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***In Situ* Porous Structures: A Unique Polymer Erosion Mechanism in Biodegradable Di-peptide-based Polyphosphazene and Polyester Blends Producing Matrices for Regenerative Engineering**

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Abstract

Synthetic biodegradable polymers serve as temporary substrates that accommodate cell infiltration and tissue in-growth in regenerative medicine. To allow tissue in-growth and nutrient transport, traditional three-dimensional (3D) scaffolds must be prefabricated with an interconnected porous structure. Here we demonstrated for the first time a unique polymer erosion process through which polymer matrices evolve from a solid coherent film to an assemblage of microspheres with an

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interconnected 3D porous structure. This polymer system was developed on the highly versatile platform of polyphosphazene-polyester blends. Co-substituting a polyphosphazene backbone with both hydrophilic glycylglycine dipeptide and hydrophobic 4-phenylphenoxy group generated a polymer with strong hydrogen bonding capacity. Rapid hydrolysis of the polyester component permitted the formation of 3D void space filled with self-assembled polyphosphazene spheres. Characterization of such self-assembled porous structures revealed macropores (10-100 μm) between spheres as well as micro- and nanopores on the sphere surface. A similar degradation pattern was confirmed *in vivo* using a rat subcutaneous implantation model. 12 weeks of implantation resulted in an interconnected porous structure with 82-87% porosity. Cell infiltration and collagen tissue in-growth between microspheres observed by histology confirmed the formation of an *in situ* 3D interconnected porous structure. It was determined that the *in situ* porous structure resulted from unique hydrogen bonding in the blend promoting a three-stage degradation mechanism. The robust tissue in-growth of this dynamic pore forming scaffold attests to the utility of this system as a new strategy in regenerative medicine for developing solid matrices that balance degradation with tissue formation.

Keywords

Polymeric materials; Composite materials; Thin films; Tissue engineering; Biomedical applications

1. Introduction

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

In bulk erosion, the polymer undergoes degradation with significant decrease in molecular weight and the corresponding material properties (such as mechanical properties) as a function of degradation time. As illustrated in Scheme 1a, the matrix dimension remains constant until the structure fails catastrophically during hydrolytic degradation. Bulk-eroding polymers have been widely used in orthopaedic applications. Unfortunately, these polymers prematurely fail and do not generate porous structures during the degradation process which necessitates the prefabrication of the materials into three-dimensional (3D) porous structures with interconnected porosity for regenerative applications. In creating porous structures many of the prefabricated scaffolds possess significantly lower mechanical properties compared to the bulk material. These structures may also deform and collapse,

thus compromising the 3D porous structure and their ability to function, particularly as bone graft substitutes. This limits the rate of nutrient and oxygen diffusion through the porous structures which are crucial for complete cell infiltration and tissue formation. Furthermore, these prefabricated structures lack predictable scaffold properties post-implantation that could lead to rate mismatch between scaffold degradation and tissue in-growth.^[3] In the case of hydrophobic polymers that undergo surface erosion, degradation occurs at the implant surface with insignificant decrease in the molecular weight of the bulk material. The matrix becomes smaller but maintains its original geometric shape as a function of degradation time until the structure is completely eroded as indicated in Scheme 1b. Thus, the surface-eroding polymers also fail to form porous structures during the degradation process and also require material prefabrication into 3D interconnected porous structures for tissue regeneration.

Our studies presented here have demonstrated for the first time a unique degradation process for biodegradable biomaterials through which it forms 3D interconnected porous structures. As indicated in Scheme 1c, the polymer system exhibits an erosion mechanism by which the polymer evolves from a solid coherent film to an assemblage of microspheres with interconnected porous structures. This could have significant implications towards developing biodegradable materials as an *in situ* forming 3D tissue engineering scaffold. The polymer system is made of polyphosphazene-polyester blends where the degradation products of the polyphosphazene neutralize the acidic degradation of the polyester.^[4, 5] The blend approach is attractive since it imparts the ability to tailor the polymer properties by simply changing the blend composition.^[6] In addition, biodegradable polyphosphazene-PLGA blends exhibit several distinct advantages. Food and Drug Administration has approved several products comprised of PLGA for a variety of biomedical applications.^[7] The synthetic flexibility of polyphosphazene allows us to design specific side group chemistry that enables both inter- and intra-molecular interactions such as hydrogen bonding.^[8] The degradation mode and pattern of the polymer can also be tuned efficiently by incorporating different side groups.^[9] For example, amino acid ester side groups confer hydrolytic instability to the polymer while addition of phenylphenoxy side groups increases its hydrophobicity. By examining nature's own chemistry hydrogen bonding significantly contributes to formation of unique and highly defined architectures in biological systems such as in the folding of proteins.^[10] The hydrophobicity that phenylphenoxy groups exert can retard the degradation of polyphosphazene and maintain structural integrity. Accordingly, a mixed-substituent polyphosphazene co-substituted with glycylglycine dipeptide and with phenylphenoxy side groups exploited these properties in this work. We also hypothesized that the combination of glycylglycine dipeptide with the hydrophobic phenylphenoxy group would lead to a suitable degradation time frame of 12-24 weeks. Furthermore, we hypothesized that the low molecular weight polyester with relatively high molecular weight polyphosphazene in the blend would favor the 3D porous structure formation and multi-phase degradation kinetics. Finally, the degradation pattern as well as *in situ* control of matrix morphology and structure for tissue regeneration processes can be achieved via blend compositional changes.

2. Results

2.1. Design and Fabrication of Blend Matrix

The blend matrices consist of a mixed-substituent polyphosphazene and commercially available PLGA polymer with a 50:50 lactide to glycolide ratio ($M_w=34$ kDa). The mixed-substituent polyphosphazene was co-substituted with the ethyl ester of glycylglycine dipeptide and with the phenylphenoxy group in a 50:50 ratio namely poly[(glycine ethyl glycinato)₁(phenylphenoxy)₁phosphazene] (PPHOS). The polymer was synthesized according to a standard two-step polymerization/substitution route (Fig. 1).^[11] The two side groups for the PPHOS were selected based on the following rationale: (i) the dipeptide was

incorporated to provide multiple hydrogen bonding sites (two proton donors per monomer unit) for coordination with PLAGA and an intramolecular hydrogen bonding network;^[8] while the phenylphenoxy group was beneficial for maintaining mechanical function and hydrophobicity;^[12, 13] (ii) the glycylglycine dipeptide ester is known to be biocompatible *in vivo* and can be hydrolyzed to glycine units, one of the common amino acids in the body;^[14, 15] (iii) the combination of the two side groups in a 50:50 ratio will result in a complete degradation time of 12-24 weeks.^[8, 16] Selection of this specific PLAGA was based on a criterion of having less steric hindrance from the α -CH₃ groups in order to achieve a higher probability of hydrogen bonding interactions between the two polymers (Fig. 1).^[8, 13] In addition, this specific PLAGA has a relatively fast degradation rate among the commercially available PLAGA family, which is advantageous to induce morphology change and pore formation. The thermal ring opening polymerization of (NPCl₂)₃ followed by nucleophilic substitution reactions of the resultant poly(dichlorophosphazene) produced the yellow polymers of PPHOS (Fig. 1). The structure and side-group ratios of PPHOS were confirmed by ¹H NMR and ³¹P NMR spectroscopies (Table S1). The detailed characterization and physical properties of the PPHOS polymer (M_w =767 kDa) are described elsewhere.^[17] Blend matrices were fabricated using a mutual solvent method.^[18] Two different weight ratios of PPHOS and PLAGA, namely 25:75 and 50:50, yielded Matrix1 (light yellow) and Matrix2 (yellow), respectively. Therefore, by taking advantage of the difference in degradation rates of the two parent polymers, PLAGA was used as a “dynamic porogen” in the 3D void space formation and evolved into the pores during the blend degradation.

2.2. Blend Matrix Characterization

The miscibility of the blend matrices has been confirmed by differential scanning calorimetry (DSC), attenuated total reflection infrared spectroscopy (ATR-IR), scanning electron microscopy (SEM), and high-resolution solid-state ¹³C NMR. For example, the blend matrices showed a single glass transition temperature (T_g) intermediate between two parent polymers (Fig. S1a). The IR spectra also showed that the C=O stretching vibrations of PPHOS and PLAGA occurred at ~1743 and ~1749 cm⁻¹, respectively. In addition, for Matrix1 and Matrix2, a second band developed at ~1677 cm⁻¹, which indicated hydrogen bonded carbonyl groups. Both blend surfaces (Fig. S1b) and cross-sections (Fig. S1c) were found to be smooth and uniform indicating absence of phase segregation. Furthermore, ¹³C NMR has also been widely used to study the intermolecular hydrogen-bonding interactions. It is known that the ¹³C nuclei show some downfield shifts when they are involved in hydrogen bonding.^[19, 20] As shown in Fig. 1, a significant downfield shift of the carbonyl carbon resonance was detected in the high-resolution solid-state ¹³C NMR spectra of blends. Thus, all the evidence supported the formation of miscible blends via intermolecular hydrogen bonding between amide and amine protons in the dipeptide units of PPHOS and the carbonyl groups in PLAGA. Furthermore, the water-in-air contact angle of Matrix2 (80.3 ± 0.1°) was significantly higher than Matrix1 (70.9 ± 0.2°) ($p < 0.05$), indicating the presence of the hydrophobic phenylphenoxy groups on the surface.

2.3. In Vitro Degradation Study

The most unique phenomenon about the polymer erosion of the dipeptide-based polyphosphazene-PLAGA blend system lies in the morphological change of the matrix as a function of time. Fig. 2 shows the representative SEM images of blend matrices 0.5 mm × 10 mm (thickness × diameter, or T × D) during *in vitro* hydrolysis in aqueous media as a function of time. At week zero, both blend matrices appeared to be smooth and uniform (Fig. 2a, b). After 4 weeks of hydrolysis, the PLAGA component had experienced significant molecular weight loss and retained only 7.87 ± 0.38% and 9.39 ± 0.08% of its original molecular weight in Matrix1 and Matrix2, respectively (Fig. 3a). Such a rapid

degradation of the PLAGA phase resulted in spherical structures on the blend surface (Fig. 2c, d). Following 7 weeks of continued rapid bulk hydrolysis of the PLAGA, more spherical structures were formed on the surface of the blend matrices as evidenced by SEM and only a small amount of the PLAGA component was found on the surface as indicated in Fig. 2e and f. During the first 7 weeks, the percentage of mass loss for Matrix1 and Matrix2 was $77.05 \pm 1.07\%$ and $56.58 \pm 1.01\%$, respectively (Fig. 3b). However, after the rapid PLAGA hydrolysis phase, the degradation rate of the blend matrices significantly decreased beyond 7 weeks. During the remaining 5 weeks of the degradation study only ~8% and ~18% mass loss was observed. Thus Matrix1 and Matrix2 experienced a total mass loss of $85.26 \pm 0.27\%$ and $74.21 \pm 0.49\%$ after 12 weeks of degradation, respectively (Fig. 3b). Such multi-phase degradation profiles of blend matrices would allow accommodating gradual tissue in-growth during the different stages of bone healing.^[21] By 12 weeks, completely open 3D continuous architecture with macropores (10-100 μm) between spheres in combination with micro/nanopores on the sphere surfaces formed with well packed polymer spheres (Fig. 2g, h). This unique *in situ* created porous structure has significant potential for cell in-growth and tissue regeneration. Furthermore, the 3D porous structure can be modulated by altering the composition of the matrix, enabling the potential to dynamically control the matrix *in situ*. For instance, a higher PLAGA content in Matrix1 resulted in faster degradation as well as smaller spheres than in Matrix2. Such uniform porous structure formation was found throughout the matrix as evidenced by the SEM images of matrix cross-sections (Fig. S2, S3). Furthermore, the increase of blend degradation time resulted in formation of porous structure with a greater amount of polymer spheres throughout the matrix (Fig. 2, S2). The spherical phase of the degraded blend was confirmed to be primarily polyphosphazenes through the use of energy dispersive x-ray spectroscopy (Fig. S4) and solid-state NMR. Furthermore, the molecular weight loss of the polyphosphazene component followed a similar trend to that of PLAGA during the matrix degradation (Fig. 3c). The blend matrices degraded much slower than pristine PLAGA over 12 weeks (Fig. 3b). It was also observed that an increase in the ratio of polyphosphazene in the blend resulted in a slower degradation rate (Fig. 3a-c). In summary, SEM images of the blend matrices during degradation in aqueous media revealed the formation of *in situ* 3D porous structure that is suitable for cell infiltration and tissue in-growth (Fig. 2). The SEM images also showed that the spheres were imbued with micro- and nanopores.

Another major advantage of the blend matrices resides in their self-neutralizing ability where the buffering degradation products of the polyphosphazene neutralize the acidic PLAGA hydrolysis products.^[5] The self-neutralizing ability of the blend matrices was assessed by recording the pH of the degradation media throughout the study. In general, the pH of the media with blend matrices was higher than that of pristine PLAGA (Fig. 3d). For example, the pH decreased dramatically to ~3.1 after 4 weeks for pristine PLAGA, which was due to the faster degradation of PLAGA to lactic acid and glycolic acid. On the other hand, the pH of degradation media was 14% and 22% higher with Matrix1 and Matrix2 than with PLAGA after 4 weeks of degradation, respectively. The increase in the pH of the degradation media with blends was maintained until the end of the hydrolysis study. This suggests that the acidity, which originated from the bulk degradation of PLAGA, was buffered by the phosphates and ammonia produced from the PPHOS backbone. This buffering effect further reduced the auto-catalyzed PLAGA hydrolysis. In anatomical defect sites where there is less body fluid, such as articular cartilage, the resultant low pH environment from the degradation of the polyester materials is detrimental to implant functions. With the use of blend materials, the resulting pH environment can be well tuned for better tissue regeneration by simply adjusting the blend composition. In addition, the changes of molecular weight for both the PLAGA and polyphosphazene components in the matrix during degradation further confirmed that the two components degraded in a similar pattern (Fig. 3a, c). Initially, the acidic hydrolysis products from PLAGA accelerated the

degradation of itself as well as PPHOS. As a consequence, the degradation of PPHOS neutralized the acidity and retarded the degradation of PLAGA.

2.4. Rat Subcutaneous Implantation Model

To examine their performance under physiological conditions, blend matrices with dimensions of 0.5 mm × 10 mm (T × D) were implanted in Sprague-Dawley rats subcutaneously.^[16, 22] PLAGA was used as the control due to its well-characterized biodegradability and recognized biocompatibility. The degradation was quantified by measuring matrix mass loss and molecular weight change. The molecular weight for both the PLAGA and polyphosphazene components in the blend system was analyzed using GPC. The morphology and porosity of the matrix was characterized and analyzed using a variety of techniques including SEM, microcomputed tomography (micro-CT), and histological staining. The hydrogen bonding changes during polymer degradation was monitored by ATR-IR. PLAGA was completely degraded whereas the blend matrices were so infiltrated with collagen tissues that it is inappropriate to characterize the mass loss after 7 weeks of implantation. Both the blend matrices were well tolerated throughout the duration of the 12-week of subcutaneous implantation period. No acute inflammation, tissue necrosis, or abscess formation were observed around either polymer matrix.

The *in vivo* degradation rate of the blend matrix was examined by measuring the mass loss of polymer matrices. No residual PLAGA was found after 7 weeks of implantation, whereas Matrix1 and Matrix2 showed $75.10 \pm 0.64\%$ and $55.17 \pm 4.14\%$ mass loss, respectively (Fig. 4a). Both the PLAGA and polyphosphazene components in the blend experienced a similar degradation pattern as characterized by the molecular weight loss (Fig. 4b, c). The number and weight average molecular weights (M_n and M_w) of both the PLAGA and polyphosphazene components decreased exponentially with degradation time throughout the implantation period. Furthermore, the apparent degradation rate, K , can be obtained according to the following exponential relationship between molecular weight and degradation time: $\lg M = \lg M_0 - Kt$. For example, the apparent degradation rates based on M_w were calculated to be 0.1014 week^{-1} and 0.0859 week^{-1} for the PPHOS component in Matrix1 and Matrix2, respectively. This indicates that PPHOS degraded faster in Matrix1 due to higher PLAGA composition. A similar trend in the apparent degradation rates was found for the PLAGA component in pristine PLAGA, Matrix1, and Matrix2. This suggests that the PLAGA degraded the slowest in Matrix2 due to the higher PPHOS composition. These results agree well with our *in vitro* observations that the PLAGA hydrolysis accelerated the PPHOS degradation, whereas the degradation of PPHOS retarded the hydrolysis of PLAGA.

Blend matrices were obtained at 2, 4, 7, 10, and 12 weeks for characterizing morphological changes by SEM. *In vivo* polymer degradation also resulted in the formation of 3D porous structures *in vivo*. Specifically at 12 weeks post-implantation, completely open 3D porous structures formed with well packed polymer spheres (Fig. S5a, b). These *in situ* polymer spheres were less than 100 μm in diameter, which potentially generates high surface area for cell-material interactions. Furthermore, the size of *in situ* formed spheres increased significantly with the increase of polyphosphazene ratio in the blend system. The spherical phase of the degraded blend was confirmed to be primarily polyphosphazenes through the use of ^{13}C solid-state NMR (Fig. S5c, d). In addition, SEM images revealed the intricate pore structure with great surface area and 3D space within the polymer sphere (Fig. S6).

Representative 2D micro-CT images in Fig. 5a-j revealed the progression of morphological and structural changes within the blend system. The lighter and darker regions indicate the polymer phase and the *in situ* formed pores, respectively. Quantitative measurements of *in-situ* porosity ((total volume-polymer volume)/total volume) of the matrix were performed

based on the 3D micro-CT reconstructions during the polymer degradation (Fig. 5k). Prior to implantation, both the blends were characterized to be uniform with a continuous polymer phase of 0% porosity (Fig. 5a, b). These observations coupled with the results from other analytical techniques such as ATR-IR, NMR, DSC, and SEM demonstrated a homogenous blend phase. During the first two weeks of implantation, only a small percentage of mass loss in the blends occurred and resulted in a porosity of ~14% and ~12% for Matrix1 and Matrix2, respectively (Fig. 4a and 5c, d, k). At weeks 2 through 10, dramatic mass losses from fast degradation of both components led to a significant porosity increase to ~86% and ~82% for Matrix1 and Matrix2, respectively (Fig. 4 and 5g, h, k). At weeks 10 through 12, the degradation rate of the blends decreased dramatically and the porosity was maintained at ~87% and 82% (Fig. 4 and 5i, j, k). Since the PLAGA component served as a “dynamic porogen”, the interconnectivity of the resultant porous structures was 100% as indicated in the 2D micro-CT images. In addition, it was shown that the blend composition had no significant effect on the porosity of the blend matrices. Both blend matrices had a porosity of 82-87% after 12 weeks of implantation, which is very important in providing sufficient space for cell infiltration and tissue in-growth.

As seen from both hematoxylin and eosin (H&E) and Masson's trichrome stain (TRI), implantation of the blend matrices also resulted in the formation of 3D porous structures *in vivo* (Fig. 6). As indicated by the arrows, the porous structure formed during matrix degradation demonstrated a great potential for directing and hosting tissue in-growth *in vivo*. A polymer sphere-based porous structure was formed after 7 weeks of implantation (Fig. 6a, b). These observations are in line with *in vitro* data discussed previously. Cellular infiltrates were found to fill in the void space between spheres as indicated by the arrows. Thereafter, the 3D porous structure continued to evolve to promote robust tissue in-growth. By 12 weeks, collagen tissues were guided to surround the polymeric microspheres and filled in the pore space between spheres (Fig. 6c, d), which is consistent with the *in vivo* findings using sintered microsphere scaffolds for tissue repair.^[23, 24]

Matrix tissue compatibility was characterized by the extent of inflammation and fibrous capsule formation.^[22] Both the blend matrices were found to elicit minimal inflammation with improved biocompatibility over PLAGA during 12 weeks of implantation.^[17] A minimal inflammatory response indicated an apparent lack of local toxicity, which could be due to the non-toxic and near-neutral pH degradation products. It was also observed that an increase of polyphosphazene content in the blend resulted in both reduced inflammatory response and fibrous capsule formation. Reduction in the fibrous capsule thickness is highly advantageous to improve mass transfer between the implants and surrounding tissues.

3. Discussion

One of the limitations for the use of biodegradable polymers as temporary substrates is the predictability of the degradation time to provide an appropriate physical, chemical, and biological function for a specific application. For a polymer blend, miscibility is required in generating a binary system with predictable degradability. For example, blending of two polymers with different degradation kinetics results in a predictable degradation pattern only when they constitute a miscible system. In general, the miscibility of any two polymers is a result of strong intermolecular interactions such as hydrogen bonding, dipole-dipole interactions, and/or van der Waals forces. In a polyphosphazene-polyester blend, hydrogen bonding between the amino groups of polyphosphazenes and the ester groups of PLAGA is predominant over dipole-dipole and van der Waals forces of attraction. High-resolution solid-state ¹³C NMR confirmed the formation of miscible blends through the hydrogen bonding between the amide and amine protons in the dipeptide units of PPHOS and the carbonyl groups in PLAGA, which is in line with our observations through SEM, DSC, and

ATR-IR. Furthermore, blending of PLAGA with PPHOS resulted in a single T_g of $\sim 35.9^\circ\text{C}$ and 40.3°C for Matrix1 and Matrix2, respectively. For a miscible blend system, the T_g of the blend can be predicted by the Wood equation:^[25] $T_g = w_1 T_{g1} + w_2 T_{g2}$ where w_i and T_{gi} are the weight fraction and the T_g of polymer i ($i=1, 2$ designating PPHOS and PLAGA, respectively). The glass transition temperatures estimated using the Wood equation for Matrix1 and Matrix2 are 34.1°C and 40.4°C , respectively, which are very close to the experimental values from DSC thermograms (Fig. S1a).

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

In this study, the degradation of the PLAGA-PPHOS blends was investigated both in aqueous media (pH=7.4) as well as under physiological conditions via a rat subcutaneous implantation over 12 weeks. The rationales for using aqueous media over phosphate buffered saline were: 1) phosphate containing buffer solutions would interfere with the ^{31}P NMR spectrum of the polyphosphazene hydrolysis products; 2) whether the PPHOS hydrolysis can neutralize the acidic pH resulted from the hydrolysis of PLAGA. For example, it was found that the pH of the degradation media decreased from 7.4 to 3.1 after 4 weeks of degradation due to the rapid hydrolysis of the PLAGA alone, whereas the pH of the degradation media with Matrix2 was 3.8. The decrease in pH also accelerated the polyester hydrolysis into carboxylic acids through catalysis. The polyphosphazene component in the blend also showed a similar molecular weight decline, which indicates that the polyphosphazene hydrolyzed to buffer the medium to some extent. The pH of the aqueous media with blends decreased dramatically during the first four weeks which is a consequence of rapid acidic hydrolysis of the PLAGA component (Fig. 3a, d). It further declined slightly through week 7, by which the majority of PLAGA had completely degraded. Thereafter, the pH was maintained until the study was concluded at week 12. The acidity neutralization of degradation media differs from our previous degradation studies with polyphosphazene-PLAGA blends in which a significant buffering effect was detected.^[5, 8] As evidenced from Fig. 3c, the polyphosphazene component underwent a $\sim 93\%$ decrease in original molecular weight during 12 weeks of degradation. The number of polymer chains degraded was much less than that for PLAGA component due to the 23-fold difference in original molecular weight. Furthermore, there are still $\sim 15\%$ and 26% of mass remaining for Matrix1 and Matrix2 which were primarily composed of polyphosphazenes as confirmed by energy dispersive x-ray spectroscopy and solid-state ^{31}P NMR (Fig. 3b, S4). Therefore, the degradation byproducts of PPHOS were not enough to compensate the acidic hydrolysis of PLAGA. The slower degradation of PPHOS is also partially due to the steric effects from the bulky phenylphenoxy group that hinders water intrusion.

The erosion pattern of a polymer matrix *in vivo* is an important measure for new materials to be used in tissue engineering applications due to the additional external factors (such as enzymes, phagocytes, lymphocytes and fibroblasts) in the physiological environment. A rat subcutaneous implantation model was used in the present study in order to characterize the degradation kinetics of the blend matrix under physiological conditions. The mass loss profiles during the implantation suggested the degradation rate to be PLAGA > Matrix1 > Matrix2 (Fig. 4a). The molecular weights of both PLAGA and polyphosphazene

components in the blend decreased in a similar trend throughout the 12-week implantation period (Fig. 4b, c), which is in line with *in vitro* findings. These observations are consistent with literature reports for degradation of PLAGA and PLAGA-polyphosphazene blends *in vivo*.^[26] In addition, a minimal inflammatory response characterized by the presence of few neutrophils, erythrocytes, and lymphocytes was found for both blend matrices.^[16, 17] This suggests an apparent absence of local toxicity that demonstrates the non-toxic nature of blend degradation products. Furthermore, the fibrous capsules surrounding the blend matrices were much thinner than with PLAGA and other biocompatible polymers. For example, the thickness of the fibrous capsule surrounding the widely used polyesters has been reported to be more than a few hundred microns.^[22, 27] In addition, Matrix2 produced less inflammatory reactions with thinner fibrous capsules than Matrix1, indicating the tunability in blend biocompatibility by changing the blend composition. Most interestingly, both blend matrices were robustly infiltrated with collagen tissue after 12 weeks of implantation. This demonstrates that the *in situ* formed porous structures are capable of accommodating cell infiltration and tissue in-growth.

A widely used method to tune the polymer degradation rate is to introduce a second monomer into the polymer chain. For example, the degradation rate of poly(lactide-co-glycolide) depends on the copolymer composition. The molecular weight loss during hydrolysis is accelerated with an increase in glycolide content. This is attributed to greater absorption of water into the polymer matrix. Therefore, the significant difference in degradation rates between Matrix1 and Matrix2 suggests that the degradation rate of the blend matrix can be effectively controlled by changing blend composition. For example, a slower degradation rate can be obtained both *in vitro* and *in vivo* by increasing the polyphosphazene composition. In addition, the increase of hydrophobicity indicated by the significant increase of contact angle of the polymer system was also attributed to the slower degradation rate.

The absence of an interconnected porous structure during the polymer bulk or surface erosion often necessitates prefabrication of the polymers into 3D structure with interconnected pores for tissue engineering applications. A variety of matrix fabrication techniques including particulate leaching,^[28] gas foaming,^[29] phase separation,^[30] and sphere sintering^[31] have been developed to produce 3D porous matrices for skeletal tissue regeneration during the past two decades. It has been demonstrated that a porosity of ~90% is highly desirable for an ideal scaffold by providing sufficient space for extracellular matrix (ECM) synthesis, a high surface area for cell-material interactions, and minimal diffusion constraints. There have also been quite a few reports on the diffusion and cell migration limitations of the closed pore system resulting from the prefabrication processes.^[3] Therefore, porosity and interconnectivity are key factors in the success of a matrix for promoting tissue in-growth and integration.^[1, 32] In addition, the optimal pore diameters for neovascularization, osteoid, and mineralized bone formation have been reported to be in the range of 5-350 μm .^[33] Interestingly, the hydrolysis of the dipeptide-based polyphosphazene-PLAGA blends resulted in the formation of 3D porous structures with 100% interconnectivity and with pore sizes less than a hundred microns. The morphology of such *in situ* formed porous structure resembled our sintered microsphere matrix, which is a biomimetic 3D pore system resulted from fused polymer microspheres.^[31] In particular, a sintered microsphere matrix from PLAGA has attracted significant interest as a load-bearing scaffold for orthopaedic tissue engineering.^[23, 34] However, for large porous structures the use of a bioreactor might be essential for complete cell infiltration, because the preformed structures present nutrient and oxygen diffusion limitations.^[35] A 12-week of blend degradation *in vivo* resulted in a porosity of 82-87% which is structurally similar to cancellous bone. Furthermore, the sphere size increases with the increase of polyphosphazene composition in the blend, whereas no significant changes to porosity were

observed. This offers an advantage for maintaining a highly porous structure while fine-tuning the pore system for facilitating cell infiltration and tissue in-growth.^[36] For example, it has been verified by image analysis that the average pore size increases with the sphere diameter. This is consistent with our earlier observations for the sintered microsphere matrix.^[31] In addition, the intricate porous structure within the polymer spheres provides additional surface area and space for promoting cell-material interactions. In the blend matrix, the fast degradation of PLAGA facilitated pore formation while the polyphosphazene microspheres maintained the matrix structural integrity during the tissue in-growth. Therefore, this unique *in situ* porous structure has significant potential for cell in-growth and tissue regeneration. By contrast, the porosity and structural integrity of prefabricated porous matrices are often compromised during the course of polymer degradation which limits cell infiltration and tissue in-growth.^[21, 37]

As illustrated in Fig. 7, one proposed mechanism model for the unique erosion process includes three stages: intermolecular hydrogen bonding dominant state (*Stage I*, 0 week), intermediate state (*Stage II*, 0-4 weeks), and intramolecular hydrogen bonding dominant state (*Stage III*, 4-12 weeks). Initially, a completely miscible blend matrix forms through strong intermolecular hydrogen bonding between PLAGA and PPHOS. In *Stage II*, phase separation that occurs during blend hydrolysis is the result of combined water ingress and hydrolysis of PLAGA, which disrupts hydrogen bonding between the two polymers. At the final *Stage III*, microspheres of the polyphosphazene are formed from the relatively intact polyphosphazene molecules due to the strong intramolecular hydrogen bonding and coalescence of hydrophobic side groups. Representative IR spectra for the blend matrix during polymer degradation confirm the three-stage process. In both degradation environment (aqueous media and *in vivo*), intermolecular hydrogen bonding (peak~1677 cm^{-1}) was found for the blend matrix at 0 week. No hydrogen bonding peaks were detected for 2 weeks and 4 weeks due to the disruption of the intermolecular hydrogen bonding resulted from fast degradation of PLAGA. After 4 weeks, PPHOS spheres formed through intramolecular hydrogen bonding (peak~1655 cm^{-1}) at 7 weeks, 10 weeks, and 12 weeks. These observations are in line with earlier literature reports where polymeric particles and aggregates were formed via disruption in hydrogen bonding interactions.^[38-40] Such a three-stage model is also supported by the intrinsic differences in the degradation rates of both PLAGA and PPHOS components from our earlier discussions. The detailed mechanism for the unique erosion process of such a polymer system into spheres and interconnected porous structure is still under investigation. Nevertheless, this represents a novel hydrolysis sequence when hydrogen bonding and hydrophobic entities are present. We believe that the dynamic pore formation process accompanying the matrix erosion will significantly enhance cell infiltration and tissue in-growth compared to the prefabricated matrices. In contrast to the compromised porosity and structural integrity observed for 3D prefabricated matrices during polymer degradation, the blend gradually evolved into a 3D porous structure with interconnected pores during degradation which enables accommodation of the gradual cell infiltration and tissue in-growth. In addition, the blend matrices have the ability to self-neutralize to achieve a near neutral-pH environment with a multi-phase degradation pattern. The blend properties such as physico-chemical properties and *in vitro* and *in vivo* biocompatibility can also be well tuned by simply varying the blend composition.^[17, 41] The results presented in this study suggest that the blend matrices hold the promise of creating a new paradigm in scaffold-based tissue regeneration.

4. Conclusions

The co-substituted polyphosphazene synthesized with hydrophilic glycylglycine dipeptide ester and hydrophobic phenylphenoxy groups showed an exceptional ability to form inter- and intra-molecular hydrogen bonds with a tunable degradation pattern. This co-polymer

resulted in a completely miscible blend system with the polyester through intermolecular hydrogen bonding. By altering the blend composition it was possible to control blend physico-chemical properties and degradation pattern. Additionally these blends inherited the ability to self-neutralize acidic degradation products of the polyester. In all the blend compositions both PLAGA and PPHOS components exhibited a similar molecular weight loss degradation pattern. Unique polymer erosion process was evident in all the blend compositions in which a solid blend film degraded to form a self-assembled polyphosphazene sphere-based 3D porous structure with interconnected porosity. Characterization of *in vivo* degradation samples at 12-week revealed the formation of 3D interconnected porous structures with 82-87% porosity. Furthermore, it was evident that these *in situ* formed 3D interconnected porous structures enabled cell infiltration and collagen tissue in-growth throughout the void space between spheres. This study for the first time reports a biodegradable and biocompatible blend system with unique erosion mechanism through which self-assembled spherical porous structures are formed. Such an observed three-stage degradation mechanism was supported by inter- and intra-molecular hydrogen bonding interactions and variations in the degradation rates of both blend components. These findings are highly encouraging to develop and control 3D scaffolds for regenerative purposes with initial higher mechanical properties that reduces over time as polymer degrades while generating interconnected porous structures to allow tissue in-growth and matrix integration. Studies are currently underway to optimize dipeptide-based polyphosphazene side group composition to achieve higher mechanical properties suitable for load-bearing applications. We are also evaluating this blend system as a factor delivery vehicle and tissue engineering scaffold for orthopaedic applications.

5. Experimental

Fabrication and Characterization of Blend Matrices

Blend matrices were prepared using a mutual solvent method [18]. Briefly, the polymers with two weight ratios of PPHOS to PLAGA (25:75 and 50:50) with a total weight of 5 g, were dissolved in 20 mL of mutual solvent (chloroform) to obtain a homogeneous solution. Samples of the polymer solution were subsequently poured into petri dishes lined with Bytac paper and the solvent was allowed to evaporate slowly at 4°C for 48 hours followed by freeze drying. Finally, circular disks of 10 mm diameter and 0.5 mm thickness were bored from the films.

The surface morphology of the blend matrices was examined by SEM using a JEOL 6700F scanning electron microscope (JEOL, Boston, MA, USA) after being coated with Au/Pd using a Hummer V sputtering system (Technics Inc., Baltimore, MD). The glass transition temperatures (T_g 's) were determined by differential scanning calorimetry (DSC) using a TA DSC Q1000 instrument with Thermal Analysis software. Polymer samples were heated from -40°C to 100°C at a heating rate of 3°C per minute under a nitrogen atmosphere. The T_g was determined from the half height point of the heat capacity change in the thermogram. Attenuated total reflection infrared (ATR-IR) spectra of polymers and blend matrices were recorded using a Nicolet iS10 FT-IR spectrometer coupled with an ATR accessory (Thermo Scientific, Inc., Waltham, MA) at a resolution of 4 cm⁻¹ and with an average of 16 scans.

High-resolution solid-state NMR

¹H-¹³C cross polarization magic angle spinning (CPMAS) spectra with total suppression of spinning sidebands (TOSS) were obtained at room temperature on a Bruker AV-300 solid state NMR spectrometer operating at 75.55 MHz for ¹³C and 300.43 MHz for ¹H. 12288 or 10240 scans were accumulated for each sample. A linear ramped contact pulse was used for ¹H and a rectangular contact was used for ¹³C, with a contact time of 1.5 msec. The

spinning rate was 5000 Hz, and the relaxation delay was 5 seconds. Spinal ^{128}H decoupling was used during acquisition. The spectra were referenced indirectly to the aromatic ^{13}C shift of hexamethylbenzene ($\delta = 132.2$ ppm). ^{31}P magic angle spinning spectra were obtained with ^1H decoupling at 121.61 MHz, and referenced indirectly to 85% phosphoric acid (0.0 ppm).

In Vitro Degradation

Blend disk matrices with dimensions of $0.5\text{ mm} \times 10\text{ mm}$ ($T \times D$) and weighing ~95 mg each were incubated in 10 mL of distilled water at pH 7.4 up to 12 weeks at 37°C [8]. The vials were maintained at 37°C in a shaker water bath for 12 weeks at 250 rpm. At specific time points (2, 4, 7, 10, and 12 weeks), the matrices were removed from distilled water and were dried under vacuum for 2 weeks. The results were reported as percentage mass loss versus time as calculated from the equation: Percentage mass remaining = $w_t / w_0 \times 100\%$ where w_t is the dry weight of the matrix at predetermined time points, and w_0 is the initial matrix dry weight. The molecular weight of the degraded matrices was also determined using GPC and reported as percentage molecular weight remaining: Percentage molecular weight remaining = $M_t / M_0 \times 100\%$ where M_t is the molecular weight of polymer component at predetermined time points, and M_0 is the initial molecular weight of polymer. Samples were visualized by SEM. The media were also collected and the pH values were recorded by a pH meter.

Rat Subcutaneous Implantation Model

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

Scanning Electron Microscopy

The surface morphology of the blend matrices was examined by SEM using a Hitachi TM-1000 tabletop microscope.

Characterization of Degradation Kinetics

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

Micro-CT analysis

Total pore volume within the scaffolds was quantified using conebeam micro-focus X-ray computed tomography (μCT40 , Scanco Medical AG, Brüttisellen, Switzerland). Samples were imaged at 55 kV and 145 micro-amperes, collecting 2000 projections per rotation at 300 msec integration time. 3D images were reconstructed using standard convolution and back-

projection algorithms with Shepp and Logan filtering, and rendered within a 12.3 mm field of view at a discrete density of 4,629,630 voxels/mm³ (isometric 6 micrometer voxels). Threshold segmentation of scaffold from background was performed in conjunction with a constrained Gaussian filter to reduce noise, applying a hydroxyapatite-equivalent density threshold of 7 mg/cm³. The volume fractions of solid versus pore were measured directly within volumetric regions of interest approximately 50 mm³.

Histological Staining

At specific time points (2, 4, 7, 10, and 12 weeks), the implants and the surrounding tissues were excised and fixed in a 10% formalin solution (Surgipath, USA) for 7 days. The samples were embedded in paraffin and sectioned using a microtome to about 4-5 μ m thickness, followed by staining with hematoxylin and eosin (H&E) and Masson's trichrome stain (TRI). The thickness of the inflammatory zone (H&E) and collagen deposition (TRI) was characterized according to a reported protocol [22].

Statistical Analysis

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. High-resolution solid-state ^{13}C NMR spectra for PLAGA, PPHOS, and the blend matrices demonstrating the hydrogen bonding interactions between the dipeptide units of PPHOS and the carbonyl groups in PLAGA. Thermal ring opening polymerization of $(\text{NPCl}_2)_3$ followed by nucleophilic substitution reactions of the resultant poly(dichlorophosphazene) produced the yellow polymers of PPHOS.

Figure 2.

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

Figure 3.

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

Figure 4.

In vivo degradation profiles of blend matrices and pristine PLAGA over 12 weeks. (a) Percentage of mass remaining; (b) Percentage of PLAGA molecular weight remaining in the pristine PLAGA and blend matrices during 12 weeks of implantation; and (c) Percentage of polyphosphazene molecular weight remaining in the blend matrices during 12 weeks of implantation. The mass loss profiles during the implantation suggested the degradation rate to be PLAGA > Matrix1 > Matrix2. The molecular weight for both PLAGA and polyphosphazene components in the blend decreased in a similar trend throughout the 12-week implantation period.

Figure 5. Micro-CT analysis for the blend matrices during *in vivo* implantation. (a-j) Representative 2D micro-CT images illustrating the progression of morphological and structural changes within the blend system, scale bar 100 μm . The darker and lighter regions indicate the *in situ* formed pores and residue polymers, respectively; and (k) *In-situ* porosity ((total volume-polymer volume)/total volume) of the matrix based on the 3D micro-CT reconstructions during the polymer degradation. As illustrated in (a) and (b), both the blend matrices showed a continuous and homogeneous phase. Quantitative analysis demonstrated 0% porosity for the blends prior to implantation (T=0). Since the PLGA component served as a “dynamic porogen”, the interconnectivity of the resultant porous structures was 100% as indicated in the 2D micro-CT images. In addition, it was shown that the blend composition had no significant effect on the porosity of the blend matrices. Both the blend matrices had a porosity of 82-87% after 12 weeks of implantation.

Figure 6.

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

Figure 7.

A proposed three-stage model for the unique erosion process: *Stage I* represents the initial blend matrix where intermolecular hydrogen bonding between PLGA and PPHOS is dominant; *Stage II* is the intermediate state where disruption of intermolecular hydrogen bonding occurs that results from the fast degradation of PLGA; *Stage III* represents the polymer sphere state where intramolecular hydrogen bonding between PPHOS chains is dominant. Representative IR spectra for the blend matrix during polymer degradation illustrate the three-stage process. In both degradation environment (aqueous media and *in vivo*), intermolecular hydrogen bonding (peak~1677 cm⁻¹) was found for the blend matrix at 0 week. No hydrogen bonding peaks were detected for 2 weeks and 4 weeks due to the disruption of the intermolecular hydrogen bonding resulted from fast degradation of PLGA. After 4 weeks, PPHOS spheres formed through intramolecular hydrogen bonding (peak~1655 cm⁻¹) at 7 weeks, 10 weeks, and 12 weeks.

Scheme 1.

Schematic illustration of different types of polymer erosion. (a) Bulk erosion; (b) Surface erosion; and (c) A unique polymer erosion through which the polymer changes from a solid coherent film to an assemblage of microspheres with an interconnected porous structure.