution or high molecular weight polymer [38, 39, 40]. Applied voltage is another important parameter which determines fiber size and morphology as it affects the shape of the Taylor cone [41, 42]. Usually, for a given polymer solution with other parameters constant, a window of electric potential exits in which the polymer can be electrospun. If the applied voltage is increased within this window, a decrease in fiber size is observed. However, if electrospinning is performed above this critical value a dramatic increase in number of beads is observed.

Electrospinning of polymer I was attempted from three different solvent systems: chloroform, tetrahydrofuran and 1:1 mixture of tetrahydrofuran and methanol. Methanol was mixed with tetrahydrofuran to increase the polarity of the mixed solvent system. The concentration of the polymer solution was kept constant at 10 wt/vol, flow rate at 1ml/hr and spinning distance at 20 cm. Electrospinning from polymer solutions in tetrahydrofuran and tetrahydrofuran/methanol mixture did not afford a stable taylor cone, even though the spinning voltage was varied from 15 to 25 kV. On the other hand, chloroform solution afforded a stable taylor cone at the spinning voltage of 20 kV and thus this solvent was selected for further optimization.
Electrospinning a 10 wt/vol solution of polymer 1 in chloroform formed both beads and fibers as shown in Figure 5-4 a. Thus further optimization was required to form bead free fibers and parameters such as a voltage, spinning distance, concentration and flow rate were varied. Decreasing the spinning voltage below 20 kV formed an unstable taylor cone. On increasing the voltage to 25 kV, a decrease in the fiber diameter and number of beads was observed (b, Figure 5-4). The voltage could not be increased beyond this point due to instability of the taylor cone. Increasing the distance between the spinneret and collector screen from 20 cm to 30 cm reduced the amount of fibers collected. Increasing the concentration of the solution from 10 wt/vol to 15 wt/vol to 20 wt/vol drastically reduced the number of beads and regular fibers were obtained (c, d Figure 5-4). However, increasing the solution concentration also increased the fiber diameter. The average diameter for fibers spun from 10 wt/vol solution was 348 nm and for 20 wt/vol solution was 1 µm. Thus, to reduce the fiber diameter, flow rate of the polymer solution was changed from 1 ml/hr to 0.5 ml/hr. This brought the average fiber diameter down to 400 nm (e, Figure 5-4). Thus optimized electrospinning conditions of chloroform as solvent, solution concentration of 20 wt/vol, flow rate at 0.5 ml/hr, spinning distance at 20 cm and spinning voltage of 25 kV gave regular, nanosized fibers of polymer 1.

In addition to poly[bis(ethyl alanato)phosphazene], nanaofibers of other degradable polyphosphazenes have also been produced by electrospinning. These polymers include, poly[bis(ethyl glycinato)phosphazene], poly[(ethyl alanato)₁ (p-phenyl phenoxy)₁ phosphazene], poly[(ethyl alanato)₁ (p-methyl phenoxy)₁ phosphazene], poly[(ethyl glycinato)₁ (p-methyl phenoxy)₁ phosphazene] and poly[(ethyl glycinato)₁ (p-methyl phenoxy)₁ phosphazene]. Fiber diameter in the range of 40 nm to 1000 nm can be
readily produced from common organic solvents such as tetrahydrofuran, chloroform and acetone. Thus electrospinning is a convenient method to produce nanofibers of degradable polyphosphazenes.
<table>
<thead>
<tr>
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<tr>
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<tr>
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<td>20 cm</td>
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<tr>
<td>d</td>
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<td>20 wt%</td>
<td>1 ml/hr</td>
<td>20 cm</td>
<td>1 µm</td>
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<tr>
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<td>0.5 ml/hr</td>
<td>20 cm</td>
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Figure 5-4: Optimization of electrospinning conditions for polymer 1 using chloroform as solvent
5.3.3 Hydrolytic Degradation of Nanofibers

Hydrolysis of nanofibers of Polymer 1 was studied in phosphate buffer saline solution at 37 °C by monitoring the molecular weight decline as well as the mass loss over a period of 4 weeks. As a comparison, hydrolysis of solution cast bulk films of polymer 1 were also studied under similar conditions. The basis of this study was to evaluate the effect of material size on the degradation behavior of the polymer. In general, the smaller the material size, the faster is the degradation rate. However, some polymers such as polyesters show the opposite trend. For example, degradation of poly(lactic acid) produces acidic byproducts which can induce a bulk autocatalytic effect, and thus the rate of degradation for such polymers is dependant on the rate of diffusion of the degradation byproducts [43]. In thin films or smaller size particles, the acidic byproducts can diffuse out at a much faster rate compared to thicker films or larger particles and thus the autocatalytic effect is reduced [44, 45].

Figure 5-5 illustrates the decline in weight average molecular weight, and Figure 5-6 illustrates the mass loss that was recorded for nanofibers and bulk films of polymer 1. Both type of samples showed a steady decline in molecular weight, with a higher loss recorded for the nanofibers. At the end of 4 weeks, polymer 1 nanofiber films recorded a 73 % decline in original molecular weight and polymer 1 bulk film recorded a 62 % decline in original molecular weight. No mass loss was recorded for the bulk film samples at the end of 4 weeks which indicated that the cleaved polymer chains were still above the critical value for dissolution in water. On the other hand, the nanofiber samples recorded approximately 10 % mass loss at the end of 4 weeks. A slight jump in mass loss
was noticed at the end of 2 weeks. Thus, the molecular weight decline and mass loss study indicated that the rate of hydrolysis was accelerated for polymer 1 in a nanofiber form compared to bulk film and this was attributed to an increased surface area for nanofibers.

The morphological changes of nanofibers and bulk films of polymer 1 during degradation were recorded with scanning electron microscopy and the results are shown in Figure 5-7 and Figure 5-8. In accordance with the mass loss results, the surface of the bulk films did not show any signs of degradation (S0 [0 weeks], S2 [2 weeks] and S4 [4 weeks] in Figure 5-7). On the other hand, significant morphological changes were recorded for the nanofiber samples. Within one week of placement in PBS solution, polymer 1 fibers shrank in size. The average fiber diameter decreased from 498 nm to 414 nm. However, at this stage, no signs of degradation were detected (E1 in Figure 5-7 and e1 in Figure 5-8). The reduction in fiber size could be attributed to a thermally induced relaxation of polymer chains as the temperature of the medium was well above the glass transition temperature of the polymer [22]. At the end of two weeks, surface corroded fibers or collapsed fibers were observed (E2 in Figure 5-7 and e2 in Figure 5-8). These features intensified with time and at the end of 4 weeks, completely disintegrated fibers were observed (E4 in Figure 5-7 and e4 in Figure 5-8). Thus the SEM results also indicate an enhancement in the rate of degradation for the nanofibers of polymer 1 compared to its bulk film sample.
Figure 5-5: Molecular weight decline recorded for electrospun and solution cast films of polymer 1
Figure 5-6: Mass loss for solution cast and electrospun films of polymer 1.
Figure 5-7: Scanning electron micrographs of electrospun polymer 1 fibers after (E0) 0 week, (E1) 1 week, (E2) 2 weeks, (E3) 3 weeks, (E4) 4 weeks and solution cast films after (S0) 0 week, (S2) 2 weeks and (S4) 4 weeks in PBS solution, at 37 °C.
Figure 5-8: Scanning electron micrographs of electrospun polymer 1 fibers after (e0) 0 week, (e1) 1 week, (e2) 2 weeks, (e3) 3 weeks and (e4) 4 weeks in PBS solution, showing degradation of fibers.
5.3.4 Polymer/Hydroxyapatite Nanofiber Composites

The last section in this chapter will deal with the electrospinning of polymer nanofiber/ hydroxyapatite composite scaffolds. These types of scaffolds are especially suited for bone tissue engineering applications. Hydroxyapatites have been extensively studied as bone repair materials due to their biocompatibility, osteoconductivity, resorbability and their ability to interact with surrounding bone [46, 47, 48]. Thus incorporation of hydroxyapatite into the polymer scaffold will improve the biocompatibility of the scaffold. Also, the hydroxyapatite particles can act as nucleating sites for apatite growth, in vivo, thus reducing the high energy of activation required for the mineralization process to begin [49].

Hydroxyapatites (HA) are calcium phosphate salts classified as stoichiometric or calcium deficient HA, depending on the Ca/P ratio. Stoichiometric HA is $Ca_{10}(PO_4)_6(OH)_2$ with a Ca/P ratio of 1.67 and fully calcium-deficient HA is $CaHPO_4(PO_4)_3OH$ with a Ca/P ratio of 1.5 [50]. It is believed that calcium deficient HA is more biocompatible than stoichiometric HA due to its similarity to biological apatite and its ability to be remodeled by native bone [51]. Composites of HA and polymers can be prepared either by mixing HA particles with the polymer or by mixing HAp precursor particles with polymer and allowing an in situ formation of HA. These self-setting composites present an advantage over composites with preformed HA as they are more moldable and also have the potential for forming an interlocked interface with the surrounding bone during HA formation. Thus, in this study, composite nanofiber scaffolds of calcium deficient HAp precursor and polymer 1 were prepared. XRD, SEM
and porosity measurements were conducted to characterize these novel composite scaffolds.

Figure 5-9 shows the scanning electron micrographs of polymer 1 / HAp precursor composite scaffolds with 31 wt%, 63 wt% and 94 wt% of HAp precursor particles. The HAp precursor used to make these composites was CDSHAp. CDSHAp is composed of tetracalcium phosphate (TetCP, Ca₄(PO₄)₂O) and dicalcium phosphate anhydrous (DCPA, CaHPO₄) which can react together to form calcium deficient HA with a Ca/P ratio of 1.6.

The porosity of the composite scaffolds and native polymer scaffold was determined with the help of a mercury porosimeter. Table 5-1 lists the results that were obtained. The total porosity of polymer 1 nanofiber scaffold was 85 % with 91µm as the average pore diameter. These numbers decreased as micron sized HAp particles were incorporated within the polymer scaffold. However, an increase in the total specific area was observed for the composite scaffold due to the added surface area of the HAp particles.

The composite scaffolds were placed in water to convert HAp precursor to HA. Figure 5-10 shows the X-ray diffraction pattern of the HA formed at 37°C after 24 hours of incubation, from the precursors. A broad peak from 31° to 34° (2θ) was observed for the composites, indicating the formation of poorly crystalline HA. Crystalline HA shows three sharp peaks at 31.83°, 32.10°, and 32.90° (2θ). SEMs of the composite scaffold showed that the fibers retained their morphology after HA formation.
Figure 5-9: Scanning electron micrographs of electrospun polymer CDSHAp composite mats
Figure 5-10: Composite scaffolds of polymer 1 and CDS HAp. Formation of hydroxyapatite from its precursors is followed by XRD.
Table 5-1: Porosity measurements on electrospun Polymer 1 / Hydroxyapatite composite scaffolds.

<table>
<thead>
<tr>
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<th>Polymer 1 nanofiber mat</th>
<th>Polymer 1/CDS HAp(93 wt%) Mat</th>
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<tbody>
<tr>
<td>Total specific surface area (m^2/g)</td>
<td>31.315</td>
<td>68.216</td>
</tr>
<tr>
<td>Average pore diameter (micron)</td>
<td>91.90</td>
<td>41.412</td>
</tr>
<tr>
<td>Total porosity (%)</td>
<td>85.50</td>
<td>60.805</td>
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</table>
5.4 Conclusions

Non-woven, nanofiber scaffolds of poly[bis(ethyl alanato)phosphazene] can be readily produced by electrospinning. The optimized processing conditions of 20 wt/vol chloroform solution, 25 kV applied potential, 0.5 ml/hr flow rate and 20 cm as the distance between the needle tip and collection screen, produced fibers of polymer 1 with average diameter as 400 nm. The fibers formed showed an enhancement in the rate of hydrolysis when compared to bulk solution-cast films of the same polymer and this was attributed to an increased surface area of the nanofibers. During immersion for 4 weeks in phosphate buffer saline solution, nanofibers of polymer 1 underwent a 73 % decline in molecular weight and a 10 % decline in mass. SEM analysis also indicated significant morphological changes during the degradation period such as shrinkage in size and corroded fiber surface. On the other hand, the solution cast bulk films did not record any mass loss or morphological changes during the duration of the study.

In addition to polymer nanofiber scaffolds, electrospinning was also successful in producing composite scaffolds of hydroxyapatite precursors and polymer 1. The hydroxyapatite precursor in these composites could be converted, in situ, to hydroxyapatite under physiological conditions. The porosity of these developed scaffolds was higher than 60% with average pore size greater than 41 micron. Thus, in this study, highly porous, biodegradable polymer scaffolds were produced that may find applications in fields such as tissue engineering or drug delivery.
5.5 References

14. Huang, Z.M.; Zhang, Y.Z.; Kotaki, M.; Ramakrishna, S. *Composite Science Technology* 2003, 63, 2223-2253.


18. Adapted from http://www.che.vt.edu/Wilkes/electrospinning/electrospinning.html


Chapter 6

Tyrosine-Bearing Polyphosphazenes

6.1 Introduction

There is an ongoing search for polymeric materials that can be used in biomedical applications. Materials that incorporate α-amino acids have generated considerable interest because they offer certain advantages over conventional polymer systems. They are structurally diverse, relatively nontoxic, tissue-compatible, and the degradation products may be nontoxic and easily metabolized by living tissues [1]. Synthetically-derived poly(α-amino acids) have been investigated for applications in degradable sutures, artificial skin, membranes for artificial kidney, wound dressing, tissue engineering and controlled drug delivery [1, 2, 3, 4, 5]. However, these materials have some undesirable properties which include thermal degradation on melting, insolubility in common solvents, and a high cost of production. For these reasons, other methods have been examined for the incorporation of amino acids into polymers to form new materials which might have improved material properties and overcome the undesirable physicomechanical characteristics associated with some conventional poly(α-amino acids).

This has led to the development of two classes of amino acid derived organic polymers. The first class includes pseudo-poly(α-amino acids), which are synthetic polymers composed of α-amino acids linked by nonpeptide bonds such as ester, carbonate, or urethane linkages [1]. Examples of pseudo poly(α-amino acids) have been
developed by Kohn, et al. [6, 7] based on tyrosine-derived polycarbonates. These materials show promise as temporary scaffolds for tissue regeneration and as drug delivery polymers. The second class consists of copolymers which contain amino acids and non-amino acids within the same polymer backbone. A widely investigated example of this class includes copolymers formed between poly(ethylene glycol) (PEG) and amino acids [8, 9]. The PEG component of the polymer increases the solubility of the poly(amino acids) in water.

In recent years, certain polyphosphazenes have also shown promise as prospective materials for various biomedical applications [10, 11, 12, 13]. Polyphosphazenes possess a backbone of alternating phosphorus and nitrogen atoms with two organic side groups attached to each phosphorus atom. These polymers are synthesized by the reactions of poly (dichlorophosphazene) with organic nucleophiles, such as, alkoxides, aryloxides or amines. Amino acid ester functionalized polyphosphazenes are biodegradable and have been shown to possess tunable degradation rates for targeted applications [14]. One such polymer, poly[bis(ethylglycinat-N-yl)phosphazene] (I, R=H), readily degrades in aqueous media to form phosphates, ammonia, glycine, and ethanol, all of which can be metabolized or excreted by the body [15, 16, 17]. Hydrolysis of the phosphazene backbone is affected by many factors and a rough correlation exists between hydrolytic instability and side group leaving ability. Amine-linked side groups with relatively low pK\textsubscript{a} values (~ 7-9) render the backbone more susceptible to hydrolytic attack than side groups with higher pK\textsubscript{a} values (> 10). In addition, for the α-amino acids, the relative rates of hydrolysis are also affected by the bulkiness of R groups in the general formula,
H$_2$NCH(R)COOR'. The relative rates of hydrolysis of four amino acid containing polyphosphazenes are shown in Figure 6-1.

In this study, we have synthesized a group of polymers based on L-tyrosine substituted polyphosphazenes. These polymers are an addition to the broad series of $\alpha$-amino acid substituted polyphosphazenes that have been studied in our program and elsewhere [10, 11, 12, 13, 14, 15, 16, 17]. The development of tyrosine-functionalized polyphosphazenes is of special interest because, unlike the previously studied poly[(amino acid ester)phosphazenes], tyrosine derivatives can be linked to the phosphazene backbone via the amino (2) or the phenolic group (3) (Figure 6-2). In earlier work we have shown that polyphosphazenes that bear tyrosine groups linked via the amino group to the skeletal phosphorous atoms together with glycine ethyl ester cosubstituents are hydrolytically erodible. The rates of hydrolysis depend on the ratio of the two side groups, with the slowest rate being associated with the highest concentration of tyrosine [18].

The attachment of tyrosine to the phosphazene backbone via the phenolic group could impart pH-sensitive solution or gel properties to this system. The presence of both acidic and basic ionizable groups should yield a material that undergoes one or more phase transitions in aqueous media over a wide range of pH values. Moreover, the pendent carboxylate groups can be utilized to form ionic hydrogels in the presence of di- or trivalent cations. Polymers with pH-sensitive solution properties have been of current interest due to their potential use in biomedical applications such as drug delivery, tissue engineering and biological membranes [19]. Naturally derived pH sensitive materials are based on alginates, dextrans and chitosan-derived materials [20, 21]. Ionic hydrogels are
interesting materials because their equilibrium degree of swelling is affected by changes in pH or ionic strength [22, 23]. This behavior makes them suitable for use in membranes and microcapsules, and in biocompatible materials. In a previous study it was shown that ionic hydrogels are formed by the Ca$^{2+}$ cross-linking of aqueous solutions of sodium poly[bis(carboxylatophenoxy)phosphazene], [NP(OC$_6$H$_4$COONa)$_2$]$_n$. The resultant hydrogels are useful for biological microencapsulation and complexation to other metal cations [24, 25].
Figure 6-1: Relative hydrolysis rates for poly[(amino acid ester)phosphazenes]
Figure 6-2: Tyrosine functionalized polyphosphazenes
6.2 Experimental Section

6.2.1 Reagents and Equipment:

The synthesis reactions were carried out under an atmosphere of dry argon using standard Schlenk line techniques. Hexachlorocyclotriphosphazene (Ethyl Corp. and PCS) was obtained from a trimer-tetramer mixture by sublimation (30 °C / 0.2 mm Hg). Poly(dichlorophosphazene) was prepared by the ring-opening polymerization of hexachlorocyclotriphosphazene in an evacuated Pyrex tube at 250 °C. Tetrahydrofuran (THF) was distilled from sodium benzophenone ketyl under a dry argon atmosphere. 1-Propanol (Aldrich) was distilled over calcium hydride. All other solvents were obtained from Aldrich and were used without further purification. N-t-BOC-L-tyrosine methyl ester (all from Sigma), sodium hydride (60% dispersion in mineral oil, Fluka), sodium (Aldrich), potassium tert-butoxide (Aldrich), trifluoroacetic acid (99% Aldrich), and buffer solutions (Aldrich) were used as received. All $^{31}$P NMR (145 MHz), $^1$H NMR (360 MHz) and $^{13}$C NMR (90.27 MHz) data were obtained with use of a Bruker 360 MHz spectrometer. $^{31}$P NMR chemical shifts are reported in ppm relative to 85% H$_3$PO$_4$ at 0 ppm. Gel permeation chromatography was carried out with use of a Hewlett-Packard HP-1090 liquid chromatograph fitted with an HP-1037A refractive index detector and a Polymer Laboratories gel 10-μm column. Glass transition temperatures were determined with use of a Q10 DSC apparatus, with a heating rate of 10 °C/min under a nitrogen atmosphere.
6.2.2 Synthesis

Synthesis of Polymers 5-7. These reactions were carried out in a similar manner. The procedure for the synthesis of polymer 7 is given as a typical example. Details for the synthesis of polymer 5 and 6 are listed in Table 6-1. Poly(dichlorophosphazene) (4) (2.00g, 17.3 mmol) was dissolved in 400 ml of THF. In a separate reaction vessel, 1-propanol (1.26 gms, 20.9 mmol) was added to a suspension of sodium hydride (0.76 gms, 18.98 mmol, 60% dispersion in mineral oil) in 50 ml of THF and was allowed to react for 24 hours. Sodium propoxide solution was then added slowly to the polymer solution via an addition funnel. The reaction was allowed to proceed at room temperature for 24 hours. N-t-BOC-L-tyrosine methyl ester (7.86 gms, 26.6 mmol) was then added to a suspension of sodium hydride (0.97 gms, 24.15 mmol, 60% dispersion in mineral oil) in 50 ml of THF and allowed to react for 24 hours, at room temperature. After the reaction was complete, this solution was added dropwise via an addition funnel to the reaction mixture that contained the partially substituted polymer. The reaction solution was then heated at reflux for 48 hours. The completion of the reaction was checked by $^{31}$P NMR spectroscopy. The polymer was purified by precipitation from heptane (2 xs), and by dialyses against methanol for 72 hours. $^{31}$P NMR (D$_2$O), ppm: δ -7.6, -12.5, -18.3. $^1$H NMR, ppm: 0.6 (3H), 1.3 (2H), 1.4 (9H), 3.0 (2H), 3.5 (2H), 3.7 (3H), 4.1 (1H), 7.01 (4H). $^{13}$C NMR, ppm: δ 180 (1C), 158 (1C), 152(1C), 135 (2C), 132 (2C), 122 (1C), 81 (1C), 71 (1C), 59 (1C), 53 (1C), 39 (1C), 29 (3C), 25 (1C), 11 (1C). IR, cm$^{-1}$: 3400-3200 (NH), 1738 (C=O), 1687 (NHC=O), 1393, 1366 (t-butyl), 1214 (P=N/P-O), 1168 (OCH$_3$).
Synthesis of Polymer 7b. Polymer 7a (1.0 g, 2.5 mmol) was allowed to swell in 50 ml of THF. Potassium tert-butoxide (3.928 g, 35 mmol) was dissolved in 50 ml of THF. The solution was cooled to 0 °C and a catalytic amount of water was added. The resultant solution was added slowly to the polymer suspension in THF, via an addition funnel. This reaction was allowed to proceed at room temperature for 24 hours. Cold water was added to the mixture and the polymer was precipitated by the addition of concentrated hydrochloric acid. The polymer was then dialyzed against deionized water for 48 hours. The solvent was evaporated and the polymer was dried under vacuum. The dried polymer was then treated with a 50 % trifluoroacetic acid / methylene chloride solution (20 ml) and the reaction was allowed to proceed for 8 hours. Excess acid was neutralized by the addition of a saturated solution of sodium bicarbonate. The polymer was then dialyzed against deionized water for 2 days after which time the solvent was evaporated to yield polymer 7b. The polymer was further dried under vacuum. $^3\text{P NMR}$ (D$_2$O), ppm: δ -7.6, -12.5, -18.3. $^1\text{H NMR}$, ppm: 0.6 (3H), 1.3 (2H), 3.0 (2H), 3.5 (2H), 4.1 (1H), 7.01 (4H). $^{13}\text{C NMR}$, ppm: δ 174 (1C), 151(1C), 132 (2C), 131 (2C), 121 (1C), 70 (1C), 56 (1C), 36 (1C), 24 (1C), 10 (1C). IR, cm$^{-1}$:3500-2600 (NH$_2$, COOH), 1724 (C=O), 1591, 1401 (COO$^-$), 1218 (P=N/P-O).

Ionic Crosslinking Reactions with Polymers 5b, 7b. Polymers 5b and 7b were dissolved in 0.2ml of deionized water. To each of the polymer solutions, was added an aqueous solution of CaCl$_2$ (0.006 mmol). The solutions were stirred for 1 min to produce the cross-linked gels.
Table 6-1: Reaction conditions for synthesis of polymer 5 and 6

<table>
<thead>
<tr>
<th>Polymer</th>
<th>[NPCl]₂ (mmol)</th>
<th>NaOR (mmol)</th>
<th>Solvent (reaction time, days)</th>
<th>t-BuOK⁺ (mmol)</th>
<th>TFA (ml)</th>
<th>³¹P NMR (ppm)</th>
</tr>
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<tbody>
<tr>
<td>5</td>
<td>17.2</td>
<td>a17.2</td>
<td>THF (3)</td>
<td>24.4</td>
<td>20</td>
<td>-7.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-13.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-18.2</td>
</tr>
<tr>
<td>6</td>
<td>17.2</td>
<td>b17.2</td>
<td>THF (1)</td>
<td>24.4</td>
<td>20</td>
<td>-9.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-12.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-15.3</td>
</tr>
</tbody>
</table>

R' : N-t-BOC-L-tyrosine methyl ester

R^a : methoxyethoxyethanol

R^b : trifluoroethanol
6.3 Results and Discussion

Among all the known polyphosphazenes, poly[(amino acid ester) phosphazenes] are probably the most popular biodegradable polymers due to their proven biocompatibility. Traditionally, these polymers have been synthesized by attaching an ester derivative of naturally occurring amino acid to the phosphazene backbone via the amino terminus. In this work we have investigated the effect of, attaching an amino acid side group to the phosphazene backbone through two different functional sites. Tyrosine-functionalized polyphosphazenes were synthesized and their hydrolytic stability, pH-sensitive behavior, and hydrogel forming capabilities were investigated. The physical and chemical properties of the polymers varied with the type of linkage between the tyrosine unit and phosphazene backbone. Poly[(ethyl glycinat-N-yl) (ethyltyrosinat-N-y1)phophazenes] (linkage via the amino group of tyrosine) were found to be hydrolytically erodible [18, 26]. The rate of hydrolysis was dependent on the ratio of the two side groups, with the slowest rate being associated with the highest concentration of tyrosine. For example, a 20-25% mass loss was detected for a polymer with 8% tyrosine units after 30 days, whereas a polymer with 83% tyrosine units underwent ~ 4% mass loss in the same time span. This is consistent with the behavior of other poly[(amino acid ester)phosphazenes], where bulkier groups linked to the α-carbon of the amino acid residue yield materials that are more resistant to hydrolysis. The hydrolysis products were identified as phosphates, tyrosine, glycine, ammonia, and ethanol derived from the ester group.
Although the synthesis of fully O-linked tyrosine substituted polymer 3 was attempted, complete chlorine replacement did not occur. For this reason, two-reagent reactions were carried out with smaller co-substitutents to yield fully substituted polymers 5a, 6a and 7a. The mixed substituent polymers were synthesized by the initial treatment of poly (dichlorophosphazene) (4) with the less hindered nucleophile, followed by the addition of an excess of sodium salt of N-t-BOC-L-tyrosine methyl ester. The synthetic outline is shown in Figure 6-3. The less sterically hindered alkoxy-based side group was introduced first to avoid potential reactions of the alkoxide with the tyrosine ester groups. To obtain free carboxylic and amino groups, deprotection reactions were carried out with the use of potassium-tert-butoxide (to remove the ester group) and trifluoroacetic acid (to remove the t-butoxycarbonyl group).

The first of three alkoxy-based side groups used was the methoxyethoxyethoxy (MEE) unit. Single-substituent polymers with this side group are readily soluble in water and polar organic solvents, and possess interesting solution and biomedical properties [27]. Copolymer analogs have shown promise in drug delivery systems [22]. The alkyl ether side group also provides sites that can be cross-linked by gamma irradiation [22]. Polymer 5b, with 55% MEE and 45% tyrosine was synthesized. The pH sensitive solubility behavior of this polymer was examined over the pH range of 2-12. Polymer, 5b, did not show pH-dependant solubility behavior. This was attributed to the highly hydrophilic alkyl ether side groups which enhance the water solubility of the polymer. Thus, the hydrophilic properties of the alkyl ether side groups, coupled with the presence of only 45% tyrosine groups, presumably dominate the solution behavior and contribute to solubility in aqueous media over a wide range of pH values. The solubility behavior of
polymer 5b in selected solvents is summarized in Table 6-2. The polymer precipitated from aqueous solution when CaCl$_2$ was added. This demonstrated that polyphosphazene-based ionic hydrogels can be obtained with the use of tyrosine side units. The proposed structure is shown in Figure 6-4.

To prepare a polymer with a more amphiphilic character, mixed-substituent polyphosphazene of structure 6b was synthesized. The trifluoroethoxy co-substituent was chosen for its hydrophobicity. This polymer was insoluble over the entire range of pH values tested (2-12). This insolubility was attributed to the combination of hydrophobic trifluoroethoxy side units and amphiphilic tyrosinyl groups which appears to prevent dissolution in either hydrophilic or hydrophobic solvents.

Polymer 7a was then synthesized to incorporate the less-hydrophobic propoxy side groups. Figure 6-3 summarizes the synthetic strategy adopted. The first step was a macromolecular substitution in which poly (dichlorophosphazene) was allowed to react with the sodium salt of propanol followed by the sodium salt of N-t-BOC-L-tyrosine methyl ester. This yielded the fully substituted polymer 7a. This polymer was a brittle, brown-colored powder that was soluble in methanol. The methyl ester units were then hydrolyzed by treatment with potassium tert-butoxide followed by acidification with hydrochloric acid to form the carboxylic acid. The partially deprotected carboxylic acid polymer was insoluble in water. Thus the polymer was neutralized with sodium bicarbonate to obtain the sodium salt derivative which was water soluble. Trifluoroacetic acid was employed for the removal of the BOC protecting group. Characterization data for polymer 7b are discussed in Table 6-3.
Unlike polymer 5b and 6b, polymer 7b showed a pH-dependent solubility behavior in aqueous media. The ability of polymer 7b to form hydrogels following the addition of divalent cations was also investigated. The addition of a CaCl$_2$ solution to the aqueous polymer solution at pH 7.4 caused precipitation of the polymer via the formation of ionic crosslinks. The proposed crosslinked structure is similar to the crosslinked structure shown for polymer 5b in Figure 6-4.

6.3.1 pH Studies

Polymer 7b showed pH-dependent solubility behavior in aqueous media that indicated the presence of free amino and carboxylic acid groups. Polymer 7b was soluble in basic aqueous media. At approximately pH 4, the polymer precipitated from solution. Below pH 2, 7b redissolved.

Comparison of this polymer with the corresponding species with oligo-ethyleneoxy or trifluoroethoxy cosubstituent groups, suggests that the propoxy units are a main factor that tip the balance to forming an amphiphilic polymer. The pH-responsive solubility behavior is controlled by the extent of ionization of the pendent carboxylic and amine functionalities. Within the tyrosinyl groups a relatively high degree of hydrogen bonding is present at low pH values (3-4) as a result of the presence of $\alpha$-COOH units. This, in turn, decreases the solubility of the polymer, which corresponds to the behavior of other polyphosphazenes with pendent carboxylic acid groups. Although the pK$_a$ value of $\alpha$-COOH in free tyrosine is 2.20, the insolubility at pH 4 indicates that enough
protonated acid groups are present to override the expected water solubility expected from the influence of the quaternized amine groups [27].

The effect of temperature and side group ratio on the pH-sensitive solubility behavior of polymer 7b was also investigated. Within the temperature range of 25 °C – 37.2 °C, polymer 7b showed a similar dependence of solubility on the pH of the media, as discussed above. However, with a change in the ratio of the two side groups, the solution properties of the polymer changed. A decrease in the amount of tyrosine as a cosubstituent yielded polymers that were no longer soluble in water. Instead, they formed hydrogels which showed a pH-dependent swelling behavior. A polymer with 55% tyrosine and 45% propoxy groups existed in a contracted state between pH 3-4. Below pH 3 and above pH 4, the polymer underwent considerable swelling. At pH 7.4, the percentage of water absorption corresponded to 410% (Figure 6-5).
Figure 6-3: Synthesis of polyphosphazenes with tyrosine and alkoxy units.
Figure 6-4: Hydrogel formation of tyrosine-bearing polyphosphazenes in the presence of Ca\(^{2+}\).
Table 6-2: Solubility of polymers in select solvents.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>THF</th>
<th>Methanol</th>
<th>Other Alcohols&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Water</th>
</tr>
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<tbody>
<tr>
<td>5a</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>5b</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes (all pH values)</td>
</tr>
<tr>
<td>6a</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>6b</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>7a</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>7b</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes(&lt;pH 3, &gt; pH 4)</td>
</tr>
</tbody>
</table>

<sup>a</sup> ethanol and isopropanol.
Table 6-3: Characterization data for poly[(60%L-tyrosinyl) (40%propyl)phosphazene]

<table>
<thead>
<tr>
<th>Polymer</th>
<th>$^{31}$P NMR, ppm</th>
<th>$M_w$&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PDI</th>
<th>$T_g$ (&lt;sup&gt;0&lt;/sup&gt;C)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>$T_d$ (&lt;sup&gt;0&lt;/sup&gt;C)&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>7b</td>
<td>-12.5</td>
<td>1.3</td>
<td>4.4</td>
<td>19.2</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>-18.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Determined by GPC (x $10^5$), using polyethyleneoxide standards.

<sup>b</sup>Determined by DSC analysis.

<sup>c</sup>Determined by TGA analysis.
Figure 6-5: pH-Dependent swelling behavior of poly [(ethyl tyrosinat-O-yl) 40% (propyl) 60% phosphazene] in aqueous media, at 25°C.
6.4 Conclusions

In this study, a series of tyrosine functionalized polyphosphazenes were synthesized and characterized. The phenolic linked tyrosine derivatives were prepared from N-t-BOC-L-tyrosine methyl ester and alkoxy-based cosubstituents. Mixed-substituent tyrosine/ oligo-ethyleneoxy polyphosphazene were soluble in aqueous media from pH 2 to 12. This polymer formed hydrogels in the presence of divalent cations. Based on previous work, radiation crosslinking of this polymer should yield hydrogels that are acutely responsive to pH changes.23 A polyphosphazene with propoxy and tyrosinyl cosubstituents showed pH-sensitive solubility behavior. Polymers with more than 55 % L-tyrosine attached to the phosphazene backbone were soluble in aqueous media, at pH 2, and were also soluble from pH 5-12. At pH 3-4, the polymers were insoluble. This is the first polyphosphazene to show a complex room temperature pH-dependent solubility behavior at various pH intervals between pH 2 and 12. This system also offers the possibility to form hydrogels via ionic crosslinks formed in the presence of divalent ions. Polymers with less than 50% tyrosine cosubstituent formed hydrogels with pH sensitive solution properties. The hydrogels showed considerable swelling below pH 3 and above pH 4.

Thus, the tyrosine functionalized polyphosphazene system offers the opportunity to incorporate properties such as bioerosion or pH sensitive behavior into one material by structural variations at the molecular level. Polyphosphazenes that bear tyrosine groups linked via the amino group are hydrolytically erodible and are suitable for tissue
engineering applications. The phenolic-linked tyrosine derivatives show pH-sensitive solution properties and may be useful in applications such as controlled drug delivery.

6.5 References


3. Huang, S.J.; Ho, L-Hua; Macromolecular Symposia 1999, 144, 7.


Appendix A

Development of Physiological Temperature Setting Polymer-Ceramic Composites for Bone Tissue Engineering

A.1 Introduction

One of the more interesting and challenging problems that exits today in the biomedical field is the development of bone repair and replacement materials. Autografts, which have been the gold standard in orthopedic treatments, suffer from disadvantages such as limited availability and donor site morbidity [1]. Allografts, which are donor bones from another source, overcome the problems with autografts as they are more readily available. However these materials have the potential of transmitting diseases or eliciting an unfavorable immunogenic response [1]. Synthetic materials such as metals, ceramics and polymers are also used in treating bone defects but their current properties are far from being ideal for this application [2, 3]. Among the various techniques that are currently under investigation, bone tissue engineering seems to be the most promising [4, 5]. This technique aims at developing biodegradable material scaffolds that can initiate the repair and regeneration of damaged bone.

Bone is a ceramic–polymer composite of approximately 70% hydroxyapatite (the mineral component) and 30% collagen (the polymeric component). Thus, one of the approaches in bone tissue engineering is to make bone like composites of hydroxyapatite and a biodegradable polymer [6]. Hydroxyapatites (HA) are calcium phosphate salts classified as stoichiometric or calcium deficient HA, depending on the Ca/P ratio.
Stoichiometric HA is $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ with a Ca/P ratio of 1.67 and fully calcium-deficient HA is $\text{CaHPO}_4(\text{PO}_4)_5\text{OH}$ with a Ca/P ratio of 1.5 [7]. Hydroxyapatites have been extensively studied as bone repair materials due to their biocompatibility, osteoconductivity, resorbability and their ability to interact with surrounding bone [8, 9, 10]. However, factors such as brittleness, low tensile loading and low impact resistance have limited their use. To overcome these disadvantages, hydroxyapatites are combined with polymers to improve upon their mechanical properties and also form structures that closely mimic the structure of natural bone [11, 12].

Synthetic biodegradable polymers such as poly(lactide-co-glycolide) (PLAGA) have been used to make composites with HA [13,14,15,16]. PLAGA is commercially available and also has FDA approval for some biomedical applications. However, PLAGA is known to degrade by a bulk-erosion mechanism which leads to a loss of over 50% of its mechanical strength in less than two months [17,18]. Also, due to acidic degradation products, the use of PLAGA can cause some biocompatibility concerns [17, 18]. Natural polymers such as collagen, chitosan and gelatin have also been used to form composites with HA [14, 19, 20]. However, since these polymers are derived from an animal source, problems of immunogenicity and batch-to-batch variations limits the usefulness of these materials. As an alternative approach to this problem, we have investigated the possibility of forming composites of degradable polyphosphazenes with HA for bone tissue engineering application.

Polyphosphazenes are hybrid polymers with a backbone of alternating phosphorus and nitrogen atoms and with two organic side groups attached to each phosphorus atom [21]. These polymers are synthesized by the reactions of alkoxides, aryloxides or amines.
with a highly reactive macromolecular intermediate, poly(dichlorophosphazene). Because a large number of different side groups can be introduced in these reactions, a wide range of properties may be generated with this polymer system [21]. With respect to bone tissue engineering application, polyphosphazenes offer the following advantages. First, biodegradable polyphosphazenes hydrolyze into nontoxic small molecule products which can be easily removed from the body. The hydrolysis products include phosphates and ammonia from the backbone, thus forming a pH buffered system [22]. Second, the synthetic versatility of polyphosphazenes allows good control over properties such as degradation rate and strength of the polymer [23, 24]. Third, ionizable side groups can be introduced along the polymer backbone which can interact with hydroxyapatite, thus forming an intimately reinforced composite.

Tenhuisen et al. have studied composites of poly[bis(carboxylatophenoxy)phosphazene] with HA [25]. Stoichiometric HA was formed in situ, under physiological conditions, in presence of polymer particles. Greish et al. further developed this system by forming composites of HA and derivatives of poly[bis(carboxylatophenoxy)phosphazene] [26, 27, 28]. They showed that the carboxylic acid units on the polymer could interact with the calcium ions from HA, thus forming an intimately reinforced composite. However due to the non-degradability of the phosphazene polymer used in these studies, this composite system has limited use in bone tissue engineering. Thus, in this study we have investigated the feasibility of forming composites of HA with degradable polyphosphazenes.

The polymers selected for this study were amino acid ester functionalized polyphosphazenes. These polymers are biodegradable and biocompatible [24, 29, 30].
The hydrolyzed ester units of the amino acid side group also present sites for interaction with the calcium ions of the HA phase, thus resulting in a better reinforcement of the composite. Three polymers used in this study were poly[bis(ethyl alanato)phosphazene] (1), poly[(ethyl alanato)\(_2\)(p-methyl phenoxy)\(_4\)phosphazene] (2) and poly[(ethyl alanato)\(_2\)(p-phenyl phenoxy)\(_4\)phosphazene] (3) (Figure A-1). These polymers were selected on basis of their \textit{in vivo} biocompatibility [Chapter 4]. Several research groups have reported on the biocompatibility of poly[bis(ethyl alanato)phosphazene]. It has been used successfully as tissue engineering membranes in treatment of periodontal disease and as nerve guide conduits for nerve regeneration [32, 33]. Polymers 2 and 3 have 50% of the alanine side groups replaced by aryloxy groups forming polymers with improved mechanical properties and a slightly slower degradation rate compared to polymer 1 [23].

Calcium deficient HA is believed to be more biocompatible than stoichiometric HA due to its similarity to biological apatite and its ability to be remodeled by native bone [28]. Also, self-setting composites present an advantage over composites with preformed HA as they are more moldable and also have the potential of forming an interlocked interface with surrounding bone during HA formation. Thus in this study, polymer-HA precursor composites were synthesized followed by the in situ formation of calcium deficient HA under physiological conditions. The polymer-ceramic composites were characterized by XRD, pH measurements, SEM, mechanical property evaluation and porosity measurements. The \textit{in vivo} response to the composites was tested in a unicortical rabbit model.
Figure A-1: Polymer structures of L-alanine co-substituted polyphosphazenes. 1: poly[bis(ethyl alanato)phosphazene]; 2: poly[(ethyl alanato)\textsubscript{1} (p-methyl phenoxy)\textsubscript{1} phosphazene]; 3: poly[(ethyl alanato)\textsubscript{1} (p-phenyl phenoxy)\textsubscript{1} phosphazene].
A.2 Experimental Section

Polymer synthesis and characterization and preparation of polymer/hydroxyapatite composites were carried out in Dr Allcock’s laboratory.

Synthesis of hydroxyapatite precursors and pH measurements and mechanical property evaluation of polymer/hydroxyapatite composites was carried out in Dr Brown’s laboratory.

XRD Analysis, SEM and in-vivo biological testing of the polymer/hydroxyapatite composites were carried out in Dr Laurencin’s laboratory.

A.2.1 Reagents and Equipment

Synthesis reactions were carried out under an atmosphere of dry argon using standard Schlenk line techniques. Hexachlorocyclotriphosphazene (Ethyl Corp. and PCS) was obtained from a trimer-tetramer mixture by recrystallization from heptane followed by sublimation (300°C / 0.2 mm Hg). Poly(dichlorophosphazene) was prepared by the ring-opening polymerization of hexachlorotriphosphazene in a sealed evacuated Pyrex tube at 250°C. The same batch of poly(dichlorophosphazene) was used in the synthesis of polymers 1-3. Ultra pure, anhydrous tetrahydrofuran (THF), toluene and triethylamine were obtained from solvent dispensing system designed by J C Meyer. L-Alanine ethyl ester hydrochloride (Chem Impex International Inc), 4-methylphenol, 4-phenylphenol (all from Aldrich), and sodium hydride (60% dispersion in mineral oil, Aldrich) were used as received. Spectra/Por regenerated cellulose dialysis membranes with a molecular weight cut-off of 12,000-14,000 were used for purification of the polymers. 31P NMR (145 MHz)
and $^1$H NMR (360 MHz) data were obtained with use of a Bruker 360 MHz spectrometer. $^{31}$P NMR chemical shifts are reported in ppm relative to 85% H$_3$PO$_4$ at 0 ppm. Gel permeation chromatography (GPC) was carried out with use of a Hewlett-Packard HP-1090 liquid chromatograph fitted with an HP-1047A refractive index detector and two phenogel 10-µm linear columns (Phenomenex, CA), calibrated with polystyrene standards (Polysciences, PA). The samples were eluted at 40°C with a 0.1 wt% solution of tetra-n-butyl ammonium nitrate (Aldrich, WI) in THF (EM Science, NJ). Glass transition temperatures were determined from a TA Instruments Q10 differential scanning calorimeter (DSC) apparatus with a heating rate of 10°C/min.

**A.2.2 Polymer Synthesis**

*Synthesis of Polymer 1.* L-Alanine ethyl ester was prepared by treatment of alanine ethyl ester hydrochloride (106.04 g, 0.690 mol) in refluxing THF (500 mL) with triethylamine (288 mL, 2.071 mol). After the solution had been stirred for 24 hours, the reaction mixture was filtered and the filtrate was added to a stirred solution of poly(dichlorophosphazene) (20.00 g, 0.173 mmol) in THF (2000 mL). The reaction mixture was then stirred at room temperature for 48 hours. The insoluble salts were removed by filtration and a white fibrous polymer was obtained by precipitation of the viscous polymer solution into hexanes. Purification of the polymer was accomplished by repeated precipitations from THF into hexanes (3X), followed by dialysis against a THF/methanol (50/50) mixture for 3 days. $^{31}$P NMR (CDCl$_3$), ppm: $\delta$ -3.5; $^1$H NMR
(CDCl$\textsubscript{3}$), ppm: $\delta$ 4.1-4.08 (3.6H), 1.4-1.27 (3H), 1.29-1.19 (3H). $M_n = 89000$ g/mol, $M_w = 196000$ g/mol, PDI = 2.2. $T_g = -10^\circ$C

*Synthesis of Polymer 2.* Poly(dichlorophosphazene) (20.0 g, 0.173 mol) was dissolved in THF (2000 ml). In a separate reaction vessel, p-cresol (20.53 g, 0.190 mol) was added to a suspension of sodium hydride (4.36 g, 0.173 mol) in THF (250 mL) and the reaction was allowed to proceed for 24 hours. Sodium p-methylphenoxide solution was then added slowly to the polymer solution via an addition funnel. The reaction was allowed to proceed at room temperature for 24 hours. L-Alanine ethyl ester (79.54 g, 0.518 mol) in THF (700 mL) was then added to the reaction mixture that contained the partially substituted polymer. The reaction solution was then heated at reflux for 48 hours. The polymer was purified by repeated precipitations from THF into hexanes (3X) and methanol (2X). $^{31}$P NMR: (CDCl$\textsubscript{3}$), ppm: $\delta$ -5.8, -7.7, -18.1; $^1$H NMR (CDCl$\textsubscript{3}$), ppm: $\delta$ 7.7-6.4 (4H), 4.2-3.9 (3.8H), 2.2-1.8 (3H), 1.1-0.7 (6H). $M_n = 2,219,000$ g/mol, $M_w = 4,608,000$ g/mol, PDI = 2.076. $T_g = -6^\circ$C

*Synthesis of Polymer 3.* The synthesis of polymer 3 was accomplished in a similar manner to polymer 2. A stoichiometric amount of the more bulky side group, sodium salt of p-phenyl phenol (32.31 g, 0.173 mol), was added to poly(dichlorophosphazene) solution (20 g, 0.173 mol) followed by the addition of excess amounts of L-alanine ethyl ester (116.64 g, 0.759 mol). The polymer was purified by repeated precipitations from THF in to hexanes (3X) and methanol (2X). $^{31}$P NMR: (CDCl$\textsubscript{3}$), ppm: $\delta$ -5.2, -7.3, -17.97; $^1$H NMR (CDCl$\textsubscript{3}$), ppm: $\delta$ 7.8-7.2 (9H), 4.8-3.9 (3.8H), 1.3-0.6 (6H). $M_n = 1020000$ g/mol, $M_w = 1900000$ g/mol, PDI = 1.86. $T_g = 35^\circ$C
A.2.3 Synthesis of Hydroxyapatite Precursors

Synthesis of CDHAp (Ca/P – 1.5) and CDSHAp (Ca/P – 1.6) precursors:

Tetracalcium phosphate (TetCP, Ca₄(PO₄)₂O) and dicalcium phosphate anhydrous (DCPA, CaHPO₄) were reacted in varying ratios to obtain two different calcium deficient hydroxyapatite precursors. TetCP was made by ball milling CaCO₃ (Osram-Sylvania, PA) and monocalcium phosphate monohydrate (MCPM, Ca(H₂PO₄)₂·H₂O, FMC Corp., NY) at a 3:1 molar ratio for 16 hours in heptane (Alfa Aesar, Ward Hill, MA). After filtering and drying, the TetCP was fired in air at 1400°C for 1 hour and quenched rapidly. X-ray diffraction was used to confirm phase pure TetCP. The TetCP was ground by hand, sieved, ball milled, and attrition milled to reduce particle size. TetCP and DCPA were then mixed in the desired Ca/P ratio and ball milled in heptane. After synthesis, the precursor powders were stored in a dessicator under vacuum to avoid hydration. The average particle size was 2.5um, as measured by SEM.

A.2.4 Preparation of Polymer/Hydroxyapatite Composites

Preparation of Biodegradable Polyphosphazene – Calcium deficient hydroxyapatite Precursors Composites: Composites of polymers 1, 2, and 3 with CDHAp and CDSHAp precursors were synthesized by emulsion technique. Briefly, 1.5 g of polymer was dissolved in 30ml of dimethylformamide (methanol was used for polymer 1). The polymer solution was then added drop wise to a vigorously stirred suspension of 15 g of the calcium deficient HAp precursor in 1 liter of heptane (Fisher Scientific, USA) and 50ml of dimethylformamide at room temperature. The suspension
was stirred for 10 minutes and the excess solvent was evaporated to dryness using a rotary evaporator. The resultant solid was dried under vacuum at 50°C for 72 hours.

The polyphosphazenes – calcium deficient hydroxyapatite precursor powders were finely ground using a mortar and pestle (Fisher, USA). The composite precursor powders were mixed with 0.5% phosphoric acid (Acros 201140010) to form a paste. The pastes were allowed to set for 24 hours at 37°C in a humidified atmosphere to form the composite.

A.2.5 Physico-Chemical Analysis of Polymer/Hydroxyapatite Composites

X-ray Diffraction: X-ray diffraction (XRD) measurements were performed on a Scintag XDS 2000 diffractometer to confirm the formation of calcium deficient hydroxyapatite composites under the current fabrication conditions. The composites were finely ground using a mortar and pestle. The fine powder was mounted on a glass slide and analyzed between 20° and 50° (2θ) at an angular sweeping rate of 2° (2θ/min) with a step size of 0.05°.

pH-Measurements: The variation in pH over time was measured using an Orion 920 pH meter. Composite precursors were initially mixed with a small amount of water using a mortar and pestle before being placed in a double-walled glass beaker with a 35 ml of distilled, de-ionized water. The temperature of the reaction vessel was held constant at 37 °C. The liquid to solid ratio was approximately 70 to 1 and the mixture was stirred continuously with nitrogen bubbled through. The reaction pH was followed for 24 hours.
Scanning Electron Microscopy: The surface of the composites were analyzed using a scanning electron microscope, SEM (JEOL 6700F, USA). The composites were prepared and incubated as explained previously. The samples were coated with Gold/Palladium and were viewed under the SEM.

Mechanical Property Evaluation of the Composites: For compressive testing, the precursor powders were combined with water in a powder to liquid ratio of 2.5 to 1 and mixed on a glass sheet using a metal spatula. The paste was pressed by hand into cylindrical-shaped molds (0.5” in height and 0.25” in diameter), resulting in three pellets for each composite. The samples were cured in a humidified atmosphere at 37 °C for 24 hours. Compressive testing was performed on a Onstron 4202 (Instron, MA) using a cross-head speed of 0.3 mm/min. Samples were loaded to the point of failure.

A.2.6 In-vivo Biological Testing of Polymer/Hydroxyapatite Composites

Tibial Defect Model and Cement Injection: All procedures were approved by the Institutional, Animal Care and Use Committee. Thirty four healthy male New Zealand white rabbits were used and the animals were randomly divided into three different groups with 6 rabbits per groups per time point that received the composite bone cements and 5 rabbit per group per time point for the control. An incision of 10mm was made to expose the proximal and medial tibia. A 5mm unicortical defect was made by drilling burr (Synthes, USA) just medial to the tibia tuberosity. The cement paste was steriley prepared (0.5g composite in 0.5% phosphoric acid) and was injected into the defect site using a 1ml syringe (Becton Dickinson, USA). The muscle and skin was closed and the
same procedure was repeated on the contralateral limb. At predetermined time points (4 and 8 weeks) all animals were sacrificed and the limbs were excised for further analysis.

**Histology:** The tibia from the rabbits were excised immediately after sacrifice and placed in methanol (Fisher Scientific, USA) for 24 hours at 4°C and afterwards stored at room temperature for 1 week. The limbs were embedded in glycol methylacrylate (PolySciences, USA) for 3 weeks (n=3). The blocks were polymerized and sectioned 100µm inside the implant to obtain 5µm thick sections using a microtome and mounted on glass slides. The samples were stained with silver nitrate to demonstrate calcified bone (Von Kossa stain) followed by a hematoxylin and eosin counterstain.

### A.3 Results and Discussion

Polymers 1, 2 and 3 were synthesized by the macromolecular substitution route which involved two steps: thermal ring opening polymerization of hexachlorotriphosphazene at 250°C to form poly(dichlorophosphazene), followed by sequential substitution of the labile chlorine atoms of poly(dichlorophosphazene) by the sodium salt of the corresponding alcohol or by an ester protected amino acid. The polymers were characterized by NMR, GPC and DSC (experimental section).

The calcium deficient hydroxyapatite precursors were composed of tetracalcium phosphate (TetCP, Ca₄(PO₄)₂O) and dicalcium phosphate anhydrous (DCPA, CaHPO₄). The precursors were proportioned by varying the molar ratios of TetCP and DCPA to produce a Ca/P ratio of 1.5 for CDHA and 1.6 for CDSHA. Calcium deficient
hydroxyapatite can be formed from these precursors by acid-base reaction between TetCP and DCPA (Figure A-2).

The polymer-ceramic precursor composites (10 wt% polymer) were synthesized by emulsion technique. Briefly, this technique involved precipitating micro-particles of polymer in a suspension of hydroxyapatite precursors. The composite powders were then mixed with 0.5% phosphoric acid to form hydroxyapatite. The physico-chemical evaluation of the composites was done by XRD, SEM and solution property evaluation.
Figure A-2: Synthesis of calcium deficient hydroxyapatite

\[ 3 \text{CaCO}_3 + \text{Ca(H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O} \rightarrow \text{Ca}_4(\text{PO}_4)_2\text{O} \]

Tet CP - Ca/P = 2.0

Tet CP + CaHPO₄ $\xrightleftharpoons[{\text{H}_2\text{O}}]{}$ \( \text{Ca}_{10-x}(\text{HPO}_4)_x(\text{PO}_4)_{6-x}(\text{OH})_{2-x} \)

\[
\begin{align*}
x = 1.0 & \quad \text{CDHAp} & \text{Ca/P} = 1.5 \\
x = 0.4 & \quad \text{CDSHAp} & \text{Ca/P} = 1.6
\end{align*}
\]
Figure A-3 shows the X-ray diffraction pattern of six composites formed at 37°C after 24 hours of incubation from the precursors. The composites examined in this study had a broad X-ray peak from 31° to 34° (2θ). Crystalline hydroxyapatite shows three sharp peaks at 31.83°, 32.10°, and 32.90° (2θ). Thus, the X-ray diffraction showed the formation of poorly crystalline hydroxyapatite from the precursors formed at physiological temperature.

Variations in solution pH, as a function of time, were recorded during hydroxyapatite formation (Figure A-4). For the CDS composites, dissolution of TetCP and DCPA resulted in an alkaline environment with solution pH lying in the range of 9.5-10.5. This indicates that dissolution of DCPA precedes that of TetCP and the final pH is dominated by TetCP. For the CDH composites, an initial rise of pH is observed, with solution pH in the range of 8.25-9. However, after 6-7 hrs of reaction, the pH drops to 6.5-7.5 as the basic TetCP is consumed.

Figure A-5 shows the surface morphology of composites formed from the precursors at 37°C after 24 hours. The hydroxyapatite formed from all six composite precursors present a micro porous structure with no significant differences between gross morphologies. The connectivity of the spherical agglomerates, as evidenced from the SEM, leads to setting and rigidity of the cements. The gross morphologies of the composite surfaces were similar with micro porous structure of natural bone. This is important as Du et al. [34, 35] have reported that synthetic hydroxyapatite has better osteoconductivity when it resembles the mineral component of bone in composition, size, and morphology.
Figure A-3: X-ray diffraction analysis of (1) polymer 1-CDHA composite; (2) polymer 1-CDSHA composite; (3) polymer 2-CDHA composite; (4) polymer 2-CDSHA composite; (5) polymer 3-CDHA composite; and (6) polymer 3-CDSHA composite, formed at 37°C, with 0.5% phosphoric acid after 24 hrs.
Figure A-4: Solution pH as a function of time during calcium deficient hydroxyapatite formation at 37.4°C from (A) CDSHAp and (B) CDHAp precursors in presence of polymers 1, 2 and 3.
Figure A-6 shows the compressive strength of the composites and compares it with the strength of hydroxyapatite, alone. Incorporation of polymers 1 and 2 did not lead to an improvement in the strength of the composites. However, incorporation of polymer 3 did show a slight improvement for the composite made from CDH precursor. This can be attributed to the higher mechanical properties of polymer 3 compared with polymer 1 and 2 [23]. However, overall, the compressive strengths observed were far from desirable and this could be attributed to the following reasons. First, the composites are made up of only 10 wt% polymer. Thus, there is not enough polymer material in the composite to realize the true reinforcing effect of the polymer. Second, the polymer is present as micron size particles, randomly distributed in the hydroxyapatite phase. Improvement in the spatial distribution of the polymer and reduction in the size of polymer particle will probably lead to a better reinforcing effect. Third, the mechanical properties of these cement type composites are heavily dependant on the powder to liquid ratio that is used to form hydroxyapatite. An increase in compressive strength is associated with an increase in the amount of powder to liquid ratio.
Figure A-6: Compressive strength for composites of Polymer 1 (EA), Polymer 2 (MPh) and Polymer 3 (PhPh) with hydroxyapatite
A.3.1 In-vivo Biological Evaluation of Polymer/Hydroxyapatite Composites

The *in-vitro* osteocompatibility of the composites was assessed by studying MC3T3-E1 cell adhesion and proliferation [unpublished data]. Results showed that cells attach and proliferate on the composite surface and after 14 days, the number of cells are significantly higher when compared to the control, tissue culture polystyrene. Furthermore, the cells on the composites preserved their phenotype as evidenced from the expression of alkaline phosphatase activity, osteocalcin, osteopontin and collagen Type I at the gene level. The *in vivo* osteocompatibility of the composites were evaluated in an unicortical rabbit model. A 5 mm unicortical defect was made by drilling a hole in a rabbit tibia using a 5 mm burr with continuous cooling with saline solution. The defect was then filled with the composite cement paste using a syringe. The placement and positioning of the cement pastes in the defects was confirmed by radiographs. The formation of new bone in the defect was followed every two weeks till sacrifice by radiography. Figure A-7 shows the radiomicrographs of Polymer 1-CDHA composite treated and control defect after 8 weeks of implantation. In the control group no new bone formation or growth was observed as evidenced from the radiographic picture. On the other hand, the implants from all the six composites tested, were found to be integrated with the surrounding bone and were completely covered with new bone at the periostial side. No necrosis of the muscle and the surrounding tissues was observed.
Figure A-7: X-ray photograph showing (a) new bone formation after 8 weeks of implantation of Polymer 1–CDHA composite (b) no new bone formation at defect site in control group
For histological evaluation, the tibias from the rabbits were excised immediately after sacrifice. Similar results were observed for all the six implants. This paragraph explains the results observed with polymer 1-hydroxyapatite composites as a representative example. Figure A-8 shows the calcified bone formed on the surface of polymer 1-CDHA and polymer 1-CDSHA, respectively, after 4 weeks of implantation. Osteoblast cells were found along the periphery of the newly formed bone. Figure A-9 shows the deposition of osteoids by the osteoblast cells on the two matrices at the end of 4 weeks. At the end of 8 weeks, contact between the bone and the implant was difficult to identify since new bone had started to form from the sides of the defect into the implant. The polymer/HA composite group after 8 weeks demonstrated the formation of new bone tissue and cellular infiltration of predominantly osteoblast and few osteoclasts. No inflammatory response or fibrous tissue layer were observed with the calcium phosphate cement composite systems. After 4 weeks, a perfect seamless contact between the composites and the bone were observed. After 8 weeks osteoblast cells had converted into osteocytes and there was a random distribution of osteocytes in the new bone. Also few osteoclast like cells were observed in the lacunae of the newly formed bone and on the implant surface which suggests the resorption and remodeling of both the implant and bone. New bone formation was clearly evident by 8 weeks in all the 6 composites studied and the interface between the new bone and the native bone could not be identified.
Figure A-8: Von Kossa stain of (a) Polymer 1-CDHA and (b) Polymer 1-CDSHA after 4 weeks with osteoblasts [Ob] lined along the periphery of the lamellar bone [LB].
Figure A-9: Shows the osteoids [Ot] along the edge of the lamellar bone [LB] and woven bone [WB] in (a) Polymer 1-CDHA and (b) Polymer 1-CDSHA after 4 weeks.
A.4 Conclusions

Novel composites of biodegradable polyphosphazenes and hydroxyapatite were synthesized. Poly[bis(ethyl alanato)phosphazene], poly[(ethyl alanato)(p-methyl phenoxy)phosphazene] and poly[(ethyl alanato)(p-phenylphenoxy)phosphazene] were mixed with two different calcium deficient hydroxyapatite precursors. The composites formed poorly crystalline hydroxyapatite from the precursors, in situ, under physiological conditions. The formed hydroxyapatite closely resembled the mineral component of bone, in its surface morphology and composition. Incorporation of poly[(p-phenylphenoxy)(ethyl alanato)phosphazene] showed a slight improvement in the compressive strength of the composites. However, considerable improvement in the mechanical properties of these composites is desired. To achieve this goal, future work will focus on increasing the polymer concentration in the composites and improving the spatial distribution of the polymer phase within the ceramic phase by incorporating polymer as nanofibers. Biological testing showed good in-vivo osteocompatibility for the polyphosphazene/hydroxyapatite composites. The absence of inflammatory cells, excellent biocompatibility and the formation of new bone tissue demonstrate these cements as potential candidates for applications in bone repair.
A.5 References


B.1 Introduction

Scaffold based tissue engineering has made significant advancements in recent years as an alternative therapeutic strategy towards the repair or regeneration of damaged tissue. The rapid growth and development of tissue engineering can be attributed to a great extent to the development of novel biodegradable polymers [1]. The use of biodegradable polymers as scaffolds potentially allows for the replacement of damaged tissue as the biomaterial undergoes resorption to accommodate new tissue. In addition to biodegradability, materials for scaffold fabrication should satisfy a multitude of properties such as appropriate physical, chemical and biomechanical properties, degradation rate matching the intended application, and non-toxic and neutral degradation products. Furthermore, tissue engineering demands scaffolds with appropriate porous architecture i.e., with pore sizes, pore size distribution, pore interconnectivity, pore shapes and pore roughness allowing for cell attachment, and ingrowth, extracellular matrix production and nutrient transport. Studies have shown that bone tissue engineering requires scaffolds with pore sizes greater than 100 µm for osteoblast in-growth. In addition to pore structure and pore tortuosity of the scaffolds, the nature of the polymer degradation products can dramatically influence cell proliferation and differentiation in porous matrices [2].
Currently, the most extensively investigated biodegradable polymers for bone tissue engineering applications are aliphatic polyesters such as PLAGA and poly(lactic acid) (PLA) due to their established biocompatibility, controlled degradation rate and excellent mechanical properties. The PLAGA degrades via the unstable backbone ester hydrolysis into lactic and glycolic acid which are removed from the body by normal metabolic pathways. However, the acidic degradation products of PLAGA (lactic and glycolic acid) have been implicated in adverse tissue reactions in certain biomedical applications. The inactivation of sensitive molecules such as proteins by the acidic degradation products of these polymers, used as drug delivery devices, has been reported [3]. Furthermore, a recent study clearly demonstrates that the local acidic environment of porous PLAGA matrices can adversely affect cell viability and migration of cells into PLAGA scaffolds in vitro and in vivo [4]. These studies have served as an impetus to develop polymers with non-toxic and neutral degradation products with appropriate degradation rate and mechanical integrity suitable for developing scaffolds for tissue engineering.

Biodegradable polyphosphazenes form a unique class of biomedical polymers that form attractive candidates as drug delivery matrices as well as scaffolds for tissue engineering [5]. Polyphosphazenes are high molecular weight polymers with an inorganic backbone consisting of alternating phosphorous and nitrogen atoms with two organic side groups on each phosphorous atom. The synthetic flexibility of polyphosphazenes has allowed the development of wide range of polymers with a variety of physical, chemical and biological properties. Among these, amino acid ester polyphosphazenes are found to be hydrolytically sensitive with a degradation rate that
can be controlled by varying the nature and ratio of the side groups. These biodegradable polyphosphazenes undergo hydrolytic degradation yielding non-toxic and neutral products comprised mainly of amino acid ester side groups, phosphates and ammonia. Due to these properties amino acid ester polyphosphazenes are attractive candidates for a variety of transient medical applications. The efficacy of these polymers for developing controlled delivery devices for low and high molecular weight drugs has been demonstrated [6]. Further, the excellent osteocompatibility of ethyl glycine co-substituted polyphosphazenes has been demonstrated by Laurencin et al. [7, 8]

Due to the synthetic flexibility of polyphosphazenes, attempts were made to develop polyphosphazenes with lactic and glycolic acid side groups in order to synergistically combine the properties of two classes of biocompatible polymers, “PLAGA and polyphosphazenes” [5]. Thus, polyphosphazenes with different esters of lactic and glycolic acids as side groups were developed and the polymers were found to be microcrystalline and hydrolytically sensitive. Yet another elegant, simple and more practical approach to combine the properties of two different polymer systems is to physically blend the polymers.

Blending of biodegradable polyphosphazenes with PLAGA was attempted by Laurencin et al. in order to combine the beneficial features of PLAGA such as recognized biocompatibility and widespread applicability with the osteoconductivity, well tuned degradability as well as the buffering capacity of the degradation products of polyphosphazenes [9]. Blending (poly[(ethyl glycinato) (p-methyl phenoxy) phosphazene] and PLAGA (LA: GA; 50:50) in different molar ratios resulted in the formation of completely miscible blends. The degradation rate of the blends was found
to be intermediate between the rates of the parent polymers. This provides the opportunity to develop materials with adjustable degradation rate by varying the blend composition. Further, it was shown that the presence of polyphosphazene degradation products could significantly reduce the acidity of PLAGA degradation products demonstrating the buffering capacity of the blends [10]. One of our current goals is to develop novel biodegradable polymeric materials with controllable degradation rates whose degradation products minimize changes in pH of the surrounding milieus as potential candidates for bone tissue engineering. This could be achieved by blending biodegradable polyphosphazenes with PLAGA as evidenced from the previous studies.

The objective of the present study was to develop novel biomaterials by blending PLAGA with poly[bis(ethyl alanato) phosphazene] (PNEA) and evaluate the osteocompatibility of these blends in vitro as candidate materials for bone tissue engineering.

**B.2 Experimental Section**

**B.2.1 Reagent and Equipment**

Poly(lactide-co-glycolide) (PLAGA) with a lactide:glycolide ratio of 85:15 (Mw. 110,000) was procured from Alkermes Inc., USA. Hexachlorocyclotriphosphazene was obtained from Nippon Fine Chemical Co. Tokyo, Japan and purified by sublimation. L-alanine ethyl ester HCl was obtained from Aldrich Chemical Co., Milwaukee, WI, USA. All the solvents were purified by standard methods. Differential scanning calorimetric
(DSC) tracings were obtained using a TA instruments DSC Q-10 with a heating rate of 10°C/min. FTIR spectra of thin films of polymers and blends were recorded using a Bucker Vector 22 FTIR spectrophotometer at room temperature at a resolution of 4 cm\(^{-1}\) and with an accumulation of 50 scans. The scanning electron micrographs (SEM) were obtained using JSM-6400 scanning electron microscope (JEOL, Boston, MA, USA) operated at an accelerating voltage of 20 kV at various magnification.

### B.2.2 Polymer Synthesis

The synthesis of PNEA was performed according to a reported procedure and characterized by multinuclear NMR [11]. The molecular weight of PNEA was 125,100 (Mw) with a polydispersity of 2.0.

### B.2.3 Blend Fabrication

Two dimensional (2-D) blend films of PNEA and PLAGA were prepared by mutual solvent method using dichloromethane as the solvent. Blend-1 (PNEA:PLAGA 25:75) was prepared by dissolving 0.175 g of PNEA and 0.525 gm of PLAGA in 14 mL of dichloromethane. Blend-2 (PNEA:PLAGA 50:50) was prepared by dissolving 0.35 gm of PNEA and 0.35 gm of PLAGA in 14 mL of dichloromethane. The polymer solution was prepared by separately dissolving 0.7 g of PNEA and 0.7 g PLAGA in 14 mL of dichloromethane. The polymer and blend solutions were poured into petri dishes lined with Bytac Teflon paper and the solvent was allowed to evaporate slowly at –20°C.
followed by freeze drying. Circular matrices (10 mm diameter) were bored from the polymer and blend films using cork borer.

**B.2.4 Cell Viability and Proliferation Study**

Primary rat osteoblast (PRO) cells were isolated from calvaria of neonatal Sprague-Dawley rats according to standard procedure [12]. The matrices were sterilized by UV irradiation of the surface for 15 min on each side before cell seeding. Cells were seeded on the matrices (n=3) at a seeding density of 50,000 cells/well and cultured for 21 days to evaluate the long-term cell proliferation. At predetermined time (3, 7, 14 and 21 days), the adhered cells were washed with PBS and polymer matrices were transferred to new wells. The cells on polymer matrices were fixed with 1% and 3% glutaraldehyde for 1 and 12 h respectively at 4°C. After washing with distilled water, the matrices were allowed to air dry at 25°C. For MTS assay, the cells on the matrices were collected by trypsinisation and incubated with 1 mL culture medium containing 200 µL MTS for 2 h in a humidified atmosphere at 37°C and 5% carbon dioxide. At the end of incubation time, the reaction was stopped by adding 250µL of sodium dodecyl sulphate. The resulting solution was diluted in a 4:1 ratio using distilled water and the absorbance was read at 490 nm. Cell numbers were calculated based on a standard curve generated using primary rat osteoblast cells. Statistical analysis was performed using one way anova with a minimum confidence level of (p < 0.05) for statistical significance.
B.3 Results

Figure B-1 shows the surface morphology of films of Blend-1 and Blend-2. Blend 1 presents a smooth uniform surface with no evidence of phase separation. Blend-2 on the other hand has a non-uniform surface showing the presence of two different domains.

Table B-1 shows the glass transition temperatures (Tg) of the parent polymers and the blends. Both the blends showed two Tgs. However, the Tg values obtained for the blends were found to be different from the Tg’s of PLAGA and PNEA. The higher Tg value for the blends was found to be 3-5 °C lower than that of PLAGA and lower Tg obtained for the blends was found to be 4-5 °C higher than that of PNEA, showing partial miscibility of the blends.

Figure B-2 shows the FTIR spectra in the carbonyl stretching frequency region of the polymers and the corresponding blends. The IR spectra of the blends were found to be a simple combination of that of PNEA and PLAGA.

Figure B-3 shows the SEMs of multilayer of PRO cells on 2-D films of Blend-1 and Blend-2 after 21 days in culture. Figure B-4 shows the number of cells on films of PLAGA, PNEA, Blend-1 and Blend-2 compared to TCPS control as determined by MTS assay. The number of cells on the blends was found to be significantly higher than that of parent polymers.
Figure B-1: SEMs showing surface morphologies of (a) blend-1 and (b) blend 2
Table B-1: Glass transition temperatures of PLAGA, PNEA, and PLAGA/PNEA Blends as determined by DSC

<table>
<thead>
<tr>
<th>Matrix Composition</th>
<th>PLAGA Content (wt %)</th>
<th>Tg (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLAGA</td>
<td>100</td>
<td>46.32</td>
</tr>
<tr>
<td>PNEA</td>
<td>0</td>
<td>-16.75</td>
</tr>
<tr>
<td>PLAGA/PNEA (Blend 1)</td>
<td>75</td>
<td>-12.48, 42.90</td>
</tr>
<tr>
<td>PLAGA/PNEA (Blend 2)</td>
<td>50</td>
<td>-11.29, 43.87</td>
</tr>
</tbody>
</table>
Figure B-2: FTIR spectra showing the carbonyl stretching vibrations of polymers and blends
Figure B-3: Multilayer of cells on (a) Blend -1 and (b) Blend -2
Figure B-4: PRO cell proliferation on PLAGA, PNEA, Blend-1 and Blend-2 over 21 day period of culture as determined by MTS assay.
B.4 Discussion

This study examined the miscibility and in vitro osteocompatibility of novel polyphosphazene-PLAGA blends as candidates for bone tissue engineering application. Previous studies of blending glycine ethyl ester co-substituted polyphosphazenes with PLAGA showed the feasibility of developing miscible blends having controllable degradation rates and neutral degradation products. In the present study we used a more hydrophobic alanine ethylester substituted polyphosphazene (PNEA) to blend with PLAGA. The SEM of Blend-2 showed evidence of phase separation. However, Blend-1 presented a uniform smooth surface (Figure B-1). The observation of phase separation in the case of PNEA-PLAGA blends is in contrast to the blends of glycine co-substituted polyphosphazenes which showed no phase separation upon blending with PLAGA. The absence of complete miscibility of the PNEA-PLAGA blends is further confirmed by DSC measurements. Thus, PNEA-PLAGA blends showed two Tgs, which are slightly different from the Tgs of the parent polymers (Table B-1) showing only partial miscibility. The miscibility of blends originates from various interactions between constituent groups of polymer chains such as hydrogen bonding and van der Waals interactions. Among these, the presence of hydrogen bonding interactions are known to significantly enhance the miscibility of the blends compared to weak van der Waals interactions. The IR spectra can be used to estimate the extent of hydrogen bonding in carbonyl and amino group containing polymers as the hydrogen bonded carbonyl or amino groups show a red shift depending on the structure of the polymers. The IR spectra of the blends (Blend-1 and Blend-2) in the carbonyl region were found to be a
simple combination of the corresponding spectra of the parent polymers showing the absence of strong hydrogen bonding interactions between the polymers (Figure B-2).

In the glycine co-substituted polyphosphazene-PLAGA blend systems, the secondary amino groups of glycine groups were found to form strong hydrogen bonds with the ester groups of the glycolide units in PLAGA (50:50) thereby resulting in complete miscibility of the polymers. However, increasing the lactide content of PLAGA was found to decrease the miscibility of the blends as demonstrated by the formation of partially miscible blends with PLAGA (80:20) and glycine co-substituted polyphosphazene [13]. The low probability of hydrogen bonding between the polymers [PNEA and PLAGA (85:15)] in the present study can be attributed to the $\alpha$-CH3 groups of the alanine and lactide units present in the parent polymers. These bulky groups could sterically hinder the formation of hydrogen bonds between the carbonyl and amino groups of the polymers.

However, the blend films were found to strongly support osteoblast adhesion and proliferation compared to parent polymers. Multilayer of cells was found on the surface of blend films as evident from the SEMs (Figure B-3). The quantitative estimation of the cell numbers on the films showed that the number of cells on the blend membranes were significantly higher than PLAGA or PNEA (Figure B-4) after 21 days in culture. We have previously demonstrated that blending PLAGA with glycine containing polyphosphazenes could significantly alter the degradation rate of the polymers and decrease the pH change associated with PLAGA degradation. Further studies on the rate of degradation, buffering capacity, mechanical properties and in vivo biocompatibility of PLAGA-PNEA blends are currently underway.
B.5 Conclusions

Blending of PLAGA with PNEA using dichloromethane as the mutual solvent resulted in partially miscible blends having weak interactions between the constituent polymers. However, the blend membranes showed significantly higher cell adhesion and proliferation compared to the parent polymers, indicating better osteocompatibility of blended membranes compared to the parent polymers.

B.6 References


VITA

Anurima Singh

Anurima Singh, daughter of Col. (Dr.) Arun Kumar and Madhu Kumar, was born on September 10th, 1976 in Allahabad, Uttar Pradesh, India. She earned her Bachelors degree in Chemistry, with Honors, at The University of Delhi, India in 1997. Anurima earned a Masters degree in Chemistry from the Indian Institute of Technology Delhi, India in 1999. She then went on to earn a Masters degree in Polymer Science and Engineering from the Indian Institute of Technology Delhi, India in 2001. During this time, Anurima had the opportunity to travel to Germany as an exchange student at the Technical University of Dresden on a Government sponsored scholarship program. Anurima began her graduate studies at the Pennsylvania State University in 2001 under the guidance of Prof. Harry R Allcock. Upon graduation, Anurima will begin her career in polymer chemistry with a position at Dow Chemicals.