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Osteoblast Growth and Bone Healing Response to Three Dimensional Poly(ϵ -caprolactone fumarate) Scaffolds

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Abstract

Poly(ϵ -caprolactone fumarate) (PCLF) scaffold formulations were assessed as a delivery system of recombinant human bone morphogenetic protein (rhBMP-2) for bone tissue engineering. The formulations included PCLF with combinations of poly(vinyl alcohol) (PVA) and hydroxyapatite (HA). The assessments included *in vitro* and *in vivo* assays. *In vitro* assays validated cell attachment using a pre-osteoblast cell line (MC3T3-E1). Additionally, *in vitro* release profiles of rhBMP-2 from PCLF scaffolds were determined up to 21 days. Data suggested PCLF incorporated with PVA and HA accelerated rhBMP-2 release and the released protein was bioactive. For the *in vivo* study, a critical sized defect (CSD) model in a rabbit calvaria was used to test PCLF scaffolds. At 6 weeks post-implantation, significantly more bone formation was measured in PCLF scaffolds containing rhBMP-2 than in scaffolds without rhBMP-2. In conclusion, we demonstrated PCLF delivered biologically active rhBMP-2, promoted bone healing in a CSD and has potential as a bone tissue engineering scaffold.

Keywords

poly(ϵ -caprolactone fumarate); three-dimensional scaffold; rabbit calvarial critical sized defect; rhBMP-2; bone tissue engineering

Introduction

It is hypothesized that five key events must occur to produce bone regeneration: 1) recruitment of osteogenic cells, 2) osteoconduction, 3) biomechanical stability, 4) angiogenesis and 5) osteoinduction (Schmidmaier et al. 2008; Giannoudis et al. 2008). Bone morphogenetic proteins (BMPs) are a powerful group of molecules that profoundly influence these five events. BMPs such as BMP-2 and BMP-7, for example, induce *de novo* bone formation and are osteoinductive (Seeherman and Wozney 2005; Hollinger et al. 2008). Thus, BMPs are pivotal for the bone wound healing cascade and when administered

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therapeutically, will augment and accelerate bone wound healing (Winn et al. 1999). However, a challenge for BMP delivery as a regenerative therapeutic is its rapid clearance from the clinical target as a consequence of circulation, thus contributing to a decreasing localized half-life (Li and Wozney 2001; Kirker-Head 2000). In addition, *in vivo* studies where BMPs were delivered to a fracture site in a formulation buffer, low efficacy resulted, and this outcome may be attributed to a short retention time at the local site (den Boer et al. 2003; Seeherman and Wozney 2005). To address therapeutic challenges for BMP delivery, we designed a delivery system composed of biodegradable polymer poly(ϵ -caprolactone fumarate) (PCLF) to allow sustained localized retention of the rhBMP-2 at the site of osseous healing.

PCLF polymers have been developed for tissue engineering scaffolds (Jabbari et al. 2005). However, hydrophobicity of PCLF due to the hydrophobic segment (i.e., PCL) may adversely affect biomolecules due to aggregation and/or conformational changes (Zhu et al. 2000). Therefore, hydroxyapatite (HA) and poly(vinyl alcohol) (PVA) components were added to mitigate this property. Moreover, further logic for these compositions was driven by fundamental bone biology emphasizing the importance of local osteoblast attachment and subsequent bone formation. PVA is an amphiphilic polymer that can adsorb onto hydrophobic scaffolds, such as PCLF, at the interface of hydrophobic surfaces (Nguyen 1996). PVA may lower surface energies of hydrophobic scaffolds by increasing hydrophilicity. This property was therefore exploited to enhance bone cell attachment. In addition, the mineral content of bone is composed mostly of HA, onto which osteoblasts can directly deposit osteoid that subsequently mineralizes. HA in combination with polyglycolic acid (PGA) and poly-L-lactic acid (PLA) had greater osteoconductive capacity than the polymeric scaffolds without HA (Zaky and Cancedda 2009). Therefore, by including PVA and HA with the PCLF scaffold design, osteoblast attachment, bone formation and osteoconduction could be promoted at the site of implantation.

The overall **purpose** of the study was to design and develop a PCLF-based system as a bone tissue engineering scaffold that would be osteoconductive, biodegradable and locally deliver rhBMP-2. Porous 3D PCLF-based scaffolds were fabricated and characterized. The *in vitro* performance was determined by exploiting cell culture techniques using a mouse osteoprogenitor cell line (MC3T3-E1). In addition, the effects of HA and/or PVA incorporation into PCLF scaffolds on rhBMP-2 release kinetics and bioactivity of the releasates were determined. Bone regeneration was determined using standardized rabbit calvarial models developed in our laboratory and used extensively by others. (Magno et al. 2010) Surgical outcome was characterized with standard technologies including micro-computed tomography (micro-CT) and histology.

Materials and Methods

Polymer synthesis

Poly(ϵ -caprolactone fumarate) was synthesized from PCL diols with a number average molecular weight (M_n) of 2,000 g/mol using previously reported procedures (Jabbari et al. 2005). The chemical structure of PCLF is shown in Fig. 1A and the resulting PCLF had a M_n of 18,000 g/mol and polydispersity index (PDI) of 2.0. The M_n and PDI were characterized by gel permeation chromatography (GPC) using polystyrene standards. The GPC system consists of a Waters 2410 refractive index detector, 515 HPLC pump, and 717 Plus autosampler, and a Styragel HR4E column. THF was used as the eluent at 1 mL/min.

Porous Scaffold Fabrication

Porous 3D PCLF scaffolds were synthesized using previously published salt leaching techniques (Kim, Yaszemski, et al. 2009), PCLF polymer (8.0 g), and salt (32 g, 300–500 μm size particles), corresponding to 80% porosity were mixed with 15 mL acetone to form a homogenous slurry and then gently heated to evaporate excess acetone. Three mL of benzoyl peroxide in N-vinylpyrrolidinone (200 mg/mL) and 100 μL of dimethyltoluidine in N-vinylpyrrolidinone (20 $\mu\text{L}/\text{mL}$) were added to the PCLF/salt mixture, extensively mixed, poured into Teflon molds and placed in an oven at 100 $^{\circ}\text{C}$ for 1 hr. Scaffolds containing hydroxyapatite (HA) nanoparticles (average size: ~ 100 nm, Berkeley Advanced Biomaterials) were fabricated by adding 30 % (w/w) HA to the PCLF/salt mixture (namely, PCLF+HA). Cross-linked PCLF scaffolds were removed from the molds and the salt was leached out by placing the scaffolds in distilled water for at least 7 days. In preparation of 3D scaffolds for *in vitro* (10 mm diameter $\times$ 5 mm height) and *in vivo* (15.2 mm diameter $\times$ 2.5 mm height) studies, PCLF cylinders were cut to the desired thickness using a razor blade and desired diameter using a cork borer. Non-porous 2D films (thickness: ~ 0.8 mm) were also fabricated after mixing PCLF with or without HA as described above without salt. The PCLF scaffolds were then sterilized by incubating in 70% ethanol for 1 hr at room temperature, washed with phosphate buffered saline (PBS) and air-dried. For PVA incorporation into PCLF scaffolds, 100 μL of 4% (w/v) PVA (m.w.: 30,000 – 70,000, Sigma) solution was added into sterile PCLF scaffolds and air-dried.

Scaffold Characterization

The chemical compositions of cross-linked polymeric materials were characterized by ATR-FTIR on a Nicolet 8700 FTIR spectrometer. A Germanium ATR crystal was used at a resolution of 4 cm^{-1} at 1000 cm^{-1} . Spectra were obtained with a minimum of 64 scans. Mechanical testing was performed on a TA instruments 2980 dynamic mechanical analyzer. Samples were tested in compression mode under increasing force of 0 to 18 N with a preload force of 0.1 N. The compressive modulus was determined from the linear portion of the stress strain curve up to 20% strain.

Cell culture and characterization

Mouse pre-osteoblasts, MC3T3-E1 (American Type Culture Collection - ATCC, Manassas, VA) were used to assess cell attachment on the 3D scaffolds. To increase cell attachment, the substrates were pre-wetted in the cell culture media for 1 hr and the MC3T3-E1 cells were seeded on 3D scaffolds at a density of 2×10^5 cells/scaffold and cultured for 24 hrs in the α -MEM medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (ATCC), 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin at 37 $^{\circ}\text{C}$ in a 5% CO_2 atmosphere. After 24 hrs of incubation, the media in each well was replaced with the osteogenic media supplemented with 10 mM β -glycerophosphate and 50 $\mu\text{g}/\text{mL}$ of ascorbic acid and further cultured for up to 21 days. At days 1 and 4, the cell morphology and attachment were imaged using scanning electron microscopy (SEM) as described previously. (Magno et al. 2010)

rhBMP-2 incorporation and release kinetics

The rhBMP-2 (Wyeth, Cambridge, MA) was dissolved in deionized water (100 $\mu\text{g}/\text{mL}$) and 50 μL of rhBMP-2 solution was added dropwise into each scaffold, and the scaffold was lyophilized. The *in vitro* rhBMP-2 release kinetics from the scaffolds was measured by an Enzyme Linked Immuno-Sorbent Assay (ELISA) kit (R&D Systems, Minneapolis, MN). Alpha-MEM supplemented with 100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin and 1% bovine serum albumin (BSA) were used as a release medium to maintain the protein

stability.(Guan et al. 2007) The releasates were collected daily, stored at -80°C and replaced with fresh media.

Bioactivity of the releasates

MC3T3-E1 cells were plated at a density of 2×10^4 cells/cm² on 24-well tissue culture plates. After 24 h of incubation at 37°C , the media in each well was replaced with osteogenic media supplemented with 10 ng/mL of either exogenous rhBMP-2 or rhBMP-2 releasates from 3D PCLF-based scaffolds. Proliferation and ALP activity of the cells in the presence of exogenous rhBMP-2 (positive control) or the releasates from the scaffolds or in the absence of rhBMP-2 (negative control) were determined at each time point up to 21 days as described earlier (Kim, Hefferan, et al. 2009). Briefly, prior to the assays, the cell lysates were prepared as described in a previous publication (Kim, Hefferan, et al. 2009). Briefly, the cultured cells on the scaffolds were rinsed with PBS three times and 0.5 mL of 1x cell lysis buffer (Cell signaling technology, Danvers, MA) was added into each scaffold to rupture the cell membranes. The lysates were frozen at -80°C until use. On the day of the assay, the cell lysates were subject to a thaw/freezing cycle three times (30 min thawing at 37°C and freezing at -80°C for 20 min) to extract the DNA and proteins from the cell cytoplasm. To analyze cell proliferation on the scaffolds, DNA content (directly correlated to the cell number) was measured using a fluorometric Picogreen^R DNA quantification kit (Molecular Probes, Eugene, OR) according to the manufacturer's instruction. Alkaline phosphatase (ALP) activity was determined using a previously established protocol (Kim, Hefferan, et al. 2009). Briefly, 20 μL of ALP working buffer solution, 80 μL of the lysate solution and 100 μL of substrate solution (5 mM *p*-nitrophenyl phosphate) were added to a 96 well plate. The plate was incubated at 37°C for 1 h and the reaction was stopped by adding 100 μL of 0.3 N NaOH solution to each well. The absorbance of each well was measured at 405 nm and the value of ALP activity was normalized to the amount of DNA in the sample.

Rabbit surgery

All surgical procedures involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) at Allegheny General Hospital (Pittsburgh, PA). The NIH Guide for Care and Use of Laboratory Animals was observed in the treatment of all experimental animals. Research was conducted in compliance with the Animal Welfare Act Regulations and other Federal statutes relating to animals and experiments involving animals. The research adheres to the principles set forth in the Guide for Care and Use of Laboratory Animals, National Research Council, 1996.

Skeletally mature New Zealand White male rabbits weighing 3.5–4.5 kg (6 – 12 month-old) were used to assess *in vivo* performance of the materials. Eight rabbits were divided evenly between either PCLF+HA+PVA scaffolds without rhBMP-2 or PCLF+HA+PVA scaffolds with 50 μg of rhBMP-2. Critical sized calvarial defects (15 mm in diameter) were prepared in a rabbit by using a 15 mm-diameter surgical trephine with copious physiological saline for irrigation (Magno et al. 2010). After 6 weeks, rabbits were sacrificed humanely according to NIH and Institutional guidelines with an intravenous overdose of barbiturate (200 mg/kg body weight) and calvariae were recovered *en bloc*. Calvariae were placed immediately into neutral buffered formalin for micro-computed tomography (micro-CT) analysis and histology.

Micro-CT analysis

Each specimen was placed on the scanning platform of a GE eXplore Locus micro-CT (GE Healthcare, Piscataway, NJ) and 360 X-ray projections were collected (80 kVp; 500 mA; 26 min total scan time). Projection images were preprocessed and reconstructed into 3D

volumes (20 μm resolution) on a 4PC reconstruction cluster using a modified tent-FDK cone beam algorithm (GE reconstruction software). Each volume was scaled to Hounsfield Units (HU) using a calibration phantom containing air and water (phantom plastic); a plug within the phantom containing hydroxyapatite was used as a bone mimic for bone mineral/density calculations. The 3D data was processed and rendered (isosurface/maximum intensity projections) using MicroView (GE Healthcare). Volumes were imported into Matlab (R2009b, Mathworks) for automated batch analysis. Briefly, a fixed cylindrical volume of interest (VOI, 14 mm diameter, 5 mm height) was applied to each volume. Since each volume was calibrated using a fixed standard, calcium phosphate, cortical bone, trabecular/woven bone, and scaffold content were determined using predefined Hounsfield Unit thresholds (>3000, 2000–3000, 750–2000, and 300–750 respectively). To account for partial volume and beam hardening artifacts around dense calcium phosphate granules (incorrectly classified as woven bone), calcium phosphate regions were morphologically “dilated” by 5 voxels and subtracted from the trabecular/woven bone mask.

Histology

The specimens were prepared for histology as previously described (Magno et al. 2010) and stained with Sanderson's Rapid Bone Stain and counterstained with van Gieson's picrofuchsin in which soft tissue and bone are stained blue and pink/red, respectively.

Statistical analysis

All assays were performed at least in triplicate to verify reproducibility and data were reported as means $\pm$ standard deviation. Statistical analysis was performed using a single factor analysis of variance (ANOVA) model and Tukey-Kramer multi-comparison test with a significance level of $p \leq 0.05$.

Results

PCLF scaffolds

The compositions of PCLF porous scaffolds (Fig. 1A) were analyzed by ATR-FTIR. FTIR spectra of PCLF, PCLF+HA and HA alone are shown in Figure 1B. When HA and fumaric acid was incorporated in the PCLF network, the characteristic bands corresponding to the specific functional groups (PO_4 and ester group, COO) was observed in the PCLF+HA FTIR spectrum, indicating that HA and fumaric acid was successfully incorporated into the polymer. The compressive modulus of porous three-dimensional scaffolds consisting of PCLF and PCLF+HA scaffolds were determined to be 193 ± 34 and 502 ± 194 kPa, respectively (Fig. 1C).

Surface characterization

Images of 3D PCLF scaffolds (fabricated by a salt porogen leaching method, 80% porosity) exhibited irregular cubical pores with low inter-connectivity. The SEM images of PCLF +HA scaffolds suggested uniform HA particle distribution throughout the scaffold surface in contrast to PCLF without HA. While the PCLF scaffolds without HA revealed a smooth surface morphology (Fig. 2A and C), the PCLF+HA scaffolds appeared to show rough surface morphology due to HA crystal presence on the surface (Fig. 2B and D). Cell attachment on 3D PCLF scaffolds with and without HA was also robust throughout the surfaces. The cells cultured on PCLF scaffolds with HA were not evenly distributed as on PCLF scaffolds without HA, likely due to differences in the surface morphologies (Fig. 2C and D).

rhBMP-2 release kinetics

rhBMP-2 release kinetics from PCLF scaffolds were determined by measuring the rhBMP-2 concentration of the releasates collected up to 21 days (Fig. 3). The cumulative release profiles from all scaffolds were characterized by a bi-phasic release, with an initial burst release within first 5 days followed by a slow release (Fig. 3A). The total rhBMP-2 cumulative release from the scaffolds was determined to be: PCLF+HA+PVA > PCLF+HA > PCLF+PVA > PCLF scaffold. The outcome indicates that HA and PVA incorporation additively increased rhBMP-2 release. Periodic rhBMP-2 release from each scaffold was also plotted to provide the data of the released protein per scaffold at each time period with the percent release. It is clear that PCLF+HA+PVA scaffold released the greatest amount of rhBMP-2 at each time period; therefore, this scaffold formulation was chosen for the rabbit calvarial model study.

Bioactivity of rhBMP-2 releasates

The bioactivity of rhBMP-2 releasates from the PCLF-based scaffolds was determined by measuring proliferation and ALP activity of MC3T3-E1 cells cultured in the presence of the releasates and compared to exogenous rhBMP-2 (Fig. 4). DNA concentration, an indication of cell number, in the group with exogenous rhBMP-2 tends to be greater other groups throughout the study. There was no significant difference in DNA concentration of the cells among the groups with the different releasates. In contrast, ALP activity in the group without rhBMP-2 (negative control) revealed significant difference, especially at later time periods, compared to other experimental groups with rhBMP-2. Among the groups with the rhBMP-2 releasates, again there was no significant difference among the test groups. Therefore, the rhBMP-2 releasates from PCLF-based scaffolds appeared to be equally bioactive to exogenous rhBMP-2 and induced osteogenic differentiation of the pre-osteoblasts.

Bone regeneration in the rabbit calvaria

Based on rhBMP-2 release profile data (Fig. 3), PCLF+HA+PVA scaffolds were selected for *in vivo* studies. Before surgeries, the morphology of PCLF implants was analyzed by micro-CT and the 2D section and 3D reconstructed micro-CT images of the implant revealed a highly porous structure throughout the scaffolds (Fig. 5A). However, calculated porosity of the scaffold was 63%, lower than the initial porogen amounts incorporated in the scaffolds (80%). This trend was consistent with a previous report.(Kim, Hefferan, et al. 2009) PCLF+HA+PVA with or without rhBMP-2 (50 µg/scaffold) were prepared to fit into the rabbit calvarial critical-sized defect. At 6 weeks, no significant adverse tissue responses were noted at necropsy (Fig. 5B). The necropsied specimens were analyzed by micro-CT. The 2D coronal sections and 3D reconstructed micro-CT images indicated that without rhBMP-2, PCLF+HA+PVA scaffolds had minimal new bone formation, whereas rhBMP-2-containing scaffolds (50 µg/scaffold) appeared to have new bone (Fig. 6A and B).

The micro-CT images were analyzed for trabecular bone volume and it was determined the rhBMP-2-supplemented PCLF+HA+PVA scaffold induced greater new bone formation compared to the scaffolds without rhBMP-2 (Fig. 6C and D). Again, rhBMP-2-treated PCLF+HA+PVA scaffold induced greater new bone formation compared to the scaffolds without rhBMP-2. ($p < 0.01$ and $p < 0.05$ for bone volume and bone mineral density or BMD, respectively)

To confirm the micro-CT findings, histological analysis was performed (Fig. 7). The contour of the implant at 6 weeks was greater than the inner and outer tables of the calvaria, possibly due to *in situ* swelling of the implant. New bone formation along the dural surface was clearly observed in rhBMP-2-treated scaffold. It is noteworthy that the continuation of bone

formation from the recipient margins across the entire defect at 6 weeks post-implantation. Critical-sized defects treated with scaffolds alone without rhBMP-2 had fibrous tissue surrounding the defect area and there were PCLF remnants throughout the defect. This observation was consistent with micro-CT data, thus suggesting the PCLF scaffolds were degrading slower than the rate of new bone formation. However, as evidenced by the histology panel at 5x magnification, there was new cancellous bone deposition in apposition to the implant. Overall, histological and micro-CT analysis data suggested that rhBMP-2-containing PCLF scaffolds promoted new bone formation when compared to the scaffolds without rhBMP-2 at 6 weeks post-implantation.

Discussion

Previous studies have shown that PCLF is biocompatible, biodegradable, self-cross-linkable, injectable, and can be synthesized in a relatively simple and straightforward process (Jabbari et al. 2005; Kim, Hefferan, et al. 2009). The current study highlighted the potential of PCLF as bone tissue engineering scaffold and as a delivery system of rhBMP-2. To enhance the delivery capabilities of PCLF as a bone scaffold, the effects of the additives (HA and PVA) were examined.

HA is similar in composition to bone. HA therefore has been incorporated with collagen (Du et al. 1998) and poly(α -ester) (Kim et al. 2006) to improve biocompatibility as well as osteoconductivity. PVA has been used to improve biocompatibility by increasing wettability (hydrophilicity) to prevent non-specific protein adsorption onto the surface (Gholap et al. 2005).

The amount of rhBMP-2 release was greatest when PVA and HA were incorporated into the PCLF scaffold. This observation may be due to the change in hydrophilicity or hydrophobicity of the PCLF scaffolds. PCLF is hydrophobic and its main building blocks are comprised of poly(ϵ -caprolactone) and fumaric acid (Fig. 1A), both are hydrophobic. When rhBMP-2 encounters hydrophobic environments, it can aggregate irreversibly or degrade within the delivery system, preventing functional release (Park et al. 1995; Zhu et al. 2000). Therefore, by decreasing PCLF hydrophobicity using PVA and HA, aggregation and degradation of rhBMP-2 may be prevented (Yeo and Park 2004), thus significantly increased rhBMP-2 release. However, it is noted that total rhBMP-2 release after 21 days was about 40% of initial amount, thus it may need further treatments (e.g., covalent rhBMP-2 incorporation) to improve release amount. In addition, ideal carriers of rhBMP-2 should degrade in register with new bone formation to prevent complications such as bone formation obstruction, chronic inflammation or multiple revision surgeries (Hollinger and Chaudhari 1992). In the current study, as observed in micro-CT and histology images, degradation of PCLF may physically interrupt bone regeneration. No significant polymer resorption by enzymatic or cellular activity such as osteoclasts was identified. To modify PCLF degradation, porosity and pore interconnectivity need to be improved (Fig. 3). The micro-CT image analysis of PCLF+HA scaffolds revealed a discrepancy between the calculated porosity (63%) and initial porogen amount (80%) that may result from residual salt porogens being retained within the scaffold as well as a non-uniform distribution of the porogen (Kim, Yaszemski, et al. 2009; Peter et al. 1999). To mitigate against this, either other porogens or another scaffold fabrication technique may be explored, such as solid freeform fabrication (SFF) (Hollister 2005). It is noteworthy that the calculated porosity by micro-CT analysis is often more realistic to obtain actual porosity data than conventional methods as it does include dead pores or isolated pores within a scaffold which are often not determined by other methods such as mercury intrusion porosimetry (Lu et al. 2000).

In the current study we report, we used significantly less rhBMP-2 dose (50 µg/defect site) and early post-implantation period (6 weeks) in combination with synthetic polymeric delivery system and showed the selected PCLF/HA scaffolds significantly increased new bone formation in a rabbit CSD calvarial model at 6 weeks post-implantation. Previous studies with the skeletally mature rabbit CSD calvarial model have reported with rhBMP-2 dosing up to 290 µg/scaffold using collagen sponge (Smith et al. 2008) or implantation time period up to 16 weeks using coral scaffold (Hou et al. 2007). In addition, those studies have used naturally-derived materials which often lack the processibility and/or controllability of final properties such as degradation rate. A localized and more physiological dose for therapeutic effect is safer and more economical than supra-physiological mega doses. In addition, a localized minimal dose of rhBMP-2 may mitigate against ectopic bone formation (Weber et al. 2001). Furthermore, the immunogenicity of PCLF is not a concern in contrast to other delivery systems as reported for collagen sponge (Lutolf et al. 2003). In addition, unlike other conventional bone tissue engineering scaffolds such as calcium phosphate cements, it is possible to fabricate injectable 3D porous PCLF scaffolds by using hydrogel porogens as demonstrated previously (Kim, Yaszemski, et al. 2009). In the study we report, we used significantly less rhBMP-2 dose (50 µg/defect site) and early post-implantation period (6 weeks) in combination with synthetic polymeric delivery system and showed the selected PCLF/HA scaffolds significantly increased new bone formation in a rabbit CSD calvarial model at 6 weeks post-implantation. As a consequence of the shortcomings of contemporary rhBMP-2 delivery system scaffolds, we have developed a new class of poly(ε-caprolactone fumarate) (PCLF)-based scaffolds in an effort to match the biological wound healing criteria. Furthermore, the implanted materials may be considered to be *osteophyllic* as a consequence of the observations of bone apposition directly on to the implanted, indicating *in vivo* biocompatibility as evidenced by histology. Although rhBMP-2 treatment induced new bone formation in a rabbit calvarial CSD when compared to the scaffold without rhBMP-2, there was marginal evidence of osteoconduction and bone ingrowth into the scaffolds as revealed by micro-CT and histological analyses. Therefore, it is important to note that precisely calibrated chemical and physical properties of a scaffold as well as pharmacokinetics of therapeutic agents from the delivery system would be an absolute necessity for a better outcome with regard to bone wound healing as well as remodeling. Overall, despite the above addressed challenges of the current formulations of PCLF-based scaffolds, the current study is important in that this is the first study to show *in vivo* bone formation in a CSD non-rodent model with minimal dose of rhBMP-2 at relatively early post-implantation (6 weeks) and demonstrated the feasibility of the PCLF material as a bone tissue engineering scaffold as well as a delivery system of rhBMP-2.

Conclusions

PCLF formulations were synthesized and fabricated into 3D porous scaffolds. Cell attachment (MC3T3-E1) on all tested scaffolds was robust throughout the surface, regardless of the polymer compositions. HA and PVA incorporation into PCLF scaffolds significantly increased rhBMP-2 release. Furthermore, rhBMP-2-treated PCLF scaffolds appeared to induce new bone formation in a critical-sized rabbit calvarial defect model.

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Figure 1.

(A) Chemical structure of poly(ϵ -caprolactone fumarate) (PCLF), (B) FTIR spectra of PCLF, PCLF+HA, and HA, and (C) compressive moduli of PCLF and PCLF+HA. Data are reported as a mean $\pm$ standard deviation for $n=3$. * represents significant difference ($p < 0.05$) between the groups.

Figure 2. SEM images of pore architecture of (A) 3D PCLF, (B) 3D PCLF+HA scaffolds. SEM images of MC3T3-E1 cells at 24 hrs of culture on (C) 3D PCLF and (D) 3D PCLF+HA scaffolds. Yellow arrows indicate attached cells on the surface.

Figure 3A.

Figure 3B.

Figure 3.

(A) Cumulative and (B) periodic rhBMP-2 release profiles from PCLF-based scaffolds. Data are reported as means $\pm$ standard deviations for $n=4$.

Figure 4A.

Figure 4B.

Figure 4.

Bioactivity of MC3T3-E1 cells in the presence of rhBMP-2 releasates. (A) DNA concentration and (B) ALP activity of MC3T3-E1 cells cultured on TCPS in the presence of rhBMP-2 releasates and exogenous rhBMP-2 at various time periods. Data are reported as means $\pm$ standard deviations for $n=4$.

Figure 5. (A) photographic and micro-CT images of PCLF+HA+PVA implants prepared with a salt leaching method before surgeries. (B) The implants after surgically placed in a critical-sized (15 mm diameter) rabbit calvarial defect. Yellow dotted circle indicates the implant location.

Figure 6. Micro-CT images of new bone formation in critical sized rabbit calvarial defects. White arrows in the 2D coronal sectional images point to the defect site. (A) PCLF+HA+PVA without rhBMP-2 and (B) PCLF+HA+PVA with 50 μ g rhBMP-2. Blue color indicates trabecular bone formation in 3D reconstruction images. White arrows indicate the defect margins. (C) Trabecular bone formation and (D) bone mineral density. Data are reported as means $\pm$ standard deviations for n=4. * represents significant difference ($p < 0.05$) between the groups.

Figure 7.

Bone healing in rabbit calvaria with PCLF+HA+PVA scaffolds with or without rhBMP-2 treatment at 6 weeks post-implantation. M indicates remaining implant. There is no evidence of osteoconduction from the parietal bone margins (stained pink-red). The overall contour of the implant exceeds the dimensions of the inner and outer tables of the resected bone. The μ CT images do not duplicate the enlarged contour. Consequently, there is a possibility the enlargement, i.e., implant swelling, may be artefactual from the histology preparation. At the 5X magnification, there is no indication of a fibrous capsule interposed between the implant and recipient bone, thus suggesting biocompatibility.