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Evaluation of Dense Polylactic Acid/Beta-Tricalcium Phosphate Scaffolds for Bone Tissue Engineering

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Abstract

Advances in biomaterial fabrication have introduced numerous innovations in designing scaffolds for bone tissue engineering. Often, the focus has been on fabricating scaffolds with high and interconnected porosity that would allow for cellular seeding and tissue ingrowth. However, such scaffolds typically lack the mechanical strength to sustain in vivo ambulatory stresses in models of load bearing cortical bone reconstruction. In this study, we investigated the microstructural and mechanical properties of dense PLA and PLA/beta-TCP (85:15) scaffolds fabricated using a rapid volume expansion phase separation technique, which embeds uncoated beta-TCP particles within the porous polymer. PLA scaffolds had a volumetric porosity in the range of 30 – 40%. With the embedding of beta-TCP mineral particles, the porosity of the scaffolds was reduced in half while the ultimate compressive and torsional strength were significantly increased. We also investigated the properties of the scaffolds as delivery vehicles for growth factors and gene delivery vectors in vitro and in vivo. The low surface porosity resulted in sub optimal retention efficiency of the growth factors, and burst release kinetics reflecting surface coating rather than volumetric entrapment, regardless of the scaffold used. When loaded with BMP2 and VEGF and implanted in the quadriceps muscle, PLA/beta-TCP scaffolds did not induce ectopic mineralization but induced a significant 1.8 fold increase in neo vessel formation. In conclusion, dense PLA/beta-TCP scaffolds can be engineered with enhanced mechanical properties and potentially be exploited for localized therapeutic factor delivery.

Keywords

Bone; Polylactic Acid; Tricalcium Phosphate; Micro-Computed Tomography; Biomechanics

Introduction

Bone grafting is a frequent reconstructive procedure in orthopaedic surgery. While processed allografts represent an abundant choice for bone grafting, they are susceptible to post-operative fractures that have been associated with high doses of irradiation required to eliminate pathogens and the risk of disease transmission.¹ Therefore, the development of suitable bone grafting biomaterials to eliminate the need for allografts in reconstructing

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large bone defects represents an unmet clinical need that can benefit from novel innovations in tissue engineering. The three essential elements for bone tissue engineering are a porous scaffolding biomaterial (osteoconduction), inductive signals (osteoiduction), and responding stem cells (osteogenesis).² The balance between the porosity and rate of degradation of the biomaterial scaffold on one hand and maintaining its mechanical strength and osteoconductive properties on the other hand is arguably one of the main challenges in bone tissue engineering. However, many scaffold fabrication techniques emphasize that scaffolds should ideally have a degree of porosity and pore interconnectivity to facilitate cellular and vascular ingrowth, and hydrophilic surface chemistry to promote cell seeding, attachment, proliferation, and differentiation.³ As a result of this dogma, highly porous scaffolds that would allow for cellular seeding and tissue ingrowth but typically lack mechanical strength have been the subject of most of the published literature on bone tissue engineering, and the alternative approach involving low porosity scaffolds has been less studied. While highly porous scaffolds might be useful, in conjunction with rigid hardware fixation, in applications such as spinal fusion, they may not be as effective for reconstructing load bearing intercalary defects in long bones.

Poly(lactic Acid) (PLA) and co-polymers are a common biomaterial choice in bone tissue engineering. PLA is a bioresorbable polymer with a long history of safe medical use in various surgical applications including suture, plates, screws, nails, and pins in orthopaedic, and oral and maxillofacial surgery. PLA can be designed as tissue engineering scaffolds by controlling their porosity, mechanical properties, and the rate of their bulk erosion.^{3,4} Several studies have also shown that the incorporation of inorganic salts such as hydroxyapatite and tricalciumphosphate (TCP) into polymer scaffolds, including PLA, can enhance their osteoinductive properties, especially when coated with osteogenic factors such as BMPs.^{5,6} These observations suggest that acellular scaffolds can mediate controlled release of osteogenic factors and vectors, which upon release target the endogenous mesenchymal cells in the milieu of the scaffold *in vivo*. However, scaffolds with porosity >60% have inferior biomechanical properties well below typical ambulatory *in vivo* stresses experienced by cortical bone, which limits their applicability in long bone reconstruction and limb salvage procedures.

The objectives of this study were to investigate correlations between the microstructural and mechanical properties of dense PLA scaffolds (interconnected porosity of <30%), and investigate the controlled release profile of osteogenic and angiogenic factors from these scaffolds *in vitro* and *in vivo*.

Materials and Methods

Scaffold Fabrication

All scaffolds were fabricated and generously donated for this study by Kensey Nash Corporation (Exton, PA). Porous three-dimensional matrix (PTM) were fabricated from polylactic acid (PLA hereafter) or from PLA impregnated with a nominal ~15% of medical grade β TCP particles (85:15% PLA/ β TCP or simply PLA/ β TCP hereafter) using a rapid volume expansion phase separation technique. The PTM process suspends β TCP particles within an interconnected porous polymer and renders the device hydrophilic, with a high degree of pore interconnectivity even as the effective porosity is reduced. A novel aspect of this process is that the suspended β TCP particles are not enveloped by the polymer but are rather exposed to the interstitial fluids and infiltrating cells, which theoretically enhances the osteoinductive properties of the scaffold.⁵ The scaffolds were fabricated as hollow tubes with an outer diameter of 2 mm and an inner diameter of 0.71 mm (cortical thickness of ~0.65 mm) to mimic long bone geometry in the mouse model (Fig. 1). The scaffolds were characterized as per the experimental design described in Table 1.

Scanning Electron Microscopy

Slices (1 mm thick) of the scaffolds were mounted and sputter coated with a thin layer (~50 Å) of gold. A LEO 982 Field emission scanning electron microscope (FE-SEM, Carl Zeiss SMT Inc. Thornwood, NY) was then used with an accelerating voltage of 5KV to view the structure of the scaffold. Low magnification micrographs were analyzed to estimate the surface porosity and pore size distribution using manual segmentation in ImageJ (<http://rsb.info.nih.gov/ij>). High magnification micrographs were also taken to assess the mineral-polymer interface.

Micro Computed Tomography

High resolution (10.5 micron) scans of the scaffolds were obtained using a VivaCT 40 scanner (SCANCO Medical AG, Basserdorf, Switzerland), with x-ray settings of 55 kVp and 145 µA, an integration time of 600 ms and a cone beam reconstruction algorithm. About 100 slices (~1.05 mm in length) per scaffold were analyzed. Due to the significant difference in the x-ray attenuation properties between PLA and βTCP, each material was segmented using different thresholds. A threshold value for the PLA scaffold was determined to match the nominal porosity of the scaffold, which was confirmed by visual comparison of the grey scale images and kept consistent for every specimen to quantify the structural parameters including the solid volume fraction (1 – porosity), strut thickness, and strut number density, as previously described.⁷ The PLA/βTCP scaffolds were binarized first using the aforementioned PLA threshold to determine the structural parameters, and a second time using a higher threshold to determine the βTCP mineral content and average diameter of the βTCP mineral particles separately. After scanning, the scaffolds were stored at –20°C until mechanically evaluated for their compressive or torsional properties.

Mechanical (Compressive) Testing

Bone cement (PMMA) was used to attach square (1 cm × 1 cm) flat plastic plates to each end of a 4 mm long scaffold. The ends were potted while the scaffolds were vertically positioned such that the end plates remained parallel. After the bone cement polymerized for 2 hours, the samples were mounted on an 8841 DynaMight Axial Testing System (Instron Corporation, Canton, MA) and a tare load of 1N was applied to each scaffold. The scaffolds were then compressed in displacement control mode at a rate of 1 mm/minute.⁸ Load-deformation data were acquired and used to compute maximum compressive load, maximum compressive deformation, axial stiffness, energy to failure, yield load and post yield deformation. Since no well-defined yield point could be observed, the yield force was determined from the intersection of a 95% slope line with the load-deformation curve. Ultimate stress, ultimate strain, modulus of elasticity, energy to failure, yield stress and post yield strain were calculated based on cross-sectional area estimates from micro CT measurements of the scaffolds' inner and outer diameters.

Mechanical (Torsion) Testing

Ten millimeter long samples of both PLA and PLA/βTCP scaffolds were prepared for torsion by potting the ends of the scaffolds in 6.35 mm² aluminum tube holders using PMMA in a custom jig to ensure axial alignment and to maintain a gage length of 5.3±0.5 mm, allowing at least 2 mm to be potted at each end. Testing was performed on the EnduraTec TestBench™ system (200 N.mm torque cell; Bose Corporation, Minnetonka, MN) at a rotation rate of 1°/s up to 80°. Torque versus rotation data was collected and analyzed in MATLAB to determine ultimate torque, maximum rotation, torsional rigidity and torsional energy to failure.

***In Vitro* Assessment of Recombinant Growth Factors Release Kinetics**

To investigate the release kinetics of recombinant growth factors from the scaffolds, PLA and PLA/βTCP scaffolds were loaded with rhBMP-2 (R&D Systems, Minneapolis, MN) using the soak and coat method. Briefly, each scaffold was placed in 70 μl of PBS containing 200 ng of rhBMP-2 and incubated for 2 hours at 37°C. Alternatively, scaffolds were soaked in 70 μl of collagen solution (PureCol, INAMED, Fremont, CA) containing 200 ng of rhBMP-2 and incubated for 2 hours at 37°C. 9 The scaffolds were then removed and placed in 500 μl of PBS and incubated at 37°C on a rocker plate. At 2, 4, 8, 12, 24, 48, 72 and 120 hours the supernatant was removed, stored at −80°C and replaced with fresh PBS. After the final time point, the scaffolds were treated with Collagenase A (Roche Applied Sciences, Indianapolis, IN) and repeatedly washed and centrifuged to collect residual rhBMP-2 on the scaffolds. The supernatants were then analyzed using an enzyme-linked immunosorbent assay (ELISA) specific for rhBMP-2 (R&D Systems, Minneapolis, MN) to measure the retention efficiency and release rate of each scaffold. All samples were assayed in duplicates and quantified using a standard curve of a gradient of rhBMP2 concentrations.

***In Vivo* Ectopic Implant Assessment of Osteo- and Angiogenesis**

To investigate the effects of scaffold-mediated rhBMP-2 and rmVEGF₁₂₀ (R&D Systems) delivery on osteogenesis and angiogenesis in an ectopic implant model, PLA/βTCP scaffolds loaded with 200 ng of rhBMP-2 and 250 ng of rmVEGF₁₂₀ in a collagen solution, were surgically implanted in the right thigh muscle, while control scaffolds coated in collagen solution without growth factors were implanted in the left thigh muscles. To control for the effects of the collagen coating, an additional group received uncoated scaffolds.

All animal studies were reviewed and approved by the University of Rochester Committee for Animal Resources. Briefly, 13-week old C57BL/6 mice were anesthetized and aseptically prepped for surgery. An incision (8 mm long) was made and the quadriceps femoris muscle was exposed. A small pocket was created at the top of the quadriceps femoris muscle by blunt dissection for scaffold implantation in the intramuscular pocket. The incision was closed with silk sutures, and the mice were allowed to move freely after recovery from anesthesia. To evaluate ectopic bone formation, *in vivo* micro CT scans were performed immediately following surgery and at 4 and 8 weeks post implantation. A threshold of 240 was used to quantify the mineralized tissue in a standardized region of interest (ROI) encompassing the scaffold.

To evaluate scaffold vascularization, the mice were anesthetized at 8 weeks and 20 ml of heparinized 0.9% saline was slowly and steadily injected through a 26-gauge needle into the heart. This injection step was repeated with 20 ml of 10% formalin and then 4 ml of a lead-chromate contrast agent MV-122 (Flow Tech, Inc. Carver, MA). Following perfusion, the animals were sacrificed, and the contrast agent was allowed to polymerize. The samples were scanned using micro CT, decalcified with EDTA for 3 weeks, and then scanned again. The two scans were co-registered to evaluate the vessels in a standardized ROI defined by contours tracing the perimeter of the scaffold on the first scan, translating the contour from the original scans to the post-decalcification scans, and then dilating the contours 4 fold to include the area surrounding the scaffold. The vessels in this ROI were quantified as previously described.¹⁰

Statistical Analysis

Data analysis including analysis of variance and unpaired Student's t-tests were performed using Prism GraphPad 4.0 statistical software to compare the PLA and the PLA/βTCP

scaffolds' microstructure, mechanical properties, and *in vitro* and *in vivo* parameters of osteogenesis and angiogenesis. Significant differences were determined at $p < 0.05$.

Results

Scanning Electron Microscopy

SEM micrographs from the external surface and cut sections in the scaffolds were taken to calculate the average pore size (Fig. 1). The average pore size (area) of the external surface of the PLA scaffolds was $62.5 \mu\text{m}^2$ while the cut section revealed that the average internal pore size was significantly larger at $167.8 \mu\text{m}^2$ ($p < 0.001$). For the PLA/ β TCP scaffolds, the average pore size on the side surface was $122.7 \mu\text{m}^2$ while the average internal pore size in the cut sections was similar at $105.1 \mu\text{m}^2$. Compared to the PLA scaffolds, the surface pore size of the PLA/ β TCP was significantly greater ($p < 0.05$), albeit there were no significant differences between the scaffolds' internal pore size. High magnification SEM micrographs (Fig. 1E Inset) confirmed that the β TCP particles were only partially embedded to the polymer and were for the most part exposed and not coated by the polymer.

Scaffold Microstructure

The porosity of the PLA scaffolds, determined from micro CT, was significantly greater and nearly doubled the porosity of the PLA/ β TCP scaffolds (Table 2). However, there were no significant differences in microstructural parameters between the scaffolds, including the strut number density and strut thickness (Table 2). The average β TCP content in the PLA/ β TCP was estimated at 9.7% by volume, with an average size (diameter) of 42.3 microns.

Mechanical Properties

The compressive mechanical testing demonstrated significant differences between the PLA and the PLA/ β TCP scaffolds. The maximum load, yield load, and axial stiffness of the PLA/ β TCP scaffolds were nearly double the PLA scaffolds (Table 3). However there were no significant differences in the scaffolds' compressive deformation at yield or failure (Table 3), suggesting no differences in the scaffolds' ductility. When normalized by the apparent cross sectional area, the material properties of the scaffolds showed similar trends. The ultimate compressive strength, yield stress, and modulus of elasticity of the PLA/ β TCP scaffolds were also significantly greater than the PLA scaffolds, while the strains at yield and failure were not significantly different (Table 3). Multivariate regression analysis was preformed using SAS 9.1.3 (SAS Institute Inc., Cary, NC) to correlate the compressive properties with the microstructure of the scaffolds. As intuitively hypothesized, the maximum load, stiffness, maximum stress, and modulus of elasticity correlated inversely with the porosity of the scaffolds, and directly with the β TCP content (Fig. 2).

As observed with the compressive properties, the torsional properties (ultimate torque and torsional rigidity) of the PLA/ β TCP scaffolds were significantly greater than the PLA scaffolds (Table 4). However there were no significant differences in the scaffolds' normalized rotation or torsional energy (Table 4).

In vitro Kinetics of rhBMP-2 Release

Scaffolds were loaded using the soak and coat method in solutions containing rhBMP-2 to investigate the factor retention and release kinetics *in vitro*. The retention efficiency of each scaffold was estimated by dividing the sum of the total amount of rhBMP-2 released and the residual rhBMP-2 on the scaffolds after 120 hours by the total amount of rhBMP-2 used to coat the scaffold. When coated in a PBS solution containing 200ng rhBMP-2, the PLA and PLA/ β TCP scaffolds had a retention efficiency of 1.52% and 0.59%, respectively. To enhance the retention efficiency, the scaffolds were next coated in a collagen solution

containing 200 ng of rhBMP-2. This resulted in a modest increase of retention efficiency up to >3% for both scaffold types. Regardless of the coating method or scaffold type, >90% of the loaded rhBMP-2 was released within the first 24 hours. The release kinetics (Fig. 3A) were modeled using a one-phase exponential association relationship

$$M_t = M_{\infty} \left(1 - \exp \left[-\frac{t}{\tau} \right] \right)$$

where M_t is the cumulative amount released at any time t , M_{∞} is the asymptotic cumulative amount released, and τ is the release time constant. The asymptotic cumulative release M_{∞} for the collagen coated scaffolds was 2-fold and 6-fold greater than the uncoated PLA and PLA/ β TCP scaffolds, respectively. The release rate constant (τ) for the collagen coated scaffolds was 2.7-fold and 3-fold significantly lower than the uncoated PLA and PLA/ β TCP scaffolds, respectively, but there were no significant scaffold related differences (Fig. 3B).

***In Vivo* Osteogenesis and Angiogenesis in an Ectopic Muscle Implant**

We tested the scaffolds as delivery vehicles for recombinant proteins *in vivo* by implanting PLA/ β TCP scaffolds loaded with rhBMP-2 and rmVEGF₁₂₀, or control scaffolds without the growth factors in muscular pockets in the thighs of mice (Fig. 4A). These scaffolds were harvested at 4 or 8 weeks post implantation, and analyzed using micro CT to quantify the ectopic mineralization and vascularization in the milieu of the scaffolds.¹⁰ There was no appreciable mineralization in the ectopic site that could be observed and reliably quantified at either time point regardless of the scaffold type or the growth factors (Fig. 4B, C). However, vessel quantification in the perfused specimens at 8 weeks post-surgery showed a significant 81% increase in blood vessels volume of rhBMP-2 and rmVEGF₁₂₀ coated scaffolds (Fig. 4D–F). There were also trends of increased vessel number density and vessel thickness with the growth factor coated scaffolds compared to the controls, although these increases were not statistically significant.

Discussion

The use of engineered polymer scaffolds as structural bone substitutes offers a promising alternative to biological allografts. Scaffolds can be engineered to tune their chemistry and microarchitecture to optimize delivery of cells and osteogenic factors as well as to enhance their mechanical properties. The success of a scaffold as a structural bone substitute depends on achieving and maintaining a balance between the biological and mechanical (functional) requirements, both of which depend upon scaffold's porosity.

In this study, we evaluated PLA scaffolds that had a volumetric porosity in the range of 30 – 40%, which was reduced by half with the inclusion of β TCP mineral particles in the scaffolds. These porosity values are significantly lower than the typical values reported in the literature. For example, Charles-Harris et al. (2007) analyzed the microarchitecture and mechanical properties of PLA and calcium phosphate glass scaffolds¹¹ and reported porosity values in the range of 93–96.5%, and compressive moduli in the range of 0.06–0.20 MPa. Zhang et al. (1999) evaluated PLLA and PLGA scaffolds combined with hydroxyapatite particles and reported porosity values ranging from 85–95%, which compressive modulus and yield strength values in the range of 6–11 MPa, and 0.25–0.4 MPa, respectively.¹² Lin et al. (2003) designed and characterized axially oriented PLDL scaffolds with porosity in the range of 58–80%, and average compressive modulus and ultimate strength values ranging from 43.5–168.3 MPa and 2.7–11.0 MPa, respectively.⁷ When compared to typical *in vivo* ambulatory stress experienced by a mouse's bone, which was estimated to be 16.8 MPa by multiplying the modulus of elasticity of a 12 week mouse

femur 8 by the typical strains measured *in vivo* during locomotion,¹³ it is likely that scaffolds with high porosity will fall short of the functional requirements *in vivo* in this mouse model. In contrast, our PLA and PLA/ β TCP scaffolds had average maximum compressive strength of 17.8 MPa and 35.7 MPa, which exceeds the estimated *in vivo* stresses in a load bearing defect model in the mouse.

In addition to their functional load-bearing role, scaffolds can mediate delivery of osteogenic factors in bone tissue engineering. Osteogenic factors, such as BMP-2, have been proven to induce endochondral bone formation *in vivo*.¹⁴ For BMP-2 to be effective it must be delivered at an initial high dose to recruit osteoprogenitor cells and have a sustained release over an extended period to differentiate the progenitor cells. The retention dose and rate of release are determined by the microarchitecture and material characteristics of the scaffold as well as the factor entrapment technique. One common approach, which we followed in this study, is to load the protein by soaking the polymer scaffold in a buffered solution of the recombinant factor. The microarchitecture including porosity and pore size determine the surface area available for growth factor binding, while the interconnectivity of the pores can mediate uniform protein binding throughout the scaffold and can also permit fluid flow through the scaffold to enhance protein release by different mechanisms. The material characteristics of the scaffold can influence the affinity of the growth factor to the scaffold surface. Surface charge affects the binding and conformation of the bound proteins.¹⁵ In the case of PLGA polymers, the binding affinity of the factor is influenced by several material characteristics including the polymer acidity, the molecular weight, and the lactide/glycolide ratio.¹⁶

In this study, we used the soaking method to load our low porosity PLA and PLA/ β TCP scaffolds with BMP-2 and VEGF and evaluated retention and release of these factors from the scaffolds *in vitro*. Release kinetics from scaffolds can obviously be influenced by a number of factors, which in addition to the protein size (molecular weight or hydrodynamic radius) include polymer hydrophilicity (or hydrophobicity), degradation rate, swelling (water uptake) rate, and protein-polymer binding affinity. However, since our approach involved surface coating of the polymer scaffold rather than emulsion or dispersion of the protein in the polymer solution, the aqueous diffusion likely dominates release and is governed only by the molecular size of the protein¹⁷ as we expect no differential affinity or binding to the polymer. Since both BMP2 and VEGF form disulfide-linked homodimers, and their monomer molecular weights are equivalent (13 and 14 KDa, respectively), we therefore predicted that their aqueous diffusion coefficients are equivalent and assayed only for aqueous BMP-2 diffusive release. The retention (or entrapment) efficiency of the scaffolds was only 0.59 – 1.52% which may be related to the low surface porosity of the scaffolds. The fact that the porosity of the PLA scaffolds is nearly double the PLA/ β TCP scaffolds is loosely correlated with the 2.6 fold increase in retention efficiency in the PLA scaffolds. To enhance the entrapment of rhBMP-2 on the scaffolds, we suspended the rhBMP-2 in type I collagen solution into which the scaffolds were subsequently soaked, to provide a thin collagen coat and enhance the retention efficiency. Type I collagen is the most abundant type of collagen and is present in bone. Alone, type I collagen has demonstrated little effect on bone formation but improved the bony interface between the graft and the host bone. Although, when used in conjunction with rhBMP-2, type I collagen has been shown to enhance bone formation in spinal fusions.¹⁸ Using this method we were able to increase the retention efficiency of the PLA and PLA/ β TCP scaffolds by 2.1 fold and 5.3 fold, respectively, essentially equalizing the retention efficiency for both scaffolds. Since we did not attempt to gel the collagen, we posit that a thin layer of collagen coating likely was responsible for the modest enhancement of rhBMP-2 entrapment, and that effective infiltration of the collagen solution into the low porosity scaffolds was not achieved due to the viscous nature of the collagen solution. Alternatives to coating recombinant factors on

the scaffolds should be considered including dissolving the osteogenic peptides in the polymers mixture during the scaffold fabrication. Such an approach has been shown to dramatically enhance the entrapment efficiency, reaching values in the range of 38 – 81% depending on the mixture formulation,¹⁹ and can potentially overcome the limitations of the soak and coat technique.

The majority (95–98%) of rhBMP-2 was released from the PLA and PLA/βTCP scaffolds in the first 12 hours, which is significantly faster than release kinetics reported by Burdick et al from 80% porous PLA scaffolds.²⁰ The rapid release kinetics in our study supports the hypothesis that the rhBMP-2 retention was mainly due to surface coating rather than volumetric entrapment throughout the porous network. The kinetics of release of a growth factor is dictated by the mechanisms of release, which include diffusion-, swelling-, and erosion-controlled release.²¹ Diffusion-controlled systems mediate factor release from the non-degraded polymer due to a concentration gradient. Swelling-controlled systems enhance factor diffusion due to polymer swelling and opening of the intermolecular crosslinking, while erosion-controlled systems mediate factor release as a result of polymer surface erosion and degradation.²¹ Since PLA scaffolds undergo bulk erosion rather than surface erosion,³ the likely dominant mechanism of release in our scaffolds is diffusion. We empirically modeled the release kinetics using an exponential association relationship that conforms to the general form of asymptotic solutions of diffusion release described by Baker and Lonsdale²² for drug diffusion coefficient (D) from a sphere of radius r following the initial burst release phase ($M_t / M_\infty > 0.6$):

$$\frac{M_t}{M_\infty} = 1 - \frac{8}{\pi^2} \exp \left[\frac{-\pi^2 D t}{r^2} \right]$$

There was excellent agreement between the model and the experimental data (R^2 range 0.87 – 0.99 for the individual samples) confirming that diffusion-controlled release was the dominant mechanism.

To evaluate the bioactivity of released growth factors *in vivo*, PLA/βTCP scaffolds loaded with rhBMP-2 and rmVEGF₁₂₀ were ectopically implanted in muscle pouches. That the scaffolds did not induce any detectable mineralization is likely due to the poor retention efficiency that effectively resulted in loading about 6 ng of either factor per scaffold. A survey of the literature suggests that a wide range of BMP-2 doses can be effective in inducing ectopic bone formation from as low as 100 to 500 milligram²³ to as high as 50 microgram²⁴ or even greater.²⁵ However, since Aspenberg et al. demonstrated that a dose as low as 1 ng of rhBMP-2 was enough to elicit bone formation in porous coralline hydroxyapatite chambers implanted in rat tibiae,²⁶ we elected to evaluate a lower concentration than typically used in these ectopic models. The low dose combined with the burst release may not have been sufficient to induce ectopic bone formation in our model. Unlike an orthotopic defect such as the tibia²⁶ where osteoblasts and osteoprogenitors are abundant, an ectopic (muscle) model might not afford enough osteoprogenitors to respond to rapid-release of low doses of rhBMP-2, which must still be verified by detailed analysis of the cellular and tissue histology in the milieu of the implanted scaffolds.

Contrary to the lack of osteogenesis in our ectopic model, a similar dose of rmVEGF₁₂₀ resulted in a significant 1.8 fold increase in vessel volume in the milieu of the scaffolds. The vessels were mostly external to the scaffolds although some limited vascular ingrowth was observed within the scaffolds. Previous studies have shown that doses as low as 250 ng of rmVEGF₁₂₀ induce ectopic vessel formation.²⁷ The increased ectopic vessel formation with low doses of VEGF in our study and others is likely due to the availability of endothelial

progenitors in the skeletal muscle. Angiogenesis is essential in the promotion of both endochondral and intramembraneous ossification but might not be on its own sufficient to enhance osteogenesis as it has been shown that combined delivery of BMP, VEGF and mesenchymal stem cells are required to significantly enhance ectopic bone formation relative to any single factor,²⁸ while controlled, sequential VEGF and BMP-2 release from acellular composite polymers has been shown to enhance bone regeneration in ectopic implants and orthotopic defects.²⁹

Conclusions

In summary, we demonstrated that dense PLA scaffolds with limited porosity could be designed to withstand *in vivo* stresses. While the embedding of exposed β TCP mineral particles reduces the porosity, it results in increased compressive and torsional properties. We also demonstrated that the scaffolds could be processed for delivery of growth factors, *in vitro* and *in vivo*, although these processing techniques must still be optimized to enhance the biological outcome. These observations merit further investigation in clinically relevant, intercalary defect models of long bone reconstruction.

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Figure 1.

Micro computed tomography (micro CT) and Scanning electron microscopy (SEM) assessment of the microstructure and porosity of the scaffolds. Micro CT rendering of the PLA (A) and PLA/βTCP (D) scaffolds, respectively (Red in D represents the embedded βTCP mineral particles). SEM micrographs demonstrate the low porosity of the external surface of the PLA (B) and PLA/βTCP (E) scaffolds, compared to the internal (cross-section) porosity (C and F, respectively). Inset in E shows a magnified micrograph of the exposed mineral surface of a βTCP particle partially embedded (yellow arrow) within the PLA polymer scaffold.

Figure 2.

Multivariable linear regression analysis of the compressive mechanical properties with the scaffold porosity and mineral content. Adjusted R² and the regression equations are indicated on each graph. The dotted diagonal line represents the identity line (predicted=measured). The solid line represents the regression line.

Figure 3.

The cumulative release kinetics of rhBMP-2 from PLA and PLA/βTCP into PBS in vitro. The scaffolds were loaded using the soak and coat method in PBS or collagen type I solutions containing 200 ng of rhBMP-2. (A) The release kinetics from each scaffold was determined by measuring the concentration of rhBMP-2 in the PBS supernatant using ELISA. (B) The release time constant (τ) is estimated by modeling the release data over time using the equation $M_t / M_\infty = 1 - \exp(-t/\tau)$. Data presented as mean $\pm$ SEM (n=3). Asterisks indicate significant differences ($p < 0.05$) between the collagen coated and non-coated scaffolds, for both scaffold types.

Figure 4.

Ectopic assessment of scaffold-mediated delivery and in vivo biologic activity of rhBMP-2 and rmVEGF120. PLA/ β TCP scaffolds loaded with rhBMP-2 and rmVEGF120, or control scaffolds without the growth factors were implanted in muscular pockets in the thighs of mice, as illustrated by x-ray (arrow in A). The constructs were harvested at 8 weeks post-implantation and analyzed using micro CT to quantify the vascularization in a standard ROI containing the construct and immediate surrounding soft tissue. Representative 3D renderings of the mineralized non-loaded (B) and loaded (C) scaffolds, and the lead-chromate perfused contrast in the surrounding vessels are shown. Rendering of the vessels in the ROI after subtracting the scaffolds demonstrates the differences in vascularity between the non-loaded (D) and loaded scaffolds (E). (F) The mean vessel volume in the ROI of the control vs. VEGF treated constructs is presented as mean $\pm$ SEM (n=6). Asterisks indicate significant differences ($p < 0.01$) between the growth factor loaded and non-loaded scaffolds.

Table 1

Experimental Design

Group	Scaffold	GF Loading/Scaffold	Analysis	Sample Size
<i><u>Scaffold Characterization</u></i>				
1	PLA	None	SEM	n=3
2	PLA/βTCP	None	SEM	n=3
3	PLA	None	μCT + Compressive Biomech	n=13
4	PLA/βTCP	None	μCT + Compressive Biomech	n=13
5	PLA	None	μCT + Torsion Biomech	n=6
6	PLA/βTCP	None	μCT + Torsion Biomech	n=7
<i><u>in vitro Growth Factor Loading and Release</u></i>				
7	PLA	200 ng BMP-2 / 70 μl PBS	GF Release Kinetics	n=3
8	PLA/βTCP	200 ng BMP-2 / 70 μl PBS	GF Release Kinetics	n=3
9	PLA	200 ng BMP-2 / 70 μl Collagen	GF Release Kinetics	n=3
10	PLA/βTCP	200 ng BMP-2 / 70 μl Collagen	GF Release Kinetics	n=3
<i><u>in vivo Assessment of Ectopic Bone and Vessels</u></i>				
11	PLA/βTCP	200 ng BMP-2 + 250 ng VEGF ^a	μCT	n=6
12	PLA/βTCP	Collagen ^b	μCT	n=6
13	PLA/βTCP	None	μCT	n=4

^aGrowth factors dissolved in 70 μl Collagen I solution

^bScaffolds were coated in 70 μl Collagen I solution without growth factors

Table 2

Scaffold microstructure parameters characterized by micro computed tomography. Data presented as mean $\pm$ standard deviation (ns indicates non significant).

	PLA	PLA/ β TCP	
Volumetric Porosity, %	39.2 $\pm$ 9.56	20.1 $\pm$ 11.9	p<0.0005
Strut Number Density, mm ⁻¹	19.4 $\pm$ 0.43	19.7 $\pm$ 0.69	ns
Strut thickness, micron	46.7 $\pm$ 6.73	53.1 $\pm$ 6.11	p<0.05
β TCP Content, %	-	9.65 $\pm$ 3.91	
β TCP Average Size, micron	-	42.3 $\pm$ 12.2	

Table 3

Compressive mechanical properties of scaffolds. Data presented as mean $\pm$ standard deviation (ns indicates non significant).

	PLA	PLA/ β TCP	
Max Load (N)	33.3 $\pm$ 14.3	63.9 $\pm$ 21.2	p<0.0005
Max Compression at Failure (mm)	0.99 $\pm$ 0.29	0.97 $\pm$ 0.23	ns
Axial Stiffness (N/mm)	76.6 $\pm$ 29.0	175.0 $\pm$ 75.3	p<0.0005
Yield Load (N)	17.4 $\pm$ 12.4	40.1 $\pm$ 18.9	p<0.005
Compression at Yield (mm)	0.73 $\pm$ 0.22	0.67 $\pm$ 0.24	ns
Ultimate Stress (N/mm ²)	17.8 $\pm$ 7.21	35.7 $\pm$ 12.3	p<0.0005
Ultimate Strain (mm/mm)	0.25 $\pm$ 0.08	0.24 $\pm$ 0.07	ns
Modulus of Elasticity (N/mm ²)	164.3 $\pm$ 65.8	394.7 $\pm$ 182.0	p<0.0005
Yield Stress (N/mm ²)	9.2 $\pm$ 6.1	22.5 $\pm$ 10.8	p<0.001
Yield Strain (mm/mm)	0.18 $\pm$ 0.06	0.17 $\pm$ 0.06	ns

Table 4

Torsional mechanical properties of scaffolds. Data presented as mean $\pm$ standard deviation (ns indicates non significant).

	PLA	PLA/ β TCP	
Ultimate Torque (N.mm)	4.46 $\pm$ 1.27	6.34 $\pm$ 1.79 *	p<0.02
Ultimate Rotation (Rad/mm)	0.04 $\pm$ 0.01	0.04 $\pm$ 0.01	ns
Torsional Rigidity (N.mm ²)	214.0 $\pm$ 57.1	332.6 $\pm$ 108.9 *	p<0.001
Torsional Energy to Failure (N.Rad)	0.12 $\pm$ 0.05	0.14 $\pm$ 0.02	ns