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Surface-modified functionalized polycaprolactone scaffolds for bone repair: In vitro and in vivo experiments

Jonas Jensen, 1 Jan Hendrik Duedal Rølffing, 1 Dang Quang Svend Le, 1 Asger Albæk Kristiansen, 2 Jens Vinge Nygaard, 2,3 Lea Bjerre Hokland, 1 Michael Bendtsen, 1 Moustapha Kassem, 4 Helle Lysdahl, 1 Cody Eric Bøunger 1,5

1 Orthopaedic Research Laboratory, Aarhus University Hospital, DK-8000 Aarhus, Denmark
2 Interdisciplinary Nanoscience Center (iNANO), Aarhus University, Aarhus, Denmark
3 Mechanical Engineering, Department of Engineering, Aarhus University, Denmark
4 Department of Endocrinology and Metabolism, Laboratory for Molecular Endocrinology (KMEB), University Hospital of Odense, Odense, Denmark
5 Department of Orthopaedics, Spine Unit, Aarhus University Hospital, Aarhus, Denmark

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Abstract: A porcine calvaria defect study was carried out to investigate the bone repair potential of three-dimensional (3D)-printed poly-ε-caprolactone (PCL) scaffolds embedded with nanoporous PCL. A microscopic grid network was created by rapid prototyping making a 3D-fused deposition model (FDM-PCL). Afterward, the FDM-PCL scaffolds were infused with a mixture of PCL, water, and 1,4-dioxane and underwent a thermal-induced phase separation (TIPS) followed by lyophilization. The TIPS process lead to a nanoporous structure shielded by the printed microstructure (NSP-PCL). Sixteen Landrace pigs were divided into two groups with 8 and 12 weeks follow-up, respectively. A total of six nonpenetrating holes were drilled in the calvaria of each animal. The size of the cylindrical defects was h 10 mm and Ø 10 mm. The defects were distributed randomly using following groups: (a) NSP-PCL scaffold, (b) FDM-PCL scaffold, (c) autograft, (d) empty defect, (a1) NSP-PCL scaffold + autologous mononuclear cells, and (a2) NSP-PCL scaffold + bone morphogenetic protein 2. Bone volume to total volume was analyzed using microcomputed tomography (μCT) and histomorphometry. The μCT and histological data showed significantly less bone formation in the NSP-PCL scaffolds in all three variations after both 8 and 12 weeks compared to all other groups. The positive autograft control had significantly higher new bone formation compared to all other groups except the FDM-PCL when analyzed using histomorphometry. The NSP-PCL scaffolds were heavily infiltrated with foreign body giant cells suggesting an inflammatory response and perhaps active resorption of the scaffold material. The unmodified FDM-PCL scaffold showed good osteoconductivity and osseointegration after both 8 and 12 weeks.


Key Words: poly-ε-caprolactone, biodegradation, scaffold, bone tissue engineering, foreign body giant cell


INTRODUCTION
Tissue engineering combines methods from materials engineering and biomedical sciences to regenerate tissue using artificial constructs. 1 Different approaches have been utilized to enhance new bone formation using scaffolds produced from synthetic and natural materials. Owing to increased research in construct material and morphology, it is now possible to implant biodegradable scaffolds which stimulate bone ingrowth directly. 2,3 These features can be accomplished by using biocompatible and biodegradable components which end up with no residual scaffold material and complete healing with autologous bone. 4

To improve scaffolds for bone regeneration, a combination of materials and surface morphology can be utilized to increase surface bioocompatibility, degradation profile of the scaffold, and mechanical properties. 5,6,7 One way to manipulate primarily synthetic polymers into a desired three-dimensional (3D) structure is using fused deposition model
(FDM), a rapid prototyping technology, which ensures each scaffold can be custom-made to accommodate challenges of different bone void dimensions without losing the desirable feature from scalable and cost-effective industrial production. Poly-ε-caprolactone (PCL) is one of the most widely used synthetic polymers in scaffold development and application within orthopedics. The polymer is highly bio-compatible and degrades into harmless by-products metabolized in the tricarboxylic acid cycle. Implementation of FDM-manufactured PCL scaffolds into clinical practice, where new bone formation is needed, has already begun.

To increase surface area for cell adhesion, a newly developed and patented procedure was used to create a nano-structured porous (NSP) PCL scaffold. A micro- and nanoporous structure was added to the macroporous FDM-manufactured scaffold by infusing the macroporous FDM scaffold with a PCL solution, which was then demixed and solidified using thermal-induced phase separation (TIPS) and lyophilization. This newly developed hierarchically structured NSP-PCL scaffold holds several hypothetical benefits compared to other scaffolds. The unique combination of a rapid prototyped and a lyophilized structure adds a surface area for cell adhesion and proliferation while mimicking the complex structure of bone to accommodate differentiation and bone formation. Furthermore, the FDM component adds a degree of mechanical strength that is unattainable using TIPS-based fabrication methods alone. High surface area also holds an advantage for functionalization with growth factors like bone morphogenetic protein 2 (BMP-2) or preseeding with stem cells before implantation.

The main aim of this study was to assess the osteogenic potential of the new scaffold in vitro and in vivo hypothesizing that enlargement of surface area via incorporation of nanostructured pores into a fusion-deposition-modeled PCL scaffold would improve its osteogenic potential. In addition, individual cells in the NSP-PCL will experience a vastly different set of mechanical cues than on a FDM-PCL scaffold. To test our hypothesis the (a) NSP-PCL scaffold was compared to, (b) a FDM-PCL scaffold, (c) a positive autograft control, and (d) a negative empty control. Another aim was to analyze the efficacy of a functionalized (a) NSP-PCL scaffold with either (a1) autologous stem cells or (a2) BMP-2 on bone healing in a calvarial bone defect model in pigs (Fig. 1).

**MATERIALS AND METHODS**

**Scaffold construction and characterization**

The scaffolds were made by FDM fabrication using a BioScaffold (SysEng GmbH, Germany). The scaffold material was the polymer PCL with a molecular weight of 50 kDa (Perstorp UK, Cheshire, UK). FDM, like any other rapid prototyping technique, allows for a precise control of the scaffolds’ porous geometry. In this study, the scaffolds were built from layered deposition of polymer strands by extruding molten PCL from an extrusion die with an inner diameter of 200 µm (final fiber diameter ~ 175 µm). To ensure thorough and stable fusion junctions between layers, the layer thickness was set to 120 µm. The center to center distance between parallel fibers in each layer was ~1000 µm and each layer was angled 105° in relation to the underlying one to allow for a tortuous and laterally interconnected porous network. These scaffolds are hereafter referred to as FDM-PCL. The scaffolds were subsequently infused with a homogenous mixture of PCL, water, and 1,4-dioxane and underwent a TIPS followed by lyophilization. The polymer concentration was 30 mg PCL/g 1,4-dioxane. Two different water concentrations were used (0.01 and 0.03 wt %). Higher water concentration resulted in larger pore sizes. The freeze-drying process resulted in a nano-structured porous PCL scaffold within the FDM element of the scaffolds [Fig. 2(D–F)]. The NSP-PCL scaffold composition was optimized with in vitro cell studies regarding pore size and morphology.

Both the FDM-PCL and NSP-PCL scaffolds were sterilized in vacuum using ethanol concentrations of 96, 70, and 50%, 30 min each, followed by rinsing in sterile water. To increase surface hydrophilicity, the scaffolds were treated with 1.25 M NaOH for 16 h, then neutralized in 1 M HCl for 1 h and rinsed in sterile water.

Characterization of scaffolds was performed using scanning electron microscopy (SEM, Nova NanoSEM 600; FEI Company, Eindhoven, the Netherlands) and microcomputed tomography (µCT; µCT 40; Scanco Medical AG, Zürich, Switzerland). The X-ray tomographic microscopy (XTM) studies were carried out at the experimental station ID19 of the European Synchrotron Radiation Facility (Grenoble, France) taking advantage of the high contrast acquired due to the high brilliance of the X-ray source realized by the synchrotron. The XTM image processing and the volume renderings were determined using isosurface representations and volume texture renderings generated by the Amira software from Mercury Computer Systems (Chelmsford, MA). The theoretical porosity of the NSP-PCL scaffold was calculated by following equation:

\[
\text{porosity}_{\text{total}} = \frac{V_{\text{dioxane}}}{V_{\text{dioxane}+H_2O+\text{PCL}}} + \frac{H_2O}{V_{\text{dioxane}+H_2O+\text{PCL}}}
\]

**In vitro assessment of scaffold construct**

NSP-PCL scaffolds and scaffolds comprising only the TIPS PCL component (h = 5 mm, Ø = 10mm) were seeded with \(2 \times 10^6\) immortalized human mesenchymal stem cells stably transduced by a retroviral vector containing the gene for the catalytic subunit for human telomerase (hMSC-TERT). The seeded scaffolds were cultured for up to 22 days at 37°C, in a humidified atmosphere of 5% CO\(_2\) in Dulbecco’s modified Eagle medium (GIBCO 31966; Invitrogen) supplemented with 10% fetal calf serum (GIBCO 10270; Invitrogen) [hereafter referred to as proliferation media], or in an osteoinductive media (OIM) consisting of proliferation media supplemented with 10 nM dexamethasone (D2915, ...
Sigma), 466 μM ascorbic acid-2-phosphate (D2915, Sigma), and 10 mM β-glycerophosphate (G-9891, Sigma).

To visualize cell ingrowth, transitional sections of scaffolds comprising only the TIPS component were cut and stained with hematoxylin and eosin (H&E). Samples of NSP-PCL scaffolds were in 70% ethanol and embedded in Technovit 9100 VR (Heraeus Kulzer, Kulzer Division, Werheim, Germany). Sections of 7 μm were cut and stained using Goldner’s Trichrome and evaluated with light microscopy to visualize mineralization. For SEM, the constructs were fixed in 2.5% glutaraldehyde containing 0.1 M sodium cacodylate buffer (pH 7.4) and dehydrated in a graded series of ethanol (50–99%) before being transferred to a desiccator for air-drying. Constructs were analyzed for attachment of cells using SEM with a low vacuum secondary electron detector.

To assess cell viability, the NSP-PCL were rinsed in phosphate-buffered saline (PBS) and stained with 2 μM calcine AM and 4 μM ethidium homodimer (EthD-1) in PBS (Live/Dead VR Viability Kit, Molecular Probes) for 45 min in the dark. Images were acquired immediately after staining using a confocal laser scanning microscope 510 Meta (Zeiss microimaging GmbH, Göttingen, Germany).

**Study design, animal model**
A paired study design comparing the intervention groups with the positive and negative control group within the same animal was chosen. A cranial bone defect model in female Danish Landrace pigs (8 months old) was utilized to compare the six groups. The calvarium was selected due to the thickness of the porcine calvaria and the trabecular bone morphology. A total of 16 pigs were equally divided between the two observation times: 8 weeks (n = 8) and 12 weeks (n = 8). Two observation times were chosen to elucidate bone healing and scaffold degradation over time. The study was approved by the Danish Animal Research Inspectorate and conformed to Danish law (application no. 2012-15-2934-00362).

**Cell extraction and cultivation**
Four weeks before surgery, autologous bone marrow was aspirated from the proximal tibia into a 50-mL syringe containing 10 mL DMEM with 50 IE heparin. A total of 30 mL bone marrow was aspirated from each animal. Mononuclear cells were purified by Ficoll gradient centrifugation (Sigma-Aldrich Co. LLC., St. Louis, MO) and thereafter
cultured in proliferation media for two passages. Subsequently, we determined the number of available cells, and we found that $1.3 \times 10^6$ cells was the largest possible number that ensured a uniform and standardized treatment. Five days before surgery, $1.3 \times 10^6$ cells/NSP-PCL were seeded and cultivated for 2 days in proliferation media followed by 3 days in OIM.

**Anesthesia**

Anesthesia during bone marrow aspiration and premedication before surgery was comprised of Zoletil mix 1 mL/10 kg (tiletamin 2.5 mg/mL, zolazepam 2.5 mg/mL, torbugesic 0.5 mg/mL, ketaminol 2.5mg/mL, and rompun 2.5 mg/mL; Virbac, DK). During the surgery procedure intravenous propofol (B. Braun Melsungen AG, Melsungen, Germany) 1 mL/kg/h, and fentanyl (Haldid, Janssen-Cilag A/S, Birkerød, Denmark) 0.5 mL/kg/h was used.

**Surgical procedure**

A sharp T-shaped, coronal-sagittal incision was made in the forehead. Periosteum was dissected and using a wire guide, three K-wires were drilled perpendicular into the bone. The middle K-wire in the intended center of the defect, and two perpendicular K-wires 2 mm lateral to the edges of the intended defects. Six nonpenetrating defects were created using a cannulated drill bit (10 mm in diameter and 10 mm in depth). Autolougous bone from the drill holes was kept aside for later use. The defects were positioned 10 mm apart to avoid biological interaction.

The defects were distributed at random using following groups: (a) NSP-PCL scaffold, (b) FDM-PCL scaffold, (c) autograft (1.12 g harvested from the drill holes), (d) empty defect, (a1) NSP-PCL scaffold + autologous bone marrow stem cells, and (a2.) NSP-PCL scaffold + BMP-2 (1 mg/scaffold from InductOs, Wyeth, Berkshire, UK). Before implantation 0.67 mL of 1.5 mg/mL BMP-2 solution was administered to the scaffold in the operation theater. The dose of 1 mg BMP-2 per defect was guided by the product information, which states that the applied concentrations should not exceed 1.5 mg/mL due to potential adverse effects, for example, localized edema. In the present study, the volume of the bone defect was 0.786 cm$^3$. The maximum applicable dose was therefore 1.18 mg/defect. Visualization of placement of holes is shown in Figure 1. The soft tissues were repositioned and sutured. The animals were treated postoperatively with systemic antibiotics (Streptocillin; Boehringer Ingelheim, Ingelheim, Germany, 1mL/10kg) for 3 days. Two animals from the 12-week group were put down due to humane endpoint complications unrelated to surgery or infections. Eight and six animals were killed after 8 and 12 weeks, respectively, using an overdose phenobarbital. Afterward, the calvaria were resected and frozen to $-80^\circ$C.

**Microcomputed tomography**

After embedding the bone specimens were scanned by high-resolution $\mu$CT scanning. Cylindrical regions of interest (ROIs), 10 mm in diameter and a depth of 8 mm were...
chosen. The cylindrical ROIs were found using the carbon fiber guide wires as markers parallel to the drill holes. The scanned images were rendered in 3D reconstruction, and computational algorithms were used to calculate bone volume to total volume (BV/TV) fractions using a calculated average threshold value of 130. Scanner integrated software was utilized to perform this analysis.

Histomorphometry
The frozen calvaria were cut in half, dehydrated in ethanol (70–99%), and embedded in methylmethacrylate. Slices with a thickness of 7 μm were made on two levels in the sagittal plane with 400 μm between each level using a microtome (Polycut E, Reichert-Jung, Heidelberg, Germany). The samples were stained with Goldner’s Trichrome stain. Staining for tartrate-resistant acid phosphatase (TRAP) was performed on NSP-PCL samples to detect osteoclast-like cells.\(^\text{15}\)

Histomorphometry was performed using Cavalieri point count technique (CAST-grid system; Olympus Denmark A/S, Glostrup, Denmark) to quantify the amounts of new bone and residual scaffold material compared with TV.

Statistics
Comparison analysis was calculated as group average with standard deviations and compared using repeated measures analysis of variance using STATA software (Ver. 12.1; StataCorp LP, College Station, TX) and PRISM (Ver. 6; GraphPad Software, San Diego, CA). Differences between groups were analyzed using Turkey's multiple comparisons test. A \(p\) value of <0.05 was considered significant.

RESULTS
Scaffold characterization
\(\mu\)CT of FDM-PCL scaffolds showed a successful layer-by-layer deposition of the polymer. SEM visualized small grooves in the surface of the PCL. The FDM manufacturing method created homogenous strings of PCL fibers in a 3D grid structure [Fig. 2(B,C)].

In the NSP-PCL scaffolds, \(\mu\)CT visualized a homogenous infusion of the NSP component within the FDM-PCL scaffold [Fig. 2(D)]. The 0.03 [wt\%] water concentration during TIPS proved most suitable, creating a range of pore sizes from 500 nm to 50 μm evenly dispersed between the plotted structures. The pore size range was estimated from XTM reconstructions made on samples from the scaffold (Fig. 3). By high-magnification SEM, even smaller pores were evident but not quantifiable [Fig. 2(F)]. The calculated theoretical porosity of the NSP-PCL scaffold was calculated to 0.9726. The microscopical porosity caused by dioxane evaporation during manufacturing was calculated to 0.9399, whereas water accounted for the nanosized pores, and a porosity of 0.0328.
Cell ingrowth and viability *in vitro*

hMSC-TERTs were seeded on the top surface of TIPS scaffolds and migration was observed during culture. On day 1, the cells adhered mainly to the seeding surface, but at day 7 migration into the scaffold was evident. After 15 days of culture, a homogenous distribution throughout the entire scaffold was observed (Fig. 4).

The viability of the distributed cells on the surface of constructs was further visualized by live/dead staining on day 7. The images showed a homogenous distribution of viable cells in the top layer of the scaffold. No dead cells were observed [Fig. 5(A)].

Mineralization nodules were observed after 22 days of culture by Goldner’s Trichrome staining, and large areas of dense matrix indicated active production of protein from cells. A gradient of increasing differentiation level was observed with elongated osteoprogenitor cells in the outermost areas surrounding round, matrix-producing and fully mineralized osteoblasts in the central part [Fig. 5(B)].

**Microcomputed tomography**

After 8 weeks BV/TV in the autograft group was 49.3 ± 12% which was significantly higher than in all other groups. Bone formation in the empty defect was 24 ± 7%, with no significant difference from the defect with FDM-PCL scaffold (25.3 ± 10%).

The defects with NSP-PCL scaffolds implanted exhibited a low BV/TV with 5.8 ± 2.4% NSP-PCL scaffold, 3.5 ± 3.1% NSP-PCL + stem cells and 2.9 ± 3.2% NSP-PCL + BMP-2 (Fig. 6).

After 12 weeks, BV/TV in the autograft group was almost the same as in the 8 weeks group at 46.5 ± 16%. This value was still significantly higher than all other groups. The empty defect also kept a steady BV/TV at 23 ± 2%. The defect with FDM-PCL increased to 29.3 ± 6%, not significantly higher than the empty defect.

In the NSP-PCL groups, no increase in BV/TV was found with the scaffold alone, BV/TV 2 ± 1.6%, or seeded with stem cells, BV/TV 3.6 ± 2.7%. The NSP-PCL + BMP-2 group saw an increase in BV/TV to 10.4 ± 10%; significantly higher than NSP-PCL alone.

No significant increase in BV was observed between 8 and 12 weeks (Fig. 6).

**Histomorphometry**

Histomorphometry from 8 weeks showed a significantly higher BV/TV in the autologous bone defect compared to the empty defect (35.9 ± 8.8% vs. 24.7 ± 5.5%). The bone morphology was however the same in both groups, albeit trabecular thickness was larger in the autologous bone.
defects. No residual autograft material was observed after 8 weeks of implantation. The new bone was particularly situated at the edges of the defects in the empty defect and more evenly distributed in the autologous bone defect. Woven bone had started to remodel into lamellar bone.

In the FDM-PCL group, new bone formation was evenly distributed throughout the defect. The PCL fibers were clearly evident throughout the defect, and filled up a great portion of what in the autologous bone and empty defect, respectively, was marrow space.

The FDM-PCL scaffold had a BV/TV of $34.7 \pm 7.8\%$, significantly higher than the empty defect. Contrary to the μCT data, no significant difference in BV/TV was found between the FDM-PCL and the autologous bone defects.

In the NSP-PCL group, a large quantity of scaffold material was present in the defects.

Most of the scaffold was infiltrated by large amounts of TRAP-positive foreign body giant cells (FBGCs) attached to the interfibrous compounds of the scaffold. The sparse bone formation was situated randomly throughout the defect. The trabecular thickness of the bone adjacent to the defect was increased. BV/TVs were $9.6 \pm 3.6\%$ NSP-PCL, $8.6 \pm 5.0\%$ NSP-PCL + stem cells, and $10.5 \pm 3.5\%$ in NSP-PCL + BMP-2 with no significant differences between the groups (Figs. 7 and 8).

In histomorphometry from 12 weeks, bone morphology and quantity in the autologous bone, empty, or FDM-PCL defects were similar to the findings after 8 weeks. However,
more lamellar bone was observed in all three groups, and the distinctive border between existing bone in the adjacent calvaria and new bone had diminished. BV/TV for the autograft group was $33.9 \pm 10.4\%$, for the empty group $24.9 \pm 4.8\%$, and for the FDM-PCL group $32.3 \pm 2.4\%$, respectively.

Again, both the autologous bone and the FDM-PCL defects had significantly higher BV/TV than the empty defect. No significant difference was found between the FDM-PCL and the autologous bone group. In the NSP-PCL groups, a significant difference in BV/TV between the NSP-PCL groups at both time points had significant lower BV/TV compared to the three other groups (****). After 12 weeks, a significantly higher BV/TV was found in the NSP-PCL + BMP-2 compared to the pure NSP-PCL (****).
(4.7 ± 1.6%) and the NSP-PCL + BMP-2 (15.0 ± 3.6%) was found. No significant difference was found between the NSP-PCL + stem cells (9.0 ± 4.7%) and the two other groups (Fig. 7).

**Scaffold degradation**

The degradation of scaffold material was quantified in the histological sections between the two time points. In vivo degradation of the scaffold material was on average 18.6% in the NSP-PCL groups, which caused a significant drop in scaffold volume. In the FDM-PCL group, only 5.4% of the scaffold volume degraded between 8 and 12 weeks with no significant difference between the time points (Fig. 9).

**DISCUSSION**

The scaffolds used in this study have been manufactured using a highly customizable backbone structure, which can be three-dimensionally restructured to enhance osseointegration. This study primarily showed a lack of new bone formation when the NSP technology was applied to a PCL scaffold. It also showed a discrepancy between bone forming potential of the NSP-PCL scaffold between in vitro and in vivo settings. The NSP-PCL scaffold showed promising results from qualitative studies using hMSC-TERT cells in an in vitro environment, but almost no bone ingrowth was evident after 8 or 12 weeks in a large in vivo bone defect. The FDM-PCL scaffolds, however, did show fine osseointegration, and better bone healing potential than the negative control, though not as pronounced as the positive autograft control.

The scaffold made purely of FDM-PCL showed a moderate osteoconductive effect after 8 weeks compared to the negative control. After 12 weeks, no significant difference in BV/TV between autograft and FDM-PCL existed, suggesting excellent osseointegration and biocompatibility on par with comparable PCL scaffolds. The large pore size of this scaffold resulted in bone ingrowth throughout the void.

It was initially suggested by Hulbert et al. in 1970, that pore sizes ranging between 100 and 400 μm was the optimal size for bone tissue ingrowth. It was later shown, that pore size can be more or less dependent on which type of progression toward ossification method the scaffold is aiming for. Small pore sizes (below 100 μm) stimulate chondrogenesis and subsequently ossification, whereas pore sizes above 100 μm stimulate direct ossification. It is known that varying pore sizes can steer mesenchymal stem cells into different directions both regarding migration, proliferation, and differentiation. We hypothesized that by making a highly variable pore size range, the scaffold would mimic the complex architecture of bone matrix, further enhancing the ossification process. The presented qualitative and unreported preliminary quantitative evaluation of hMSC-TERTs migration into the scaffold in vitro demonstrated a homogenous distribution of cells after 14 days. The hMSC-TERTs have previously been used to assess cell migration into similar scaffolds in vitro. In the in vivo study, cell migration toward the center of the NSP-PCL scaffolds was evident, but besides few bone forming cells, large quantities of multinucleated foreign body cells were present suggesting that a phagocytic response was stimulated. If this was the result of hypoxia alone, a trend toward formation of connective tissue and cartilaginous tissue would be expected. The findings however suggest that NSP-PCL elicited a foreign body response. Since scaffold degeneration was less evident in the FDM-PCL group, initiation of this phagocytic inflammatory response could be due to the morphology of the TIPS part of the scaffold. This response was so pronounced that no difference between the BV/TV in the three NSP-PCL groups were evident, despite both BMP-2 and MSCs showing additive effect toward new bone

**FIGURE 9.** Histogram showing the amount of scaffold material (PCL) relative to the total volume (PCL/TV), measured with point count technique on histological samples. Error bar represent positive SD. A relative lower amount of PCL was found when BMP-2 was added to the scaffold compared to the other two NSP-PCL groups at both time points. When mononuclear cells where added to the scaffold a relative smaller amount of PCL was measured compared to the unmodified NSP-PCL at both time points. No significant differences in PCL/TV were found between the groups. An average decrease in PCL/TV of 18.6% was found between 8 and 12 weeks in the NSP-PCL groups, compared to a 5.4% decrease in the FDM-PCL group.
formation on similar types of scaffolds.23–25 These findings suggest that scaffold morphology and ultimately low pore size can inhibit new bone formation and instead cause a sustained inflammatory response. These observations are in agreement with a previous report that describes that a pore size of 0.6 μm hinders cellular ingrowth and is likely to evoke a foreign body response.26 The same report further states that this effect is primarily observed in the FDM-PCL group. BMSCs are known to possess immunomodulatory properties which target a multiplicity of different cell types involved in the immune response.27 The immune response after implantation of NSP-PCL scaffolds may therefore differ between cell-loaded and pure scaffolds. This in vitro observation of FBGCs was of qualitative nature only. However, we did not observe an obvious difference in FBGCs or other signs of inflammation between the cell-loaded and pure NSP-PCL group. In this article, the period of in vitro cell culture before implantation of the cell-loaded scaffold was intentionally kept short to resemble the clinical setting in which time before surgery is constrained (i.e., tumor revision and trauma surgery).28 However, a longer in vitro period than the applied 2 + 3 days could potentially have enhanced cellular penetration into the scaffold before implantation. It remains speculation whether this alternative approach would have been advantageous for the outcome in vivo.

In this article, we chose to stimulate the cells with osteogenic media to augment direct ossification after implantation of the cell-loaded scaffold. The rationale behind this approach was that calvarial bone healing is theoretically mediated via intramembranous ossification rather than endochondral ossification. However, endochondral ossification has also been observed in calvarial bone healing according to Sun et al.28 Hence, prestimulation of the cell-loaded scaffolds with chondrogenic media could be worthwhile to investigate in the setting of calvarial bone healing in future studies.

The degradation of the PCL backbone of the scaffolds is intended to happen gradually over a period of 6 to 12 months. To accomplish this, we used a PCL with molecular weight of 50 kDa, which ensures a favorable degradation profile without compromising mechanical properties during bone ingrowth and remodeling.29 It is widely accepted that the primary degradation of PCL is through degradation and erosion involving hydrolytic cleavage of the polymer backbone only at its surface.30–32 A relatively high degradation (20%) of the scaffold volume was found between 8 and 12 weeks; a higher degradation rate than in similar studies.33 This could be due to the large surface area and therefore surface degradation through hydrolytic cleavage. Furthermore, it is known that macrophages take active part in the degradation of especially particulate PCL through phagocytosis.34 This could be the connection between the high degradation rate of the delicate NSP structure and the cellular response toward inflammation instead of bone formation. The relatively higher degradation of PCL in both the group with added BMP-2 and stem cells after 8 weeks suggests that a cellular response could be the cause of enhanced degradation. Theoretically, stem cells representing a larger potential phagocytic cell pool as well as the well-known potential of BMP-2 to recruit osteoclast-like cells35–37 could both explain the observed acceleration of scaffold degradation.

The pig was the animal of choice for this experiment owing to its bone regeneration rate (1.2–1.5 μm/day) being comparable to that of humans (1.0–1.5 μm/day).38 The calvarium represents a large surface, easily accessible and with trabecular bone with at thickness suitable for defects. However, drawbacks include a lack of mechanical load in the test period, and a high standard deviation within the empty-defect group suggests variability between bone healing potential depending on position of defect in the calvaria. The high standard deviation is consistent with reports by Schlegel et al.38 in this model. The dependency of bone healing capacity upon positioning of the bone defect within the same animal is currently under investigation at our laboratory.

Scaffolds created by FDM have a large potential in individualized tissue engineering. Scaffolds can be created from a 3D rendering of a CT or MRI scan to accommodate a particular bone void. However, creating bioactive constructs is a multidisciplinary challenge. Results found in the lab need to be translated into more clinical relevant studies before clinical application can be considered. The discrepancies between in vitro and in vivo results in this study emphasize the importance of animal studies in translational medicine.

CONCLUSIONS

The new NSP-PCL scaffold developed to mimic the complex multisized porous structure of bone was tested in vitro and in vivo. Osteogenic potential and high cell viability were observed in vitro, but were not found after translation into a large animal model. Instead, acceleration of scaffold degradation, probably due to infiltration of FBGCs was observed. Neither functionalization of the NSP-PCL scaffold with autologous MSCs nor BMP-2 stimulated bone formation. The unmodified FDM-PCL scaffold showed good osteoconductivity and osseointegration after both 8 and 12 weeks emphasizing this scaffold as a base for tests of other modifications in the future. This study underlines the importance of translation of new concepts into animal models before implementation into clinical trials.

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