

An initial investigation of photocurable three-dimensional lactic acid based scaffolds in a critical-sized cranial defect

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Abstract

Degradable polymer networks formed by the photoinitiated polymerization of multifunctional monomers have great potential as in situ forming materials, especially for bone tissue engineering. In this study, one specific chemistry was analyzed with respect to bone formation in a critical-sized defect model with and without adsorbed osteoinductive growth factors present. The scaffolds degraded in ~8 months and possessed an elastic modulus similar to that of trabecular bone. A porous scaffold fabricated with ~80% porosity and pore diameters ranging from 45 to 150 μm was implanted in a critical-sized cranial defect in rats. When implanted alone, the scaffolds were filled primarily with fibrous tissue after 9 weeks with only mild inflammation at the defect site. When the scaffolds released osteoinductive growth factors, statistically more bone filled the scaffold. For instance, $65.8 \pm 9.4\%$ ($n = 5$) of the defects were filled with radiopaque tissue in the osteoinductive releasing scaffolds, whereas only $24.2 \pm 7.4\%$ ($n = 5$) of the defects were filled in the untreated defects 9 weeks after implantation. These results illustrate not only the benefits of delivering osteoinductive factors when developing synthetic bone grafts, but the potential of these materials for supporting the infiltration and development of bone in large defects.

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1. Introduction

Tissue engineering is emerging as a technique that could potentially be used in the future to develop synthetic bone graft replacement materials for the treatment of large bone defects. While bone grafting is one of the most common and successful clinical treatment options, there are still many limitations. With autografts, the amount of bone available for harvest is limited, and the harvesting procedure may require a second surgery and induce morbidity at the tissue donation site [1]. While allografts circumvent these problems, higher failure rates are observed with allografts and associated with decreased integration of the donated tissue and increased potential for rejection [1]. With tissue engineered grafts, the appropriate signals

(e.g., osteoconductive surface, growth factors, and osteoprogenitor cells) can be delivered in a controlled fashion and hopefully overcome many of the limitations in current grafting treatments [2,3].

Many approaches are being investigated for tissue engineering and, specifically, for bone tissue engineering. Our group is interested in the development of in situ forming materials that are easily implanted by a surgeon, able to fill irregularly shaped defects, and provide good contact between the implant and the native tissue. In particular, photopolymerization provides control over the polymerization exotherm (i.e., temperature rise) [4], which can minimize tissue necrosis due to excessive temperature increases; spatial control during polymerization allowing the fabrication of complex structures; and a mechanism to polymerize multifunctional monomers under physiological conditions (i.e., body temperature and in the presence of body fluids) [5] for the in vivo formation of biomaterials.

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An in situ forming material for bone tissue engineering needs to be both biocompatible and osteoconductive, polymerize within clinically acceptable timescales, have controlled degradation and mechanical properties, and be easily applied to a bone defect in an in vivo environment. These requirements have led to the development of highly crosslinked degradable networks formed from multifunctional polyanhydride monomers [6,7], poly(propylene fumarates) [8], and more recently, multifunctional lactic acid based oligomers [9].

In this last category, one specific network composition, poly(2EG10LA), that is formed by grafting 10 lactic acid units on each side of a di(ethylene glycol) core, has been identified as having desirable properties for the intended application of bone tissue engineering and reacts rapidly (<5 min) to form networks with controlled degradation and mechanical properties [9]. Specifically, primary rat calvarial osteoblasts attached and functioned on surfaces of the poly(2EG10LA) similarly to control polymers of tissue culture polystyrene and 50:50 poly(lactic-co-glycolic acid) [10]. After 3 weeks of in vitro culture, the attached osteoblasts stained positive for mineral components. Additionally, when implanted subcutaneously, the inflammatory response to the poly(2EG10LA) networks was very mild and supported the infiltration of vascularized fibrous tissue [11]. Thus, many of the previously mentioned design criteria are met.

In addition to their use as in situ forming implants, these degradable multifunctional monomers may enable facile fabrication of scaffolds with complex architectures. For example, stereolithography or related techniques [12] utilize the spatial control of photopolymerization to construct elaborate objects from two-dimensional slices of three-dimensional images. Additionally, Hollister and others [13] have developed an image-based approach for designing patient-specific scaffolding materials directly from either Computed Tomography or Magnetic Resonance Image data. The negative is fabricated from a dissolvable component (e.g., wax) and is filled with a multifunctional monomer that is subsequently polymerized to form a crosslinked network. After dissolution of the dissolvable component, the desired scaffold with an optimized and controllable architecture remains.

The overall objectives of this study were to (i) characterize porous scaffolds of poly(2EG10LA) fabricated using photopolymerization and a previously developed poragen leaching technique, and (ii) analyze these prefabricated scaffolds in a commonly used critical-sized cranial defect in rats to elicit both the hard tissue response and the potential for restoring bone tissue in large defects with and without the delivery of growth factors. Without treatment, this defect will not heal properly within the lifetime of the rat and, thus, provides an appropriate animal model for bone tissue

engineering approaches. The specific objective of the in vivo study was to look at the ability of the poly(2-EG10LA) scaffold to support tissue ingrowth and to enhance bone tissue formation by loading the scaffolds with osteoinductive growth factors. Although these materials were prefabricated prior to implantation, previous studies have shown that the in vivo photopolymerization of multifunctional monomers is possible and that the photopolymerization process is not detrimental to the surrounding tissue [14].

2. Experimental

2.1. Scaffold fabrication and characterization

The multifunctional oligomer, 2EG10LA, was synthesized and purified as described previously [9]. Scaffolds were fabricated by dissolving the viscous oligomer and photoinitiator (0.5 wt% 2,2-dimethoxy-2-phenyl acetophenone, DMPA) in methylene chloride, mixing the solution with salt particles, allowing the methylene chloride to evaporate, packing the mixture in a teflon mold, and photopolymerizing for 15 min with ~ 10 mW/cm² ultraviolet light (Blackray). The salt was leached from the scaffold through several changes of deionized water over 48 h to leave a macroporous degradable scaffold. After drying and sputter coating with gold, the macroscopic architectures of these scaffolds were viewed with scanning electron microscopy (SEM, ISI SX30). Although this specific leaching technique may not be compatible in vivo, salt could be substituted with either gelatin or sugar particles to enhance the biocompatibility.

Scaffolds (1 cm diameter, 2 mm thick) were fabricated with 80 wt% salt sieved into ranges of 45–150, 150–300, or 300–600 μ m and 20 wt% of the 2EG10LA oligomer. Previous studies [11] have shown that lower than 80 wt% salt leads to pores that lack interconnectivity, whereas greater than 80 wt% leads to scaffolds with very low mechanical stability. After salt leaching and drying in a desiccator, scaffolds were degraded in phosphate buffered saline (PBS) on an orbital shaker (60 rpm) at 37°C. The PBS was changed at least once a week to maintain constant pH and sink conditions. At desired time points, scaffolds were removed, frozen at -80°C , lyophilized to remove excess water, and weighed to calculate the polymer mass loss.

The mechanical properties of cylindrical scaffolds (2:1 aspect ratio, 1 cm height, 0.5 cm diameter) were measured on a Materials Testing System 858 Mini Bionix II material tester with a maximum load of 5 kN and a 1 mm/min crosshead speed. The elastic modulus was calculated from the initial slope of the stress versus strain curve, while the yield strength was calculated as the stress at the intersection of a line drawn parallel to

the elastic modulus with an x -axis intercept at 1% strain. Five samples were analyzed for each pore size.

2.2. Drug release

Twenty microliters of a growth factor solution (equivalent to 30 μg of growth factor in deionized water) was injected into the center of the porous scaffolds and allowed to adsorb at room temperature for 1 h before immersion in 1 ml of deionized water in 24-well plates. The growth factors used in this study were isolated directly from bovine tissue and include a mixture of proteins such as bone morphogenetic proteins, transforming growth factor- β 1, and fibroblast growth factor [15]. At desired times, the porous scaffold was moved to a new well, and the solution was sampled. Protein release (i.e., release of adsorbed proteins and protein diffusion from the scaffold pores) was quantified with a total protein assay (Bio-Rad), where a differential color change in the assay reagent was quantified by measuring the absorbance at 595 nm (Perkin Elmer, Lambda 40) and related to known protein standards.

2.3. Cranial defect

Sprague-Dawley rats (Harlan, \sim 300 g) were anesthetized with isoflurane, and an incision was made through the subcutaneous tissue covering the cranium. An 8 mm defect was created using a dermal punch with care to avoid damage to the underlying dura and superior sagittal sinus vein. The craniotomy segment was removed and the defect was: (1) filled with the removed bone (positive control), (2) left open (negative control), (3) filled with a poly(2EG10LA) scaffold or (4) filled with a poly(2EG10LA) scaffold loaded with growth factors as described above. Scaffolds (8 mm diameter, 1 mm height) were fabricated as described above utilizing the salt leaching technique with 80 wt% salt sieved to 45–150 μm . After salt leaching, the scaffolds were sterilized by emerging in ethanol and overnight exposure to ultraviolet light. Prefabricated scaffolds were implanted to study the efficacy of the polymer chemistry, pore structure, and growth factor release (if loaded) to facilitate bone tissue growth. Future work will investigate in situ formed scaffolds in bone defects. All procedures were performed with IACUC approved protocols.

Animals (5 per composition per time point) were sacrificed by CO_2 asphyxiation after 5 and 9 weeks, and the crania were dissected. Contact radiographs were taken of the excised cranium to visualize mineralization in the controls and the treated defects. The radiopacity in each defect was quantified using NIH Image Software after subtracting the background from the scanned radiographs. Since the scaffolds are not radiopaque, any radiopacity is indicative of tissue formation in the

defect. Histological sections of the excised cranium were used for a qualitative analysis of mineralized and fibrous tissue growth within the three-dimensional scaffolds. After fixation, the undecalcified tissue was processed using standard methyl methacrylate embedding and sectioning techniques. Sections were stained with a Van Gieson stain to visualize mineralized tissue formation.

2.4. Statistical analysis

Statistical analysis was performed using a Student's t -test with a minimum confidence level of 0.05 for statistical significance. All values are reported as the mean and standard deviation of the mean.

3. Results and discussion

3.1. Scaffold development and characterization

A modified poragen leaching technique that utilizes a photopolymerization process was used to fabricate porous scaffolds from a potentially osteoconductive synthetic bone graft material. This technique was previously used by our group to form three-dimensional scaffolds from multifunctional monomers [10] and was used to fabricate poly(propylene fumarate) scaffolds by Fisher et al. [16]. An SEM image of a representative scaffold fabricated from the 2EG10LA monomer and salt particles ranging from 45 to 150 μm is shown in Fig. 1. With this technique, scaffolds of various macroscopic architectures are readily fabricated by changing both the ratio of polymer to the poragen and the mean diameter and size distribution of the poragen used.

The degradation behavior for scaffolds with the same chemistry (i.e., poly(2EG10LA)) and porosity, but

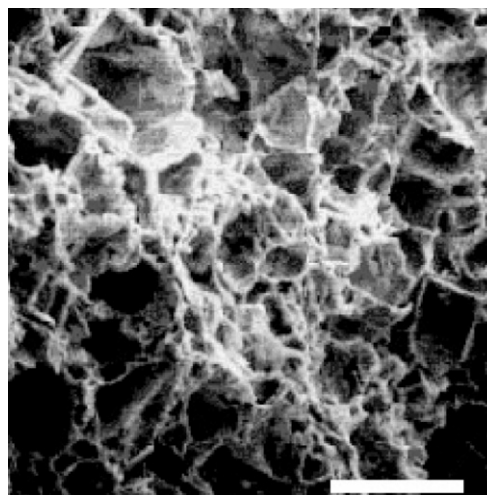


Fig. 1. SEM image of scaffold fabricated from the 2EG10LA oligomer and 45–150 μm sized salt particles (bar = 250 μm).

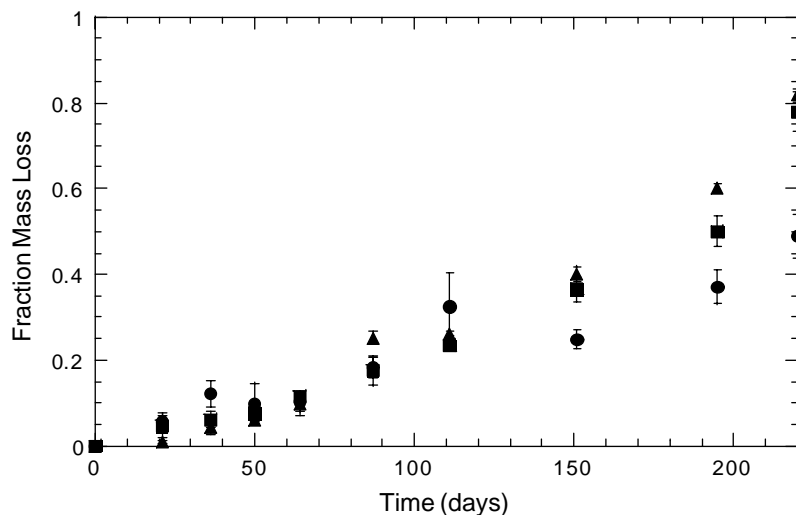


Fig. 2. Mass loss versus degradation time for scaffolds ($n = 5$ per time point) fabricated from the 2EG10LA oligomer and 80 wt% salt particles of different diameters: 45–150 μm (●), 150–300 μm (■), or 300–600 μm (▲) sized salt particles. Scaffolds degraded in PBS at 37°C on an orbital shaker.

fabricated with different pore sizes is shown in Fig. 2. This porosity was chosen based on previous studies indicating that scaffolds fabricated with less than 80 wt% poragen do not have an interconnected pore structure [11], which would limit the amount of tissue infiltration into the scaffold. In general, there was little mass loss at early degradation times. As degradation proceeded, mass loss accelerated with the scaffolds almost completely degraded after ~ 8 months. There was little difference in the mass loss profiles between the scaffolds with different pore sizes until later degradation times when the scaffolds with the highest pore sizes degraded faster than the scaffolds with the lowest pore size.

Interestingly, the polymer degraded much slower when fabricated into porous scaffolds than was reported in previous studies [9] with the same materials fabricated into solid disks. This behavior is potentially explained by an autocatalytic degradation effect, where the acidic degradation products do not readily diffuse out of the polymer and substantially lower the internal pH of the network. An acidic degradation environment has been shown to accelerate the degradation of ester bonds [17] in PLGA. When the material is porous, the thickness of the polymer walls is very thin ($\sim 10 \mu\text{m}$) and, thus, the diffusional length scale is reduced to the thickness of the pore walls. The accumulation of acidic degradation products would then be minimized compared to that of a monolithic polymer disk (1 mm thickness) of the same material. This could also explain the faster degradation of scaffolds fabricated from larger salt particles, since the walls of the pores are thicker as the pore size increases (i.e., the total pore surface area decreases). A slowed degradation (i.e., with the porous scaffolds) could also improve the tissue response to the degrading

Table 1
Mechanical properties of scaffolds fabricated with photopolymerization of 2EG10LA oligomer and various sizes of salt particles

Scaffold	Elastic modulus (MPa)	Yield strength (MPa)
80 wt% salt, 45–150 μm	3.09 ± 0.78	0.33 ± 0.04
80 wt% salt, 150–300 μm	2.89 ± 0.95	0.26 ± 0.06
80 wt% salt, 300–600 μm	7.83 ± 2.99	0.38 ± 0.11

polymers since degradation products are continuously released, which minimizes their burst release at late degradation times.

Both the elastic modulus and yield strengths of these 2EG10LA scaffolds of equal porosity but fabricated from salt particles with different diameters are listed in Table 1. The elastic modulus was statistically higher for the 300–600 μm scaffolds than the 45–150 and 150–300 μm scaffolds, but there was no statistical difference ($p < 0.05$) in the yield strengths between the different scaffolds. These values are similar to those found for photocurable poly(propylene fumarate) scaffolds of similar architectures [16]. One explanation for the higher elastic modulus with the higher pore sizes could be the increase in the thickness of the individual pore walls as greater amounts of poragens are used. Although this may increase the mechanics, there may also be a decrease in the pore interconnectivity of the scaffolds. Despite the measured mechanical properties being very close to that of other types of porous degradable scaffolds, the applications of these networks will be limited to trabecular bone applications or in combination with other treatments (e.g., metal fixation devices).

3.2. Cranial defect

In this study, prefabricated photocrosslinked porous scaffolds were implanted into 8 mm defects produced in the cranium of rats. This model is widely used as a measure of bone regeneration [18] since the critical-sized defect will not heal when left untreated. Scaffolds were fabricated from the 2EG10LA oligomer and 80 wt% salt sieved into a size range of 45–150 μm . This composition was chosen due to both the good interconnectivity of the pores and the desirable properties of the polymer chemistry with respect to cell adhesion and biocompatibility. Additionally, preliminary studies (results not shown) indicated that osteoblasts were seeded more uniformly on the chosen scaffolds than scaffolds fabricated with other pore ranges (i.e., 150–300 or

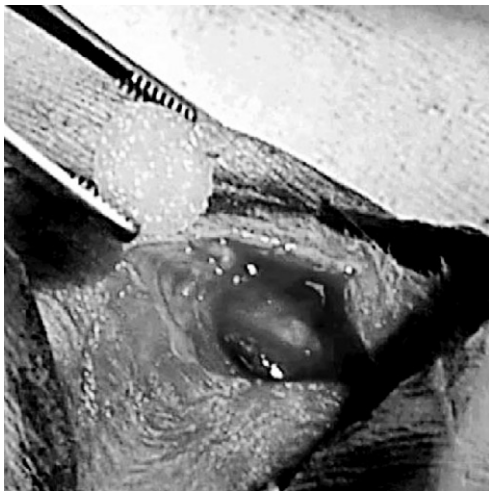


Fig. 3. Image of surgical procedure where scaffold is being placed into the 8 mm defect in a rat cranium.

300–600 μm) during in vitro studies. An image of a scaffold being implanted into the cranial defect during the surgical procedure is shown in Fig. 3.

In one of the treatments, osteoinductive growth factors were adsorbed onto the scaffold and delivered to the defect site. These growth factors were isolated directly from bovine tissue and were previously determined to induce bone formation when implanted subcutaneously [19]. The in vitro release of these growth factors from the porous scaffolds (80 wt%, 45–150 μm) is shown in Fig. 4. The majority of the growth factors ($\sim 70\%$) are released in the first 24 h followed by a slower and sustained growth factor release rate for up to approximately 6 days. Although there are more intricate methods for growth factor delivery to give a variety of release profiles with a more sustained delivery, this treatment was chosen as a simple technique to determine if the combination of an osteoconductive photocurable material with osteoinductive growth factors would be beneficial for the induction of bone regeneration.

When the implants were removed after both 5 and 9 weeks, no obvious inflammation was noted, and all of the animals survived both the surgical procedure and time until harvest. Macroscopically, there was tissue formation that entirely filled the defects in all treatments except the negative control. In the negative control, only a thin layer ($\sim 200 \mu\text{m}$) of transparent fibrous tissue had filled the defect after both 5 and 9 weeks.

Radiographs of the defect site 9 weeks after implantation are shown in Fig. 5. Interestingly, complete union in the positive control (similar to an autograft procedure) was not seen at the time of harvest. An increase in radiopacity (i.e., tissue formation) was seen only at the edges of the negative control, whereas a light radiopacity was noted throughout the whole defect when a polymer scaffold was implanted. A significant amount

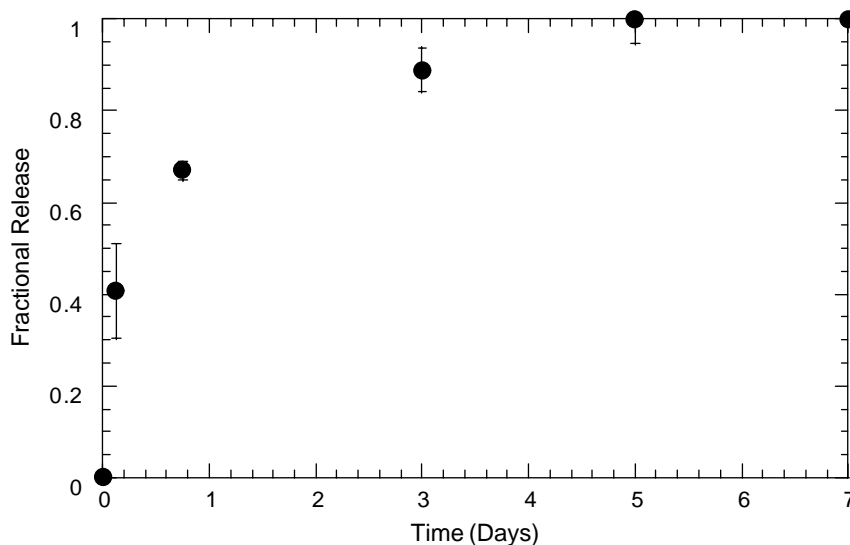


Fig. 4. In vitro fractional release versus time for the osteoinductive growth factors from the porous scaffolds.

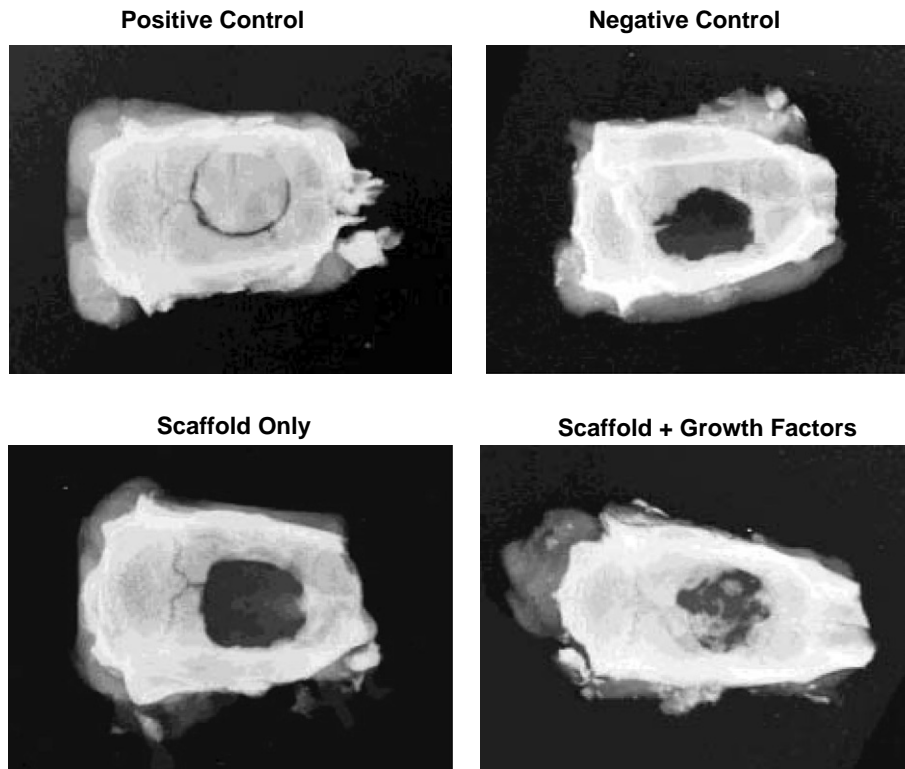


Fig. 5. Radiographs of rat calvaria 9 weeks after treatment of 8 mm critical-sized defects with the removed bone (positive control), no treatment (negative control), a scaffold only, and a scaffold plus growth factors.

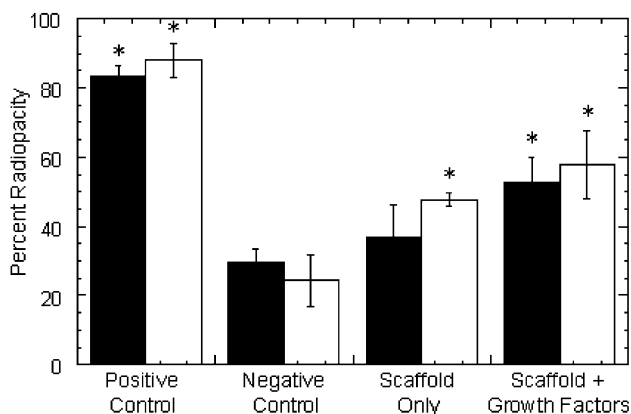


Fig. 6. Quantification of radiopacity in 8 mm critical-sized ($n = 5$ per treatment for each time point) defects 5 (black) and 9 (white) weeks after treatment with the removed bone (positive control), no treatment (negative control), a scaffold only, and a scaffold plus growth factors. (*) indicates statistical difference from the negative control at the same time point.

of bone had formed in the defects when growth factors were delivered with the scaffold. When quantified, $88.0 \pm 4.8\%$ radiopacity was seen in the positive control after 9 weeks, whereas only $24.2 \pm 7.4\%$ was found in the negative control. For the scaffold, only $47.7 \pm 1.9\%$ radiopacity was seen which was substantially increased to $65.8 \pm 9.4\%$ when growth factors were delivered with

the scaffold. These values are statistically different from the negative control at the 9 week time point. Radiopacity results for both time points are summarized in Fig. 6.

Histological sections of the excised cranium 9 weeks after implantation are shown in Fig. 7. For the positive control, interesting results were noted. In many locations between the implanted bone and the surrounding defect interface, a fibrous bridge remained with little integration of the bone tissue. This lack of integration could be due to significant fibrous tissue infiltration before union of the implanted bone tissue with the native bone. With the negative control, small amounts of bone tissue had regenerated at the edge of the defect with some fragments of bone remaining from the surgical procedure, but mainly a thin layer of fibrous tissue had filled the defects. Since little regeneration occurs without treatment, this animal model is good for tissue engineering approaches.

The inflammatory response to the implanted polymer scaffolds was very mild with few foreign body giant cells and macrophages seen within the porous scaffolds or in the surrounding tissue. While tissue infiltration was supported throughout the scaffold, the development of mineralized tissue in the absence of growth factors was minimal with only a few localized areas of mineralization noted within the pores. The scaffold was primarily

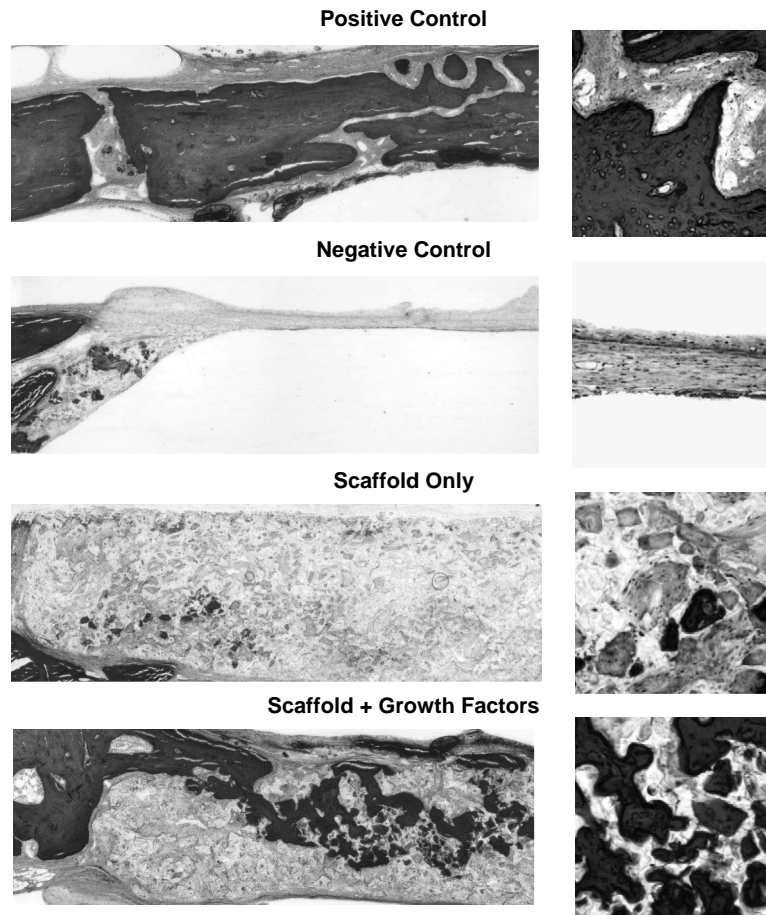


Fig. 7. Van Gieson stained histological sections of rat calvaria 9 weeks after treatment of 8 mm critical-sized defects with the removed bone (positive control), no treatment (negative control), a scaffold only, and a scaffold plus growth factors. Mineralized tissue stained red.

filled with fibrous tissue with some vascular structures present throughout. The good filling of the scaffold with tissue throughout the defect indicates that the structure was interconnected and demonstrates potential for guided tissue regeneration. The small amounts of mineralization are probably due to cells migrating from the surrounding bone tissue or signals that were secreted from surrounding cells that induced mineralization.

With the scaffolds releasing osteoinductive growth factors, a significant increase in bone regeneration was seen as a much larger portion of the scaffold was filled with mineralized tissue. The difference in mineralized tissue production between the scaffolds with and without growth factors indicates that an osteoinductive component is very important in stimulating bone regeneration in these tissue engineering approaches. Although the whole scaffold is not filled with bone, longer time points or a more sustained growth factor delivery rate (> 6 days) could potentially improve these results.

In the literature, there have been many debates over the desirable architecture (e.g., pore size and porosity) for scaffolding material used in bone tissue engineering.

For instance, Whang et al. [20] showed that scaffolds fabricated from PLGA with very small pore sizes (< 50 μm) induced bone formation due to the stabilization of hematomas, enabled by the capillary action of small scaffold pores to immobilize surrounding blood and marrow from the osseous wound, which contain the proper stimulatory growth factors for bone regeneration. In a cranial defect model, significantly more bone formed in the scaffold treated defects than in negative controls even without the external delivery of stimulatory growth factors. In a separate study, Winn et al. [21] developed bone biomimetics consisting of PLA/collagen (pores between 50 and 25 μm) seeded with both osteoblast precursor cells and recombinant BMP-2. When analyzed in a critical-sized calvarial defect in athymic rats, very little tissue formation was seen when the scaffold was implanted alone or with the osteoblast precursor cells, but significant bone regeneration was seen after 4 weeks with the addition of the BMP-2. In our study, we also saw little mineralization when the scaffolds were implanted alone, but saw a dramatic difference in mineralization when delivered with osteoinductive growth factors. Although the growth

factors were incorporated into the scaffold in different manners, these studies all enunciate the importance of both an osteoinductive component and the proper scaffold architecture in bone tissue engineering approaches.

4. Conclusions

Degradable scaffolds with various macroscopic architectures were readily fabricated from a 2EG10LA multifunctional monomer using photopolymerization and a poragen leaching technique. By changing the pore size in the scaffold, only small changes in the scaffold degradation and mechanics were noted. One particular scaffold with an interconnected porous structure and pore diameters ranging from 45 to 150 μm was analyzed in a critical-sized cranial defect model in rats and a significant increase in radiopacity was observed in treatments receiving the scaffolds over an untreated defect 9 weeks after implantation. When the scaffold released osteoinductive growth factors, a significant increase in radiopacity was seen after both 5 and 9 weeks over the negative control, and a large increase in mineralized tissue formation was seen histologically in the scaffold without growth factors. These studies indicate that porous poly(2EG10LA) scaffolds have potential as an in situ forming synthetic bone graft material to enhance current treatment options for large bone defects.

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