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Novel Factor-loaded Polyphosphazene Matrices:

Potential for Driving Angiogenesis

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Abstract

Currently employed bone tissue engineered scaffolds often lack the potential for vascularization, which may be enhanced through the incorporation of and regulated release of angiogenic factors. For this reason, our objective was to fabricate and characterize protein-loaded amino acid ester polyphosphazene (Pphos)-based scaffolds and evaluate the novel sintering method used for protein incorporation, a method which will ultimately allow for the incorporation of proangiogenic agents. To test the hypothesis, Pphos and their composite microspheres with nanocrystalline hydroxyapatite (Pphos-HAp) were fabricated via the emulsion solvent evaporation method. Next, bovine serum albumin (BSA)-containing microsphere matrices were created using a novel solvent-non solvent approach for protein loading. The resulting protein (BSA) loaded-circular porous microsphere based scaffolds were characterized for morphology, porosity, protein structure, protein distribution, and subsequent protein release pattern. Scanning electron microscopy revealed porous microsphere scaffolds with a smooth surface and sufficient level of sintering, illustrated by fusion of adjacent microspheres. The porosity measured for the PNPhGly and PNPhGly-HAp scaffolds were 23 +/- 0.11% and 18 +/- 4.02%, respectively, and within the range of trabecular bone. Circular dichroism confirmed an intact secondary protein structure for BSA following the solvent sintering method used for loading, and confocal microscopy verified that FITC-BSA was successfully entrapped both between adjacent microspheres and within the surface of the microspheres while sintering. For both Pphos and their composite microsphere scaffolds, BSA was released at a steady rate over a 21day time period, following a zero order release profile. HAp particles in the composite scaffolds served to improve the release profile pattern, underscoring the potential of HAp for growth factor delivery. Moreover, the results of this work suggests that the solvent nonsolvent technique for protein loading is an optimal one that will allow for future development of angiogenic factor loaded Pphos matrices with the capacity to invoke neovascularization.

Keywords

Polyphosphazene; Nanocrystalline; nano Hydroxyapatite; VEGF; Solvent sintering; Angiogenesis

1. Introduction

Recent studies have shown that approximately 6.3 million fractures occur annually in the US with the highest level of these fractures occurring among the elderly (65 and older) as a result of fall-related accidents [1-2]. Furthermore, due to advances in medical technologies, the number of persons aged ≥ 65 years is projected to increase to 68.1 million by 2040, which translates to projected higher numbers in bone related injuries [3].

With the increasing demand for more efficient bone tissue substitutes, biodegradable polymers have come to be a major focus in bone tissue engineering research. Studies have shown that synthetic biodegradable materials can be easily formed into mechanically stable, porous scaffolds that allow for nutrient flux and tissue ingrowth. Commonly employed α -hydroxy ester polymers, poly(lactide-co-glycolide) (PLGA) and polycaprolactone (PCL) exhibit promise as a treatment in this area; nevertheless, over time, long term use of these materials raise issues, such as accumulation of acidic degradation products, in the case of PLGA, and reduced biocompatibility in the case of PCL due to its highly hydrophobic nature. To circumvent the issues seen with these materials, other polymers have been explored.

A newly emerging group of polymers, polyphosphazenes (Pphos), shows impressive potential for use in the area of bone tissue engineering. Pphos polymers possess an inorganic backbone composed of alternating phosphorous and nitrogen atoms with organic side groups bound to the phosphorous atoms, see Figure 1a [4-5]. Furthermore, with regards to the synthetic versatility of Pphos, the side group substitution procedure allows for tailoring the polymer's physicochemical properties including - glass transition temperature, mechanical strength, degradation rate, hydrophilicity, and biocompatibility in vitro as well as in vivo [6-9]. Not only can the properties of this polymer be easily modified based upon the respective side groups, but these materials are capable of degrading into neutral, buffering degradation products of ammonia, phosphate, and the corresponding side groups [10-14].

Moreover, our lab has previously demonstrated the osteocompatibility of amino acid ester substituted polyphosphazenes [8, 21]. For this work, the amino acid ester polyphosphazene heteropolymer, poly(ethyl phenylalanato:glycinato)phosphazene (PNPhGly), with a ratio of phenylalanato to glycinato groups of 75:25, Figure 1b, is evaluated for the formation of three dimensional (3D) factor-loaded scaffolds. Incorporating low levels of ethyl glycinato side groups is expected to tune the degradability of the polymer by allowing for water to access the polymer backbone, which may otherwise be inhibited by solely involving the bulkier phenylalanato side group. Furthermore, by tailoring the degradation rate we intend to alter the rate and fashion in which future incorporated factors are released.

In addition to biodegradable polymers, bioactive ceramics have been successfully employed as bone substitutes [15]. Bioactive nanocrystalline hydroxyapatite (HAp), $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, is a calcium phosphate ceramic which comprises the mineral component of natural bone. In experiments aimed to investigate the osteocompatibility of nanocrystalline hydroxyapatite compared to microcrystalline, HAp proliferation is enhanced on nanoHAp when compared to larger sized HAp particles. Also, we expect that the presence of the nanocrystalline HAp particles will allow for tuning factor release by increasing the surface area of the proposed matrices and by further improving the potential for angiogenic molecules and protein adsorption to the surface.

Because bone is a highly vascularized tissue, the requirements needed for bone substitutes include favoring the formation of a vascular network. This is key to sustaining cell infiltration and survival. Incorporation and release of the proangiogenic macromolecule, vascular endothelial growth factor (VEGF), to promote of neovascularization presents many advantages. Vascular endothelial growth factor (VEGF), an endothelial cell mitogen that plays

a large role in both angiogenesis and vasculogenesis, is also involved in a number of other physiological and pathological processes. Numerous studies have demonstrated and supported the notion of VEGF incorporation and release for enhancing bone tissue regeneration [16-17]. An increase in the presence of VEGF during the initial hematoma phase and the later (endochondrial ossification) callus formation stage of the bone healing pathway has been demonstrated [18]. Additionally, another group, Filvaroff et al., has shown that inhibition of VEGF in mice delays bone tissue healing. Their work indicates that VEGF is required for callus formation and mineralization, two of the latter stages in bone wound healing [18]. Henceforth, by introducing angiogenic promoting factors to the defect site via the 3-D composite scaffolds, neovascularization of the synthetic composite constructs is expected, providing access for stem cells, osteogenic precursor cells and factors, as well as enhanced delivery of oxygen and nutrients.

In tying the biodegradable materials phase and proangiogenic factor components together, a novel method for sintering Pphos-based microsphere scaffolds is employed for protein loading. There are currently several methods for incorporating bioactive factors into biodegradable matrices; however, the caveat revealed with each of these methods outweighs the benefits of their use. For instance, with the double emulsion (water-in-oil-in-water) method for protein loading, low encapsulation efficiency is observed as hydrophilic drugs or proteins are leach out during microsphere fabrication and sterilization. Also, the protein adsorption/soaking method remains a costly alternative, requiring high concentrations of expensive growth factors in order to obtain a reasonable loading efficiency. Protein adsorption, not to mention, is not a suitable technique for creating scaffolds for long term use as the loaded factors tend to be released in a burst fashion [19-20]. The ideal case is to develop a system capable of zero order release. In other words, the optimal system will be one in which constant levels of drug are released over time, independent of the initial amount loaded. Furthermore, by performing both the microsphere sintering and protein loading methods simultaneously, sustained, zero order release is expected over longer durations.

2. Materials and Methods

2.1. Materials

Solvents, tetrahydrofuran (THF), hexanes, methylene chloride, and deuterated chloroform were obtained from Sigma Aldrich (St. Louis, MO). Additionally, essentially fatty acid free, $\geq 96\%$ (agarose gel electrophoresis), lyophilized powder bovine serum albumin (BSA), O-phthaldialdehyde Solution Complete (OPA), and polyvinyl alcohol (PVA), molecular weight 30-70kDa, and FITC-Albumin bovine were purchased from Sigma Aldrich (St. Louis, MO). Phosphate buffered saline (1X) (PBS) and recombinant mouse VEGF were acquired from Invitrogen (Carlsbad, CA). And nanocrystalline hydroxyapatite (HAp), 100nm, was received from Berkeley Advanced Biomaterials, Inc. (Berkeley, CA).

2.2. Polymer Synthesis

The novel polymer, poly(ethyl phenylalanato-glycinato)phosphazene (PNPhGly), substituted with 75% phenylalanato and 25% glycinato side groups, was synthesized. Pphos was fabricated using a two-step preparative protocol in which an intermediate poly(dichloro phosphazene) was first produced by the thermal ring opening cationic polymerization of the cyclic trimer at 250°C. The reactive chlorine atoms of the intermediate were then replaced, in the presence of triethylamine, by the amino acid ester groups, ethyl phenylalanine and ethyl glycine [20-24].

2.3. Polymer Characterization

Once synthesized, the PNPhGly polymer was analyzed for a number of parameters. Gel permeation chromatography (GPC) was performed by a Hewlett Packard LC 1090 unit with a

polystyrene stationary phase, to evaluate the molecular weight of the polymer. Briefly, a 5% (w/v) solution of PNPhGly in tetrahydrofuran (THF) was created and filtered into a vial. A polystyrene stationary phase (Zorbax 300S column) and a mobile phase of tetrahydrofuran with a 100uL injection volume was used. Samples were, next, analyzed by nuclear magnetic resonance spectroscopy (^1H NMR) (Bruker 360 MHz NMR spectrometer). For this, a 10% (w/v) polymer melt in deuterated chloroform (d-chloroform) was formed.

2.4. Fabrication of Polyphosphazene Microsphere Scaffolds

Microspheres were fabricated using a conventional emulsion solvent evaporation technique [25]. Briefly, a 20% (w/v) solution of PNPhGly in methylene chloride was created. The polymer solution was then steadily poured into a 1% poly(vinyl alcohol) PVA aqueous solution stirring at a constant rate of 4200 rpm, and the emulsion was stirred for 24 hours to allow for solvent evaporation. The resulting microspheres were washed 3Xs with distilled water and dried by vacuum filtration prior to sieving to the desired size range.

To form composite PNPhGly-HAp microspheres, similar techniques to those mentioned above were employed [27]. Composite microspheres were fabricated by the addition of nanocrystalline hydroxyapatite (HAp) to the polymer melt solution. First, 4g PNPhGly and 1g nanoHAp were dissolved in methylene chloride forming a 80:20 polymer-HAp ratio and a 20% (w/v) polymer solution. The PNPhGly-HAp solution was then steadily poured into a 1% (w/v) poly(vinyl alcohol) PVA aqueous solution stirring at a constant rate of 420 rpm, and the emulsion was stirred for 24 hours to allow for solvent evaporation. The resulting microspheres were then washed 3Xs with distilled water and dried by vacuum filtration prior to sieving to the desired size range.

The solvent/nonsolvent soft sintering approach for forming Pphos-based microsphere matrices has been developed and well characterized by the Laurencin labs [28-29]. Briefly, (500-700um) microspheres were placed into a stainless steel mold and a solvent/nonsolvent solution of THF and Hexanes was dripped onto the microspheres. Two ratios of solvent to nonsolvent were tested-25:75 and 30:70 THF:Hexanes. The resulting microsphere scaffolds were permitted to sit under fume hood conditions overnight, prior to being placed under vacuum to remove any residual solvent. Pure PNPhGly and composite PNPhGly-HAp microsphere discs (8mm $\times$ 2mm, diameter = 8mm in diameter, thickness = 2mm) were fabricated and further characterized by scanning electron microscopy (SEM) and porosimetry.

2.5. Scanning Electron Microscopy (SEM)

Scaffolds were coated with a gold/palladium alloy with a sputter coater. SEM (Amray scanning electron microscope, JEOL JSM-840A) was performed using an accelerating voltage of 10kV, a 27mm working distance, and a 1×10^{-10} amp probe current.

2.6. Porosimetry Analysis

Using a mercury intrusion porosimeter (Micromeritics Instrument Corporation, Norcross, GA), the pore size was calculated by measuring the pressure needed to oppose the surface tension of a known volume of mercury pushed into the sample. Briefly, PNPhGly and PNPhGly-HAp microsphere-based (500-700um) scaffolds (8mm $\times$ 2mm) were employed (n=3 per run), and data from two runs were evaluated, n=2.

2.7. Circular Dichroism (CD) Analysis

To investigate protein structural stability following interaction with solvents that will be employed for the soft solvent sintering technique, circular dichroism analysis (CD) was performed using an AVIV 215 spectropolarimeter. A 1 μM stock solution of bovine serum

albumin (BSA) reconstituted in phosphate buffered saline (PBS) was prepared. Methylene chloride (2mL) was added to 2mL of the 1mM BSA solution and the mixture was capped and shaken briefly to promote interaction of the solvent and protein. Under fume hood conditions, the solution was permitted to sit uncapped overnight to allow for complete solvent evaporation. Following solvent removal, the BSA samples were analyzed at 25°C using an AVIV 215 spectropolarimeter. The above steps were repeated for the solvents: tetrahydrofuran (THF), hexanes, acetone, ethanol, and methylene chloride.

The same procedure was repeated with Recombinant Mouse VEGF, 38.8kDa, (Invitrogen, Carlsbad, CA). The solvents investigated for this case were: tetrahydrofuran (THF), hexanes, acetone, and ethanol. A 20µg/mL stock solution of VEGF in phosphate buffered saline (PBS) was prepared and interacted with 2mL of the solvent/nonsolvent combinations. Next, the tube was capped and shaken briefly to promote interaction of the solvent and protein. Under fume hood conditions, the solution was permitted to sit uncapped overnight to allow for complete solvent evaporation. And following solvent removal, the protein samples were analyzed using an AVIV 215 spectropolarimeter.

2.8. Fabrication of BSA-Loaded Solvent-Sintered PNPhGly Microsphere-Based Scaffolds

In order to evaluate the feasibility of incorporating protein, a solvent/nonsolvent sintering approach for loading Bovine Serum Albumin (BSA) (Sigma-Aldrich, US) was tried.

A specific volume of 1mg/mL BSA was added to THF and Hexanes at a ratio of 15:75:10 THF:Hexanes:1mg/mL BSA and agitated immediately before applying to the microspheres. Next, the sintering solution was dripped over the PNPhGly and composite PNPhGly-HAp microspheres (500-700µm) forming scaffolds, that were 8mm in diameter and 2mm in thickness (8mm×2mm). The scaffolds were then placed under vacuum to allow for the complete removal of solvent and residual moisture.

2.9. Protein Release from Solvent-Sintered PNPhGly Microsphere-Based Scaffolds

To measure the release profile of BSA from the PNPhGly and composite PNPhGly-HAp microsphere scaffold (500-700µm) discs (8mm×2mm), first, scaffolds were sterilized in 70% ethanol for 15 minutes then rinsed in 1X PBS to mimic the commonly employed sterilization procedures that precede *in vitro* and *in vivo* analysis of synthetic matrices. Matrices were then placed in centrifuge tubes containing 2mL of PBS, and moved to a water bath maintained at 37°C with continuous shaking at 100 RPM. At various time points (1, 4, and 10 hours, and 1, 3, and 7days) all of the supernatant was collected from the vials and evaluated for protein content using the o-Phthalaldehyde (OPA) reagent method. After 21d, the scaffolds were dried and the solvent extraction method was performed to quantify the remaining protein [30]. Briefly, 1-2mL of methylene chloride was added to the polymer matrix and resulting mixture was vortexed for 5 minutes. After centrifuging the samples at 4500rpm for 10 minutes, the resulting polymer solution phase was decanted leaving behind a protein pellet. This procedure was repeated twice more and the resulting protein pellet was resuspended in 1mL of 1X PBS and evaluated for protein content using the o-Phthalaldehyde (OPA) reagent method. With this method of protein quantification, OPA reacts with primary amines to produce a fluorescent compound that can be excited at 360nm and detected at 465nm using a UV-Vis spectrophotometer. Approximately 300µL of OPA solution complete, containing b-mercaptoethanol and boric acid, was added to 10µL of each sample. The reaction solution was incubated in the absence of light for 5 minutes. Following, the sample fluorescence was read using a UV-Vis spectrophotometer. First, a calibration was performed to determine the fluorescence intensity for given dilution series of BSA. A standard curve was constructed; and, the concentration of BSA released at specific time points was extrapolated from the slope of

the standard curve. The amount of protein released was reported in terms of a percent cumulative release (%).

2.10. Confocal Microscopy

A Nikon PCM2000 laser scanning confocal was used to visualize the distribution of loaded FITC-BSA on the microsphere scaffolds. For scaffold fabrication, 10mg of FITC-BSA was added to 1mL of 25:75 THF and Hexanes and the solution was agitated using a vortex device plus sonication. A fraction of the resulting sintering solution was dripped over PNPhGly microspheres (500-700um) forming scaffolds, 8mm in diameter and 2mm in thickness (8mm×2mm). The scaffolds were then placed under vacuum to allow for the complete removal of solvent and residual moisture. Samples were visualized by confocal laser microscopy using a Plan-NeoFluar 10X/0.3 objective at an excitation of 488 nm.

2.11. Statistical Analysis

A Post-Hoc Tukey test was employed to establish significance between groups. Furthermore, a p-value of 0.05 will be the threshold value for determining significance unless stated otherwise.

3. Results & Discussion

3.1. Polymer Characterization

The Pphos family of polymers consists of more than 700 different polymers, each virtually unique with respect to the range of different properties that depend on the nature of the side groups present [5]. Once the novel polymer, poly(ethyl phenylalanato-glycinato)phosphazene (PNPhGly), was synthesized, the material was analyzed for a number of parameters.

Gel permeation chromatography (GPC) (GPC Series 1100, Hewlett Packard) was used to determine the molecular weight of the fabricated novel PNPhGly, n=4. The weighted average molecular weight, Mw, and number average molecular weight, Mn, were determined. The ratio of these two values, or the polydispersity index, PDI, was calculated from these two measures.

The high molecular weight distribution observed for PNPhGly, Table 1, implies that the polymer will possess good mechanical properties (i.e., compressive modulus and compressive strength). Moreover, the low polydispersity index value calculated is indicative of a narrow molecular weight distribution and verifies that the polymer synthesis reaction was successful in creating a uniform quality product. Having a product with a narrow molecular weight distribution, in addition, ensures that the material and its properties can be easily characterized.

For further characterization, samples were analyzed by nuclear magnetic resonance spectroscopy (NMR) (Bruker 360 MHz NMR spectrometer). Through this technique the chemical structure of the heteropolymer was verified. The resulting spectra shows the following expected peaks for PNPhGly (75:25): 2H's - 1ppm, 2Hs - 2.1ppm, 1H - 3ppm, 1H - 3.8ppm, 3H's - 4.2ppm, CDCl3 - 4.75ppm, 1H - 7.2ppm, 4H's - 7.1ppm.

3.2. Scaffold Architecture

A microsphere scaffold fabrication technique was used for the purposes of promoting osteoconductivity, porosity, and interconnectivity of the pore structure. It is important that the scaffolds developed be osteoconductive; in other words, the scaffolds must permit bone cell migration within and throughout the scaffold. For this reason, we employed a set of techniques to form the polymer into a three dimensional porous microsphere matrix, techniques which have been proven to enhance conductivity compared with other scaffold fabrication techniques [26,31-32].

Images of solvent sintered microsphere scaffolds were obtained using a scanning electron microscope (JEOL JSM-840A) to illustrate the level of sintering between adjacent PNPhGly-based microspheres using the solvent sintering technique, and essentially allow for visual and qualitative examination of the micro architecture of the scaffolds, (e.g. microsphere size, surface porosity and pore interconnectivity).

Two solvent to nonsolvent ratios were examined-25:75 and 30:70, to evaluate which composition would provide a satisfactory level of sintering. Visual examination of the PNPhGly and the composite PNPhGly-HAp microsphere scaffolds, Figure 2, demonstrates that a sintering solution composition of 25:75 THF:Hexanes promotes a sufficient level of fusion between neighboring spheres, generating a microsphere scaffold with an appropriate architecture and morphology. The microsphere based scaffolds formed using the 30:70 THF:Hexanes sintering solution were structurally inapt for future bone tissue regenerative applications. SEM analysis illustrates, Figures 2G - 2J, that the 30:70 THF:Hexanes sintered scaffolds lacked the required porosity and interconnective pore structure needed to allow tissue ingrowth. Furthermore, these results suggest that increasing the solvent to nonsolvent ratio increases the potency of the sintering solution in terms of sintering power. This phenomenon is similarly observed with increasing temperature or exposure time during a thermal sintering process [33].

Porosimetry (Micromeritics Instrument Corporation, Norcross, GA) was used to quantitatively evaluate the scaffold architecture (mean pore volume and porosity/pore size distribution). Because these scaffolds, in future applications, should serve as a negative template for bone, which has a measured average porosity of 70-80%, it is imperative that the solvent/nonsolvent composition employed allows for forming scaffolds with a porosity on the order of 20-30% [34].

The porosity measured for these PNPhGly and PNPhGly-HAp microsphere based scaffolds, sintered with 25:75 THF:Hexanes, Table 2, were measured to be 23 +/- 0.11% and 18 +/- 4.02%, respectively. The porosity measurements obtained for these scaffolds are ideal for bone tissue repair applications, further providing support for the use of the soft sintering approach for PNPhGly microsphere scaffold formation and factor loading.

3.3. Circular Dichroism (CD) Analysis

By exposing a protein sample to circularly polarized light, an absorbance spectra specific to the protein's configuration can be generated.

The CD spectra data were obtained to determine whether brief interaction of BSA and VEGF with solvents potentially involved in the soft sintering procedure for protein incorporation would result in alteration in the secondary structure of these proteins. Moreover, the data illustrate that interaction of BSA with the mentioned solvents does not result in significant alteration of protein structure or significant alteration of the spectra profile. The differences, however, seen in the CD spectra between VEGF exposed to the mentioned solvents and VEGF held in PBS (control) can be attributed to slight alterations in the secondary structure of the protein. Nevertheless, whether, this slight change will result in a loss of the growth factor bioactivity remains to be determined with future investigations aimed to evaluate the response of endothelial cells to VEGF-loaded Pphos microsphere based scaffolds. Furthermore, to corroborate the CD evidence, in the future we intend to conduct an *in vitro* study to investigate whether the growth factor bioactivity is maintained following factor incorporation and release from microsphere-based scaffolds.

3.4. Factor Release from Solvent-Sintered PNPhGly Microsphere-Based Scaffolds

An evaluation of BSA release was conducted to establish the capability of the solvent sintering technique for growth factor loading and for generating PNPhGly and PNPhGly-HAp based scaffolds with the capacity to deliver growth factors in a sustainable fashion. For these studies, BSA served as a model protein for VEGF. Both proteins, VEGF and BSA, exhibit high molecular weights ($M_w > 30\text{kDa}$) and a net charge at neutral pH. It is expected that during the solvent sintering/loading procedure, proteins will not merely adsorb to the microsphere surface, but will primarily enter the dissolved polymer phase between contacting microspheres and on the microsphere surface. This alternate method for protein incorporation was examined due to the pitfalls observed with currently employed systems for loading. Major drawbacks of incorporating factors during the emulsion/solvent evaporation method, used for microsphere fabrication, or even during thermal sintering procedures, used to form microsphere-based scaffolds, is the potential for incorporated soluble factors to leach out into solution during microsphere formation or to be damaged by exposure to extreme temperatures. The solvent/nonsolvent sintering method, on the other hand, proves to be a more effective method for the formation of factor-loaded polymer microsphere-based structures.

As evident in the confocal images of FITC-BSA loaded PNPhGly scaffolds, simultaneously loading FITC-BSA and sintering the microspheres allows for creating scaffolds in which the incorporated macromolecule accumulates between adjacent microspheres and within the microsphere surfaces. Figure 4 depicts FITC-BSA distribution at different orthogonal sections/planes providing support for the idea that the novel sintering method can be used to generate scaffolds in which factors are effectively incorporated. The illustration shown represents protein distribution in a $512\mu\text{m} \times 512\mu\text{m}$ area in the xy plane, at a particular slice in the z axis. Moreover, further experimentation must be performed to examine the loading efficiency of this method. The confocal image presented here merely provides evidence that the protein is being loaded effectively on the surface and in between adjacent spheres. It should be noted, that using this method for factor loading, it is expected that the majority of the protein loading should primarily occur at the surface in closest proximity to the device used to dispense the solvent sintering solution, having some effect on the uniformity of the protein distribution. Nevertheless, the release results obtained suggest that a non uniform protein distribution does not prevent acquiring a sustained controlled release of BSA from the Pphos-based microsphere scaffolds, see Figure 5.

Moreover, with the BSA-loaded PNPhGly and composite PNPhGly-HAp microsphere discs ($8\text{mm} \times 2\text{mm}$) developed using the solvent sintering method of factor incorporation, we are able to show, in Figure 5, that following exposure to an aqueous solution no ‘burst effect’ is observed. By evaluating the protein profile with various mathematical models for describing factor release, the likely mechanism for BSA release from the PNPhGly based scaffolds, with 75% ethyl phenylalanato groups, is primarily via polymer erosion. It is expected that the dominant presence of the bulky ethyl phenylalanato side groups, will lend the polymer to be more hydrophobic in nature. Moreover, for hydrophobic biodegradable polymers, degradation has been shown to occur primarily by surface erosion, explaining why this particular PNPhGly polymer is suitable for drug delivery applications.

A well known model for drug release from surface eroding polymers, developed by Hopfenberg, describes drug release from a slab geometry according to the equation shown below [35].

$$\frac{M_t}{M_\infty} = 1 - \left(1 - \frac{k_0 \cdot t}{c_0 \cdot a}\right)^n$$

Where M_t is the amount of drug released at time, t ; M_∞ is the amount of drug released when the material is abolished, k_o is the erosion kinetic constant, and n describes the matrix geometry. For a flat slab, $n=1$; while $n=2$ for a cylinder, and $n=3$ for a sphere. C_o indicates the uniform initial concentration of drug within the matrix and a is the initial matrix radius. Furthermore, by assuming constant values for k_o , c_o , a , and M_∞ , and for a slab configuration, drug release should exhibit a zero order profile, which is what is observed for this particular BSA releasing PNPhGly and composite PNPhGly-HAp scaffold system.

The slight difference observed between the two groups, PNPhGly and composite PNPhGly-HAp, is most likely due to electrostatic interactions between the nanocrystalline HAp particles on the surface of the composite microsphere scaffolds and the released protein, allowing for protein adsorption and ultimately resulting in the more controlled release pattern observed when compared to the PNPhGly group alone. The idea behind such interactions existing between mineral HAp and proteins has been supported with studies that demonstrate successful VEGF adsorption onto ceramic-based materials as a result of electrostatic forces [38]. Additionally, adsorption of proteins to HAp has been studied and reported by other groups, who claim that the drug delivery capabilities of less crystalline hydroxyapatites can be attributed to a high surface area, which was measured using the BET method [39].

More importantly, though BSA (66kDa)-loaded PNPhGly scaffolds may exhibit a slightly different release profile than VEGF (38kDa)-loaded PNPhGly-based scaffolds, due to their differing properties, the release model developed here will provide some insight into the release of macromolecules/growth factors in general from the 3D PNPhGly and PNPhGly-HAp scaffold systems. Because bolus delivery of VEGF has been found to lead to hypotension or a drop in blood pressure, and in vivo biodegradation of VEGF accounts for its short half life, there is a need for a system that delivers appropriate levels of active VEGF over a sustained period of time in order to properly promote angiogenesis [36-37]. The work described here provides a practical solution to this problem, one that is expected to allow for controlled increments of VEGF to be released over time, ensuring that the surrounding endothelial cells are repeatedly activated. To determine the efficacy of this novel release system for bone tissue engineering applications, future dose response analysis must be undertaken to provide insight into the ideal amount of VEGF to be released, a concentration capable of eliciting a proangiogenic response, promoting endothelial cell proliferation and tubule formation.

Based upon the data revealed here, composite amino acid ester Pphos-HAp microsphere scaffolds show promise as agents for angiogenic factor delivery and ultimately for neovascularization and bone tissue repair.

4. Conclusions

No other study has employed the solvent sintering method for protein loading onto biodegradable microsphere based scaffolds. Moreover, this is the first time in which poly(ethyl phenylalanato:glycinato)phosphazene (PNPhGly) and composite poly(ethyl phenylalanato:glycinato)phosphazene -nanocrystalline HAp (PNPhGly-HAp) scaffolds are being investigated for use as potential angiogenic delivery vehicles. The data generated in this study provides insight into the potential for using protein-loaded composite amino acid ester based polyphosphazene-nanoHAp matrices for bone tissue engineering purposes through promoting scaffold vascularization. Additionally, great support is shown here for using the solvent sintering process as an alternative to the other methods of bioactive factor loading (i.e., adsorption and double emulsion solvent evaporation) into biodegradable synthetic polymer matrices.

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a

b

Figure 1. Polyphosphazene Schematic

a. Structure of polyphosphazene backbone, b. Structure of ethyl phenylalanato side group, R_1 , and ethyl glycinate side group, R_2 .

Figure 2. Scanning Electron Microscopy

SEM images of solvent sintered PNPhGly and PNPhGly-HAp microsphere scaffolds sintered using 25:75 and 30:70 THF:Hexanes solutions. Accelerating voltage, 10kV; Working distance, 25mm; Probe current, 1e-10amps. Images A (500X, 45um scale bar), B (100X, 230 um Scale bar), and C (15X, 1585 um scale bar), represent three magnifications of the 25:75 THF:Hexanes sintered PNPhGly. Images D (500X, 45um scale bar), E (100X, 230 um Scale bar), and F (15X, 1585 um scale bar), illustrate three magnifications of the 25:75 THF:Hexanes sintered composite PNPhGly-HAp. The 30:70 THF:Hexanes sintered PNPhGly scaffolds are shown in images G (50X, 475 um scale bar) and H (15X, 1585 um scale bar). And, the 30:70

THF:Hexanes sintered composite PNPhGly-HAp scaffolds are shown in figures *I* (50X, 475 um scale bar) and *J* (15X, 1585 um scale bar).

a

b

Figure 3. CD spectra of VEGF (Top) and BSA (Bottom)

VEGF and BSA CD spectra generated following exposure to solvents that may be utilized for simultaneous soft sintering and protein incorporation into Pphos based microsphere scaffolds. Wavelength range, 200-260nm. For BSA, the spectra retain the same form, suggesting no significant alteration in secondary structure following interaction with the mentioned solvents.

Figure 4. FITC-BSA Distribution

A Nikon PCM2000 laser scanning confocal was used to illustrate the distribution of the FITC-BSA loaded on the PNPhGly microsphere scaffolds (8mm×2mm) formed using the solvent nonsolvent sintering approach. FITC excitation occurred at 488 nm. Three orthogonal views are depicted here; where the red line represents the 'yz' plane, the blue line, the 'xz' plane, and the green line, the 'xy' plane. FITC-BSA is shown to be accumulated both within the surface of the microspheres and between adjoining microspheres.

Figure 5. Cumulative BSA Release

Zero order release of BSA is demonstrated over the course of 21d from both the loaded PNPhGly and loaded composite PNPhGly-HAp scaffolds. Electrostatic interactions between nanoHAp and BSA are expected to have contributed to the controlled release curve shown here, ($R^2 = 0.981$ and 0.899 for the PNPhGly-HAp and PNPhGly groups respectively). Statistical significance was reached from 3d - 21d between the two groups, $n = 6$, $p < 0.05$.

Table 1

Molecular Weight Properties of PNPhGly

Property	Units (Da)
Mw	$5.88 \times 10^5 \pm 4.08 \times 10^4$
Mn	$5.05 \times 10^5 \pm 2.21 \times 10^4$
PDI	1.16 $\pm$ 0.03

Table 2

Porosimetry Results

Sample	Pore Diameter (um)	Porosity (%)
PNPhGly	97.08 +/- 14.44	23.05 +/- 0.11
PNPhGly-HAp	99.79 +/- 39.99	17.94 +/- 4.02