Effects of scaffold architecture on cranial bone healing

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Abstract

Scaffolds for bone tissue engineering should be designed to optimize cell migration, enhance new bone formation and give mechanical support. In the present study, we used polycaprolactone-tricalciumphosphate (PCL/TCP) scaffolds with two different fibre lay down patterns which were coated with hydroxyapatite and gelatine as an approach for optimizing bone regeneration in a critical sized calvarial defect. After 12 weeks bone regeneration was quantified using microCT analysis, biomechanical testing and histological evaluation. Notably, the experimental groups containing coated scaffolds showed lower bone formation and lower biomechanical properties within the defect compared to the uncoated scaffolds. Surprisingly, the different lay down pattern of the fibres resulted in different bone formation and biomechanical properties; namely 0/60/120° scaffolds revealed lower bone formation and biomechanical properties compared to the 0/90° scaffolds in all the experimental groups. The different architecture of the scaffold fibres may have an effect on nutrition supply as well as the attachment of the newly formed matrix to the scaffold. Therefore, future bone regeneration strategies utilising scaffolds should consider scaffold architecture as an important factor during the scaffold optimisation stages in order to move closer to a clinical application.

1. Introduction

In general, bone is a dynamic and multifunctional organ, capable of good healing and remodelling capacities. However, in certain cases, surgical therapeutic intervention is required due to a limited intrinsic regeneration potential[1]. Beside the conventional surgical procedures, the concept of tissue engineering has emerged as an important approach to bone regeneration research. There are two major bone tissue engineering approaches to develop novel treatment concepts involving scaffolds: cell-based and cell-free. Scaffolds serve as space holders for cells and allow in-growth of host tissues into the reconstruction site after transplantation. Thus, they provide structures that facilitate the three-dimensional proliferation, differentiation and orientation of cells in order to enable tissue-like growth *in vivo.* Scaffolds facilitate the transfer of loads to surrounding tissues and preferably allow the reconstruction site to be mechanically competent directly after insertion. Scaffolds also provide a space in which tissue development and maturation towards complex multi-cellular systems can occur.

The properties of a material's surface can directly influence single cell behaviour, in the same way the three-dimensional structure plays a critical role in the orchestration of tissue formation *in vivo.* Surface properties and microstructure of a material refers to the material at the nanoscale or microscale level, whereas scaffold architecture defines the structure of the biomaterial in space at a tissue-length scale. Scaffolds not only provide the structural basis for cells to form a three-dimensional tissue-like construct *in vivo*, but they also influence the vascularity.

To improve the mechanical and biochemical properties of the scaffolds, calcium phosphate ceramic particles have been mixed into the polymer phase directly[2, 3].

However, an important aspect which has been neglected in the context of bone tissue engineering is the interfacial properties between the ceramic and matrix phases, and therefore, limited improvements have been seen regarding the mechanical properties of polymer/ceramic composite scaffolds compared to polymeric scaffolds. Furthermore, the masking of the ceramic particles by a very thin polymer layer on the scaffold surface by the so called "skinning effect" may diminish the proliferative and osteoconductive properties offered by some bioactive ceramic particles[4-6].

Hence, to improve the proliferative and osteoconductive properties of polymer/ceramic composite scaffolds, coating the scaffolds with a layer of mineralised apatite deposit is considered an efficient approach. In this context, to improve the mechanical properties of the polymer/ceramic composite scaffolds we have developed silanized polycaprolactone/tricalciumphosphate (PCL/TCP(si)) scaffolds, which have significantly improved mechanical properties compared to standard PCL/TCP scaffolds. Moreover, to improve the osteoconductive properties of the PCL/TCP(si) scaffolds, biomimetic coating were applied. The developed biomimetic apatite coated PCL/TCP(si) showed excellent mechanical properties and promising proliferative and osteoconductive properties *in vitro*[7]. Although, it is well known that the internal pore size and the architecture of the scaffolds influence the capability for bone regeneration, the optimal properties are still much debated[8, 9]. The effect of different lay down patterns has been conducted in terms of mechanical and *in vitro* characterization[10-12]. However, the effect of different lay down pattern on bone regeneration in an *in vivo* model has not yet been tested.

To address these issues, in the present study, we investigated the use of silanized polycaprolactone/tricalciumphosphate (PCL/TCP(si)) scaffolds and carbonated hydroxyapatite coated scaffolds (PCL/TCP(si)-CHA) as an approach for optimizing the bone regenerative capabilities of the respective scaffolds in a critical sized calvarial defect model. Furthermore, in conjunction with the apatite coating, whilst keeping the overall porosities the same, we have analysed the effect of two different lay down patterns of the scaffolds, namely 0-60° and 0-90°, on bone regeneration capabilities.

We hypothesized that the scaffolds would provide a suitable substrate for cell proliferation and osteogenic differentiation *in vivo.*

2. Material and Methods

2.1. Materials

Poly(*ε*-caprolactone) (PCL) (M_n: 80,000), 3-glycidoxypropyl trimethoxysilane (GPTMS), acetic acid (CH_3COOH), calcium chloride (CaCl2), potassium hydrophosphate (K_2HPO_4), phosphoric acid (H_3PO_4, 85% solution in water), sodium carbonate (Na_2CO_3) and sodium hydroxide (NaOH) were purchased from Sigma-Aldrich, Singapore. Tri-calcium phosphate (TCP) was purchased from Progentix, The Netherlands.

2.2. Scaffold fabrication

PCL/TCP(si) composite was synthesized as previously reported[7]. In brief, surface activation of TCP was achieved using phosphoric acid at room temperature for 2 hr. The surface activated TCP was then washed with distilled water, and GPTMS (4 wt% of TCP) was added into TCP solution and refluxed at 75 ºC for 24 hr. The GPTMS modified TCP (TCP(si)) was collected by filtration and the washed TCP(Si) was incorporated into PCL solution through homogenization. The homogenized composite was dried and finally annealed to give PCL/TCP(si) composite. PCL/TCP(si) scaffolds were fabricated using an in house screw extrusion system (SES) with screw rotational speed of 25 rpm and nozzle diameter of 0.3 mm at processing temperature of 85 ºC. The scaffolds were fabricated with a size of 5 mm in diameter and 2 mm in thickness and two different laydown patterns, namely 0º/90º and 0º/60º/120º (Figure 1 A/B). The final composition for the scaffold fabrication was PCL (80%wt) and TCP (20%wt). Scaffolds used in this study showed similar characteristics with a porosity of 67-71%, a pore size of 420-500μm, and a scaffold surface area of 65-73mm^2.

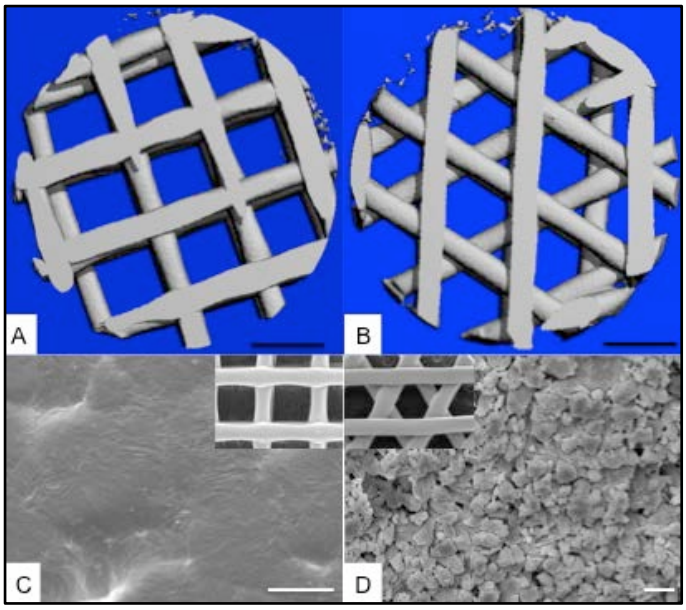

Fig. 1: 3D microCT reconstruction of the scaffolds showing the two different laydown patterns of the fibers (A/B, bar=1mm). SEM images showing the unmodified surface of the scaffold fibers (C, bar=10μm) and the hydroxyapatite coated surface of the scaffold fibres (D, bar=1μm).

2.3. Surface coating on scaffolds

The fabricated PCL/TCP scaffolds were first treated in 10 ml of 5 M NaOH at room temperature for 12 h, followed by thorough washing with deionised water to remove residual NaOH. The NaOH-treated scaffolds were then dipped alternately into calcium chloride solution and potassium hydrophosphate solution to obtain a $CaHPO_4$ coating as a nucleation site for the next CHA coating[13]. In brief, the NaOH-treated scaffolds were dipped in 20 ml of 0.2 M aqueous $CaCl_2$ solution for 10 min and then dipped in deionised water for 5 s, followed by air drying for 3 min. The sample was subsequently dipped in 20 ml of 0.2 M aqueous K_2HPO_4 solution for 10 min and then dipped in deionised water for 5 s, followed by air drying for 3 min. The whole process was repeated three times. The $CaHPO_4$-coated scaffolds were immersed in 20 ml of 0.1 M CH_3COOH and then 10 ml of 0.1 M $CaCl_2$ and 6 ml of 0.1 M H_3PO_4 (Ca/P = 1.66) were dropped slowly through separate syringe pumps under stirring. The pumps were adjusted to keep the ratio of Ca/P at 1.66. After further stirring for 30 min, 18 ml of 0.1 M Na_2CO_3 with the molar ratio of CO_3/PO_4 = 3 was gradually added. The mixture was stirred for a further 30 min and then the pH of the mixture was adjusted to 9 using 1 M NaOH. The CHA-coated PCL/TCP

scaffolds (PCL/TCP–CHA) were collected after ageing the solution for 3 h. Finally, the scaffolds were thoroughly washed with deionised water and freeze dried (Fig. 1 C/D).

2.4. Animal Surgery

Fifteen skeletally matured male Lewis rats were obtained from Animal Resources Centre, Canning Vale, WA. The rats were housed at the QUT Medical Engineering Research Facility (MERF) at the Prince Charles Hospital, Chermside. The animals received water and pelleted ration *ad libitum* throughout the experiment. The animal ethics committee of the University of Queensland approved all experiments. The rats were subjected to critical sized bone defect creation in their skull and implantation of the PCL/TCP(si) scaffolds. For each treatment group including the positive control group, two different laydown patterns of the scaffolds were used. Accordingly, the rats were assigned to five groups of six, as follows:

Experimental Groups	**Pattern: 0/60°/120°**	**Pattern: 0/90°**
PCL/TCP(si) (positive control group)	n = 6	n = 6
PCL/TCP(si)-CHA	n = 6	n = 6
Empty control group (n=6)	--	--

Table 1: The five experimental groups of the study. Every group consists of 6 animals.

2.5. Surgical procedure

All rats were operated under general anaesthesia. Buprenorphine (0.01 - 0.05 mg/kg subcutaneously) were used pre-operatively for pre-emptive analgesia and post-operatively every 6-12 hour as painkiller. General anaesthesia was provided using mixture of Ketamine and Xylazine (75-100 mg/kg Ketamine + 5-10 mg/kg Xylazine intraperitoneal in same syringe). Rats were handled briefly by hand for intraperitoneal injection of anaesthetic and then released into a separate cage until ready for surgery. The frontoparietal region was prepared by clipping hair with a delicate clipper and vigorous disinfection was achieved by application of chlorhexidine in alcohol solution. One dose of broad-spectrum antibiotic was given to

the rats before surgery as prophylaxis. In order to produce critical size bone defects, a sagittal incision of approximately 20 mm was performed over the scalp of the animal. A full-thickness bone defect (5 mm in diameter) was then trephined in the centre of each parietal bone (two defects per calvaria) using a slow speed dental drill with irrigation to prevent heat damage of the host bone. Caution was taken, when drilling down the bone, not to damage the underlying exposed dura mater (Figure 2). A strip 2-mm wide of the marginal periosteal surrounding the defect was then removed. According to the implantation plan, both bone defects in each rat were implanted with one of the treatment modalities described above. During anaesthesia, surgery and immediate post-operative period, the rats were kept warm on a heating pad and after that, they were transferred to a clean warmed cage for recovery.

Animals were kept for 12 weeks after surgery and then sacrificed by CO_2 inhalation.

To collect the implants, the skin was dissected and the entire skull containing the defects/scaffolds were removed for further analysis.

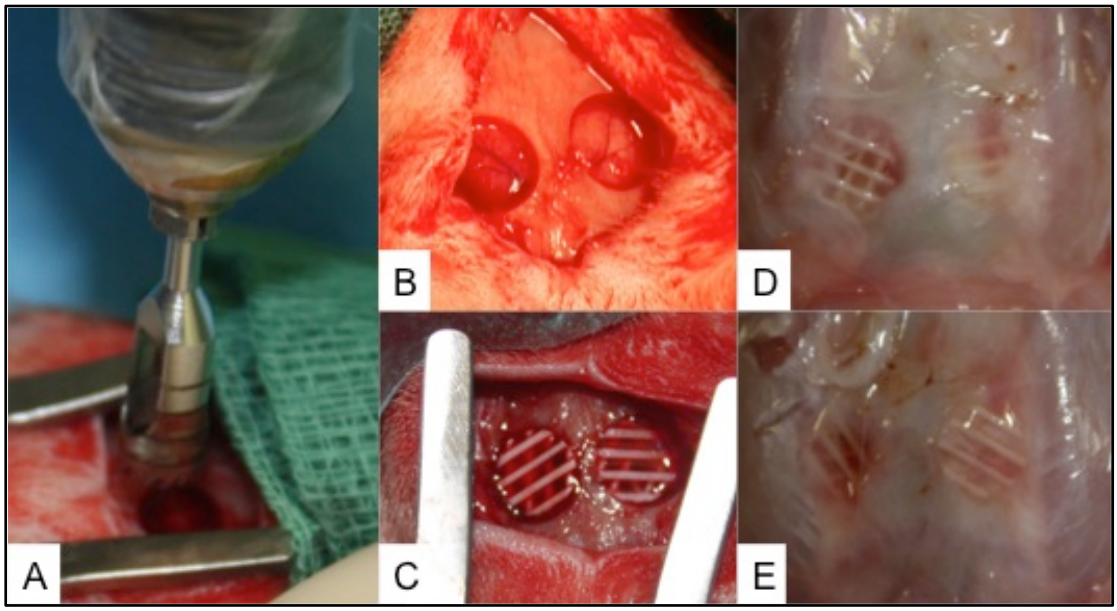

Fig. 2: The scull defects were drilled with a 5mm dental drill (A), the bone and the surrounding periosteum were carefully removed without damaging the dura (B) and the scaffolds were press fitted with good contact between the host bone and the scaffold (C). This was evident from the explanted specimens which showed excellent scaffold integration into the host bone with no fibrous capsule evident at 12 weeks in either the 0/90° laydown pattern group (D) and the 0/60/120° laydown pattern group (E).

2.6. Microcomputed tomography (MicroCT)

Twelve weeks after surgery mineralization within the constructs was quantified using a Micro-CT 40 scanner (Scanco Medical, Brüttisellen, Switzerland). Samples were scanned at an energy of 55 kVp and intensity of 145 μA with 226 ms integration time, resulting in an isotropic voxel size of 36 μm. From the scanned volume, a cylindrical region of interest (ROI), corresponding to the defect size of 5 mm diameter and at the location of the original defect was selected for analysis. After segmentation of the mineralised tissue with a threshold of 220 (equivalent to 312 mgHA/ccm), a Gauss filter width of 0.8 and filter support of 1.0, the mineralized matrix volume, was quantified throughout the entire construct and presented as bone volume in mm^3.

2.7. Mechanical testing

After microCT analysis, the samples were wrapped in wet gauze and stored at -20 C° until further analysis. Upon thawing, the rat skulls were potted into petri dishes with polymethylmethacrylate bone cement (Meliodent Rapid Repair, Heraeus Kulzer) to enable stable fixation for the mechanical testing. Non-destructive micro-compression on the calvaria defects was performed using a Micro Tester 5848 (Instron) with a 10-N load cell. An indenter probe was micro-fabricated for the test. Micro-compressions of up to 50% strain were conducted at an average of eight different locations on each defect site, and the load-displacement and stiffness (compression modulus) were determined. The probe locations were identified to be the pore spaces (between the scaffold struts) of the constructs to measure the modulus of regenerated tissue rather than scaffold material. Intact calvarial bone, soft tissue and the struts of the scaffolds were used as controls. Push-out tests were then conducted to evaluate the functional mechanical integration of the tissue-engineered constructs into the host calvaria, and were performed on the Micro

Tester 5848 (Instron) with a 1-kN load cell[14,15]. An indenter probe of 4.5 mm diameter, slightly smaller than the scaffold diameter of 5 mm, was fabricated for the test. Four to six specimens were used for each group.

2.8. Histology/Immunhistochemistry

For processing decalcified samples into paraffin, parietal bone was fixed in 10% neutral buffered formalin for 24 h and decalcified in 15% EDTA for 3 weeks at 4 °C. The samples were then serially dehydrated in ethanol in a tissue processor (Excelsior ES, Thermo Scientific, Franklin, MA, USA), and embedding in paraffin. Sections (5 μm) were taken using a microtome (Leica RM 2265). The slides were then deparaffinized with xylene and rehydrated with serial concentrations of ethanol, before being stained with haematoxylin and eosin (Sigma Aldrich) and mounted with Eukitt mountant (Fluka Biochemica, Milwaukee, WI, USA).

For immunohistochemistry, sections were deparaffinised with xylene and rehydrated with serial concentrations of ethanol. Subsequently, sections were rinsed in distilled water and placed in 0.2 M Tris-HCl buffer (pH 7.4). Endogenous peroxidase activity was blocked by incubating the sections in 3% H_2O_2 in Tris-HCl for 20 min. This was followed by three washes with Tris buffer (pH 7.4) for 2 min each. Sections were incubated with Proteinase K (DAKO, Botany, Australia) for 20 min and subsequently incubated with 2% bovine serum albumin (BSA) (Sigma, Sydney, Australia) in DAKO antibody diluent (DAKO) in a humidified chamber at room temperature for 20 min to block nonspecific binding sites. Afterwards, immunohistochemical staining was performed using a primary mouse antibody specific to the osteogenic marker type I collagen (provided by Larry Fischer, National Institute of Health, Bethesda, USA). Non-immunized rabbit IgG (DAKO) was used as an isotype control to rule out non-specific reactions of rabbit IgG with rat tissues as well as non-specific binding of the secondary antibodies and/or peroxidase labelled polymer to rat tissues. The sections were incubated with the specific antibody or negative control in humidified chambers at 4°C over night. Sections were then washed three times for 2 min with Tris buffer (pH 7.4) and incubated with peroxidase labelled dextran polymer conjugated to goat anti-mouse and anti-rabbit immunoglobulins (DAKO EnVision+ Dual Link System Peroxidase, DAKO) at room temperature in humidified chambers for 60 min. Colour was developed using a liquid 3,3-diaminobenzidine (DAB) based system (DAKO).

Kaiser's glycerol gelatin (DAKO) were used for coverslip mounting.

2.9. Statistics

Statistical analysis was performed for all the quantitative results using Student's t-test for comparing means from two independent sample groups. A confidence level of 95% was used, statistical significance was set at $p < 0.05$.

3. Results

After 12 weeks, all groups were euthanized and the heads were removed for further evaluations. No wound infection or other complications occurred during the course of the experiment.

3.2. MicroCT

The bone regeneration capacity of scaffolds with different surface modifications and different laydown patterns were assessed *in vivo* after transplantation in a rat calvarial defect. Transplants were recovered after 12 weeks, subjected to micro-CT analysis and then processed for mechanical testing. Micro-CT analysis revealed a significantly higher degree of newly formed bone matrix in all experimental groups compared to the negative control group (empty defect) (Fig. 3). The empty defects remained primarily devoid of any mineralized tissue throughout the study, demonstrating they were of critical size (non-healing within the length of the study). The CHA-coated scaffolds with a 0/90° laydown pattern showed the highest amount of bone formation. Furthermore, all experimental groups treated with mPCL/TCP-Scaffolds with a 0/90° laydown pattern of the fibres showed a tendency towards more bone formation compared to the mPCL/TCP-Scaffolds with a 0/60° configuration of the fibres. However, no significant difference could be found with regard to the mineral density of newly formed bone matrix (Fig. 3).

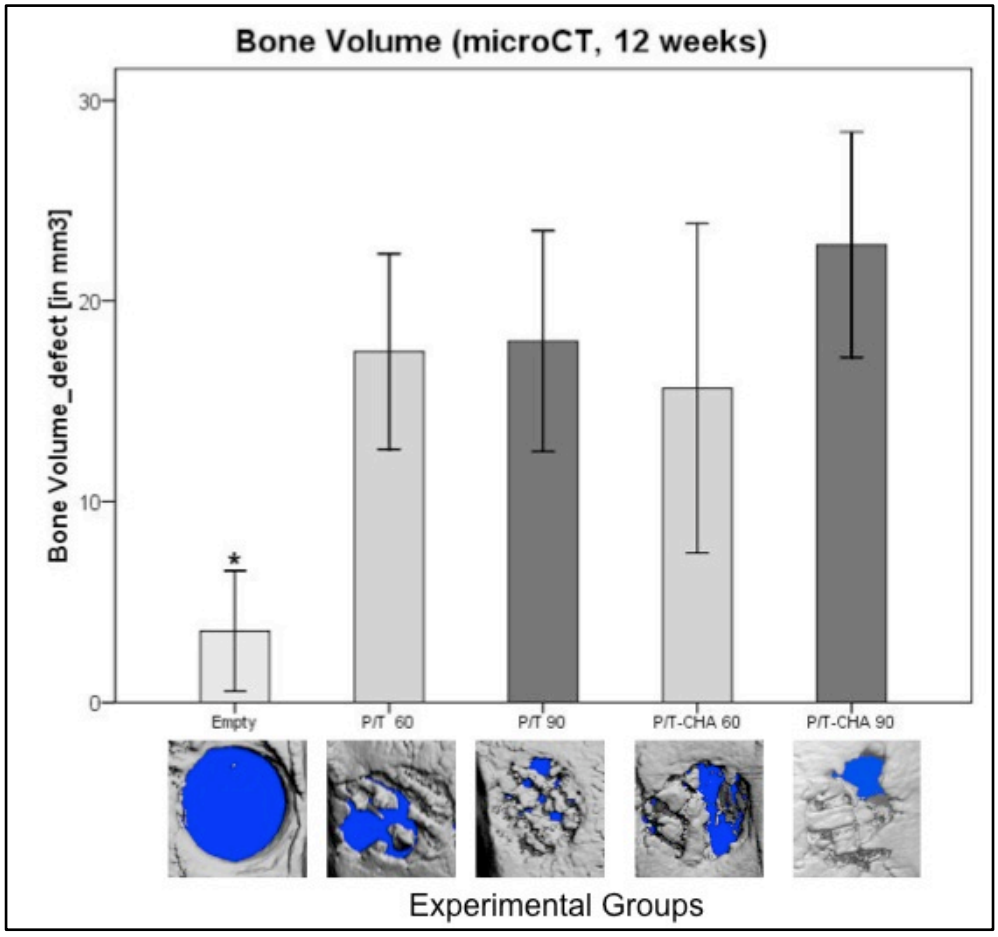

Fig 3.: MicroCT results of bone regeneration for all experimental groups. The bottom row shows representative 3D-reconstruction from the bone defects. All the experimental groups showed significantly more newly formed bone matrix within the defect compared to the empty groups (negative control). No significant differences could be detected between the experimental groups, whereas the mPCL/TCP-CHA-0/90° Scaffold-group showed the most bone formation of all groups. Furthermore, a tendency towards more bone formation in all the groups using the 0/90° pattern could be detected. Significant values are represented as *P < 0.05.

3.3. Mechanical testing

To test the mechanical integrity of the newly formed tissue within the scaffold pore space, micro-compression was performed on harvested calvaria at 8 different locations within the pore spaces of each scaffold (Figure 4A).

The tissue stiffness of the mPCL/TCP-(si)-0/90° and the mPCL/TCP-(si)-CHA-0/90° showed the highest stiffness of all experimental groups. Interestingly, all defects treated with mPCL/TCP-Scaffolds with 0/90° laydown pattern of the fibres showed a tendency towards higher stiffness compared to the mPCL/TCP-0/60°-Scaffolds.

Push-out tests were performed to evaluate the mechanical integration of the constructs within the host calvaria, an important consideration from a craniofacial treatment point of view (Figure 4B). After 12 weeks, the defects treated with mPCL/TCP-(si)-0/90° Scaffolds, showed a comparable push-out strength to that of host bone tissue, suggesting new bone was forming from the periphery of the defect edges and encouraging integration of the implant within the defect site. All the other experimental groups showed significantly lower values for the push-out tests compared to the host bone. Within the experimental groups, we confirmed our observations from the previous analyses, that the mPCL-TCP-Scaffolds with the 0/90° configuration of the fibres performed better, evidenced by higher push-out strengths, compared to the 0/60°-laydown pattern scaffolds.

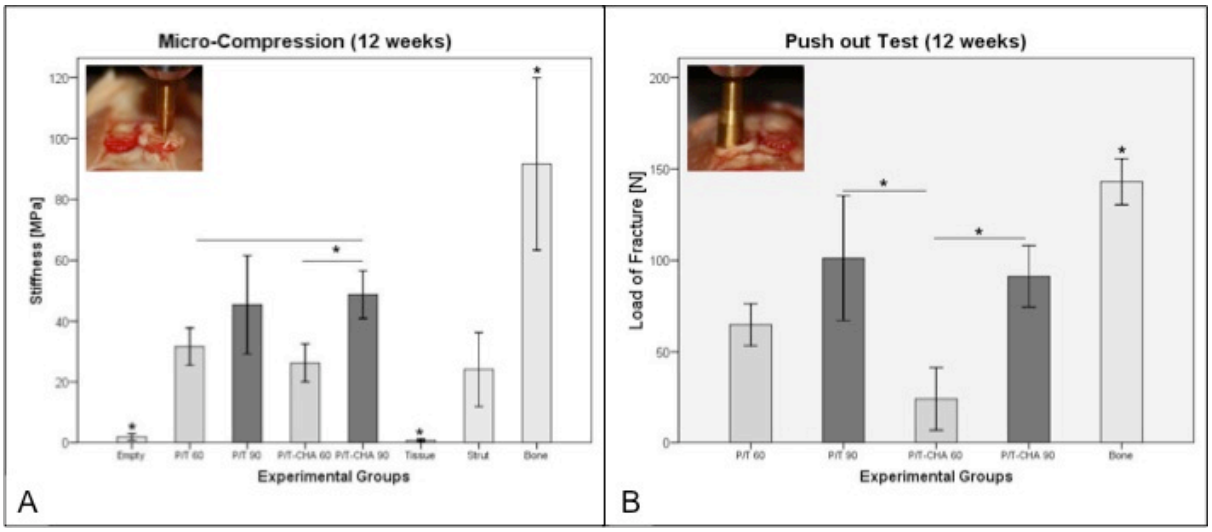

Fig. 4: Micro-compression tests were performed after 12 weeks (A). Stiffness of the samples within the defect is reported here. Regenerated tissue within the mPCL/TCP-(si)-0/90°-Scaffold group and the mPCL/TCP-(si)-CHA-0/90°-Scaffold group showed superior stiffness for all experimental groups. Significant values are represented as *P < 0.05. Push-out tests were performed after 12 weeks (B). Load of fracture of all samples are reported here. Regenerated tissue within the mPCL/TCP(si)-0/90°-Scaffold group and the mPCL/TCP-(si)-CHA-0/90°-Scaffold group showed the highest push-out strength of all experimental groups. Significant values are represented as *P < 0.05.

3.4. Histology

Histological examination of decalcified samples was performed after 12 weeks. Representative H&E staining demonstrated clear defect bridging from the host bone with a good bonding of the scaffold in all experimental groups (Figure. 5). New bone formation is observed in all experimental groups adjacent to the host bone.

Notably, the mPCL-TCP-Scaffolds with the 0/90° configuration of the fibres revealed new bone formation adjacent to the implant struts (left as empty lacunae due to the dissolution of the PCL implant by xylene during processing) which penetrated to the middle of the defect, whereas the scaffolds with the 0/60° configuration of the fibres predominantly showed new bone formation closer to the host bone with less in the centre of the scaffold.

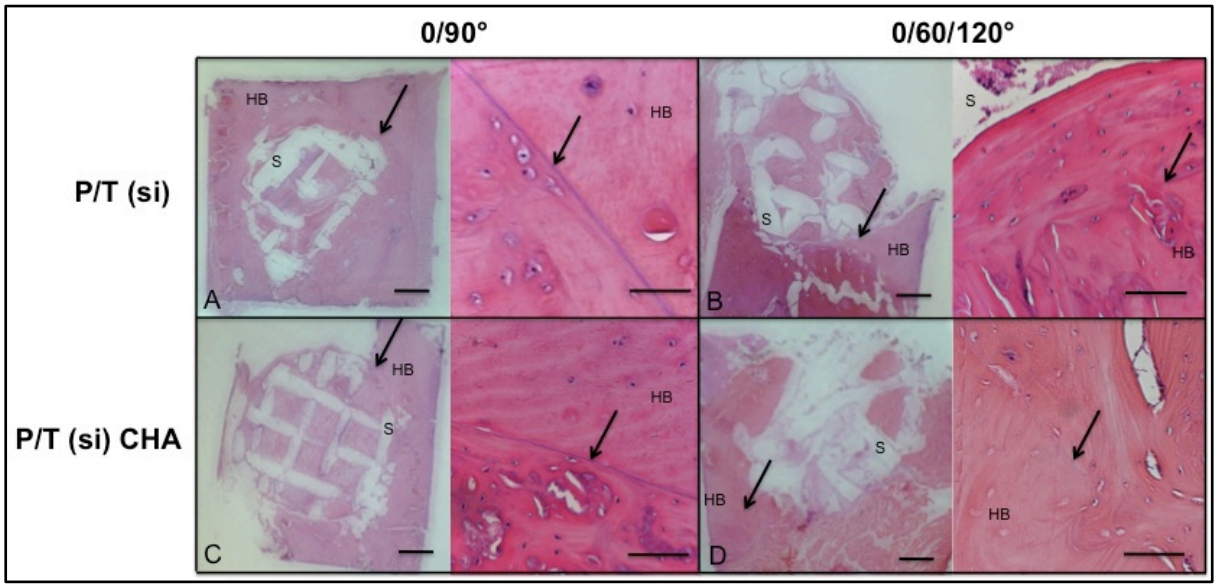

Fig. 5: The overview of the defect treated with different scaffolds showed in the H&E staining a good bonding of the newly formed tissue to the host bone (HB)(bar in the overview=1mm). Arrows indicate the edges of host bone and the area of the higher magnification (bar=50μm). The higher magnifications demonstrated the ingrowth of new bone matrix from the host bone. The solvents used during the preparation of the histological sections resulted in the mPCL–TCP scaffold material being dissolved away during the embedding. Hence mPCL–TCP struts (S) are represented in histological sections as empty lacunas of similar geometry.

New bone formation should be accompanied by the expression of osteogenic marker proteins, amongst these are type I collagen (Col-I), and osteocalcin (OC). Furthermore, markers of vascularity such as Von Willebrand factor (vWF) may be used to ascertain the vascularity at the defect site which is important to bring in nutrients and progenitor cells to the healing defect tissue. Figure 6 (A,B) shows entire defect/scaffold regions for both the 0/60/120° and 0/90° laydown patterns respectively with the magnified region probed for Col-I, OC and vWF (Figure 6 E-J). As a non-specific marker of osteoblastic differentiation during mineralisation, type I

collagen showed a greater intensity in the scaffold groups with the 0/90° laydown pattern compared to the 0/60/120° laydown pattern scaffolds, as shown in Figure 6 E-H. Clear vascularity can also be detected in both groups (Figure 6 I,J).

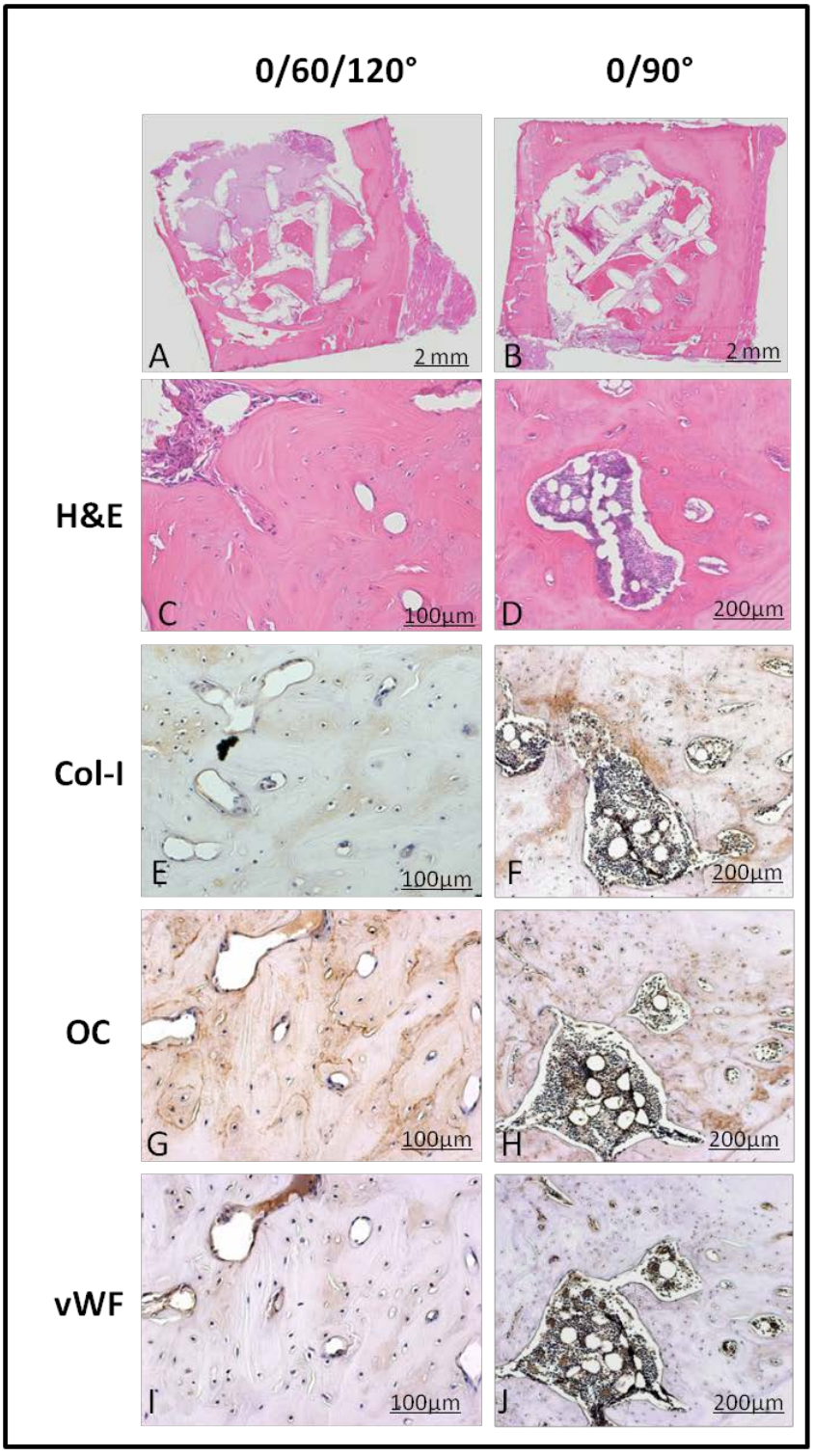

Fig. 6. Representative H&E staining of scaffolds with 0/60/120° and 0/90° configuration of the fibres (A-D). Col-I and OC staining is more prevalent in the groups with the 0/90° laydown pattern of the struts compared to the 0/60/120° scaffolds groups (E-H). All scaffold groups demonstrated vascularisation as evidenced by vWF staining (I,J).

4. Discussion

A wide number of different biomaterials have been developed for cranial defects and are subject to intense current research efforts. There are two major engineering approaches to developing novel treatment concepts involving biomaterials: cell-based and cell-free. In both cases, materials are engineered to provide optimal function for specific applications. In other words, scaffolds for cell-based therapies are intended to provide a compatible carrier for viable cells for enhanced histogenesis, function and integration within the recipient's tissue bed. Scaffolds for cell-free repair are designed to stimulate neo-histogenesis, often by mimicking signals for anabolic processes. In the past, different scaffolds have been shown to be useful supports for the reconstruction of bone, however only a handful have brought fully satisfactory clinical results[16].

The philosophy of biomimetics has provided a new direction for biomaterial design. There are several different methods to influence and optimise the properties of the scaffold for bone tissue engineering such as different materials, different surface coatings, the pore size as well as the pore geometry. To improve integration of the scaffolds within the host site, they need to be resorbable and should have a porosity or favourable texture to enable good bonding with the surrounding tissue. Coatings and addition of bioactive molecules can also improve scaffold integration. These principles have been applied with varying success to musculoskeletal repair and reconstruction, but opportunities exist for more novel procedures[17,18].

The philosophy of biomimetics has provided a new direction for biomaterial design. In this study additive manufactured PCL/TCP(si) scaffolds coated with apatite were evaluated *in vivo*. The surface coated scaffolds demonstrated excellent results in previous *in vitro* studies[7] but did not show the same trend *in vivo* compared to the uncoated scaffolds. Moreover, the CHA-gelatine coated scaffolds showed the least impressive mechanical properties and bone volume formation of all experimental groups. Though *in vitro* we have found significantly better results for the coated compared to the non-coated PCL/TCP(si) scaffolds, this did not translate to the *in vivo* situation. The surface coating of these scaffolds support the osteogenic differentiation of cells during *in vitro* conditions, but these effects were not sustained in an *in vivo* model. Clearly there are many different factors that influence bone

regeneration *in vivo* and *in vitro*, and the complex interplay occurring *in vivo* is difficult to replicate within an *in vitro* research setting. In this study the differences between the surface coated groups and the uncoated scaffolds were more distinctive in the mechanical testing especially for the CHA and gelatine coated scaffolds. The surface modification with CHA and gelatine might lead to an appropriated bone formation within the pores, but the newly formed matrix may, in fact, be connected to the gelatine coating and not to the scaffold itself. This might explain the major differences detected using mechanical tests. The difference in findings between *in vitro* and *in vivo* studies once again reminds us that, unfortunately, *in vitro* studies in isolation are not sufficient to predict the clinical outcome[17].

Beside the surface modification, the scaffolds had different configuration of their polycaprolactone fibres (0/90° and 0/60/120°). To our knowledge, no other group has reported differences in bone regeneration, with relation to the architecture of the struts in an *in vivo* model. In our study, the experimental groups with the 0/90° fibres alignment showed significantly higher bone regeneration potential compared to the 0/60°-scaffold group, with respect to the newly formed bone matrix, the stiffness of the bone matrix and the maximum load of fracture. In the 0/90°-scaffold group, a better incorporation of cells from the host, as well as a better supply of nutrients might explain better bone regeneration. The different architecture of the struts also influenced the surface volume as well as the permeability of the scaffolds. Scaffolds with the 0/90° fibre alignment showed a significant higher porosity, a larger pore size, and a lower scaffold surface compared to the scaffolds with a 0/60° fibre alignment.
Studies have demonstrated that pore size and porosity have an effect on bone tissue regeneration, with scaffold pore sizes required for bone regeneration ranging from 100 to 500μm to allow micro vessel formation and a sufficient flow of nutrients to the interior of the scaffold[9, 19].
Roosa *et al.* showed in an *in vivo* model, that the pore size of polycaprolactone scaffolds had limited influence on bone regeneration[8]. Three different scaffolds with unique architecture and pore sizes of 350, 500, and 800μm were seeded with bone morphogenetic protein-7-transduced human gingival fibroblasts and implanted subcutaneously in immuno-compromised mice. They concluded the pore size played

a limited role in bone regeneration. Similar results were shown by Fisher *et al*.[20], by implantation of scaffolds with different porosities and pore sizes subcutaneously and in cranial defects in rabbits, they also detected no statistical difference with respect to bone regeneration or inflammatory responses.

In our current study all groups comprising scaffolds with a 0º/90° configuration of the fibres showed a tendency towards more bone formation, higher stiffness and higher push-out forces compared to the scaffolds with a 0º/60° configuration of the fibres. These results can therefore not only be explained by the differences in porosity, pore size and surface area. The architecture of the struts must also play an important role for new bone formation and integration of the newly formed tissue within the host bone, which also correlated with elevated mechanical properties.

In conclusion, we have demonstrated that a surface modification of PCL-TCP scaffolds, which has shown increased osteogenic potential of scaffolds *in vitro*, did not translate into the *in vivo* situation using a rat cranial model. It showed minor regenerative potential regarding new bone formation as well as mechanical properties compared to uncoated scaffolds. In addition to studying the surface modification, we evaluated different architecture of the scaffold fibres with respect to their bone regeneration potential. From this perspective we can conclude, that a 0/90°-alignment of the scaffold fibres demonstrated a significantly improved regeneration of bone tissue, meaning more bone formation with better mechanical properties in all experimental groups. In future it remains a challenge to optimise the many scaffold properties, which effect bone regeneration to produce an appropriate scaffold for bone tissue engineering applications,but considerations of scaffold architecture are clearly an important consideration.

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5. References

1. Perry CR. Bone repair techniques, bone graft, and bone graft substitutes. Clin Orthop Relat Res. 1999; (360): 71-86.
2. Kretlow JD, Mikos AG. Review: mineralization of synthetic polymer scaffolds for bone tissue engineering. Tissue Eng. 2007; **13**(5): 927-38.
3. Wang F, Shor L, Darling A, Khalil S, Sun W, Guceri S, et al. Precision extruding deposition and characterization of cellular poly-epsilon-caprolactone tissue scaffolds. Rapid Prototyping Journal. 2004; **10**(1): 42-9.
4. Hutmacher DW, Schantz JT, Lam CX, Tan KC, Lim TC. State of the art and future directions of scaffold-based bone engineering from a biomaterials perspective. J Tissue Eng Regen Med. 2007; **1**(4): 245-60.
5. Rezwan K, Chen QZ, Blaker JJ, Boccaccini AR. Biodegradable and bioactive porous polymer/inorganic composite scaffolds for bone tissue engineering. Biomaterials. 2006; **27**(18): 3413-31.
6. Gupta D, Venugopal J, Mitra S, Giri Dev VR, Ramakrishna S. Nanostructured biocomposite substrates by electrospinning and electrospraying for the mineralization of osteoblasts. Biomaterials. 2009; **30**(11): 2085-94.
7. Arafat MT, Lam CX, Ekaputra AK, Wong SY, Li X, Gibson I. Biomimetic composite coating on rapid prototyped scaffolds for bone tissue engineering. Acta Biomater. **7**(2): 809-20.
8. Roosa SM, Kemppainen JM, Moffitt EN, Krebsbach PH, Hollister SJ. The pore size of polycaprolactone scaffolds has limited influence on bone regeneration in an in vivo model. J Biomed Mater Res A. **92**(1): 359-68.
9. Karageorgiou V, Kaplan D. Porosity of 3D biomaterial scaffolds and osteogenesis. Biomaterials. 2005; **26**(27): 5474-91.
10. Hoque ME, San WY, Wei F, Li S, Huang MH, Vert M, et al. Processing of polycaprolactone and polycaprolactone-based copolymers into 3D scaffolds, and their cellular responses. Tissue Eng Part A. 2009; **15**(10): 3013-24.
11. Moroni L, de Wijn JR, van Blitterswijk CA. Three-dimensional fiber-deposited PEOT/PBT copolymer scaffolds for tissue engineering: influence of porosity, molecular network mesh size, and swelling in aqueous media on dynamic mechanical properties. J Biomed Mater Res A. 2005; **75**(4): 957-65.
12. Moroni L, de Wijn JR, van Blitterswijk CA. 3D fiber-deposited scaffolds for tissue engineering: influence of pores geometry and architecture on dynamic mechanical properties. Biomaterials. 2006; **27**(7): 974-85.
13. Oyane A, Uchida M, Yokoyama Y, Choong C, Triffitt J, Ito A. Simple surface modification of poly(epsilon-caprolactone) to induce its apatite-forming ability. J Biomed Mater Res A. 2005; **75**(1): 138-45.
14. Dhert WJ, Klein CP, Wolke JG, van der Velde EA, de Groot K, Rozing PM. A mechanical investigation of fluorapatite, magnesiumwhitlockite, and hydroxylapatite plasma-sprayed coatings in goats. J Biomed Mater Res. 1991; **25**(10): 1183-200.
15. Dhert WJ, Verheyen CC, Braak LH, de Wijn JR, Klein CP, de Groot K, et al. A finite element analysis of the push-out test: influence of test conditions. J Biomed Mater Res. 1992; **26**(1): 119-30.
16. Woodruff MA, Hutmacher DW. The return of a forgotten polymer--Polycaprolactone in the 21st century. Progress in Polymer Science. **35**(10): 1217-56.
17. Rai B, Lin JL, Lim ZX, Guldberg RE, Hutmacher DW, Cool SM. Differences between in vitro viability and differentiation and in vivo bone-forming efficacy of human mesenchymal stem cells cultured on PCL-TCP scaffolds. Biomaterials. **31**(31): 7960-70.
18. Woodruff MA, Narayan Rath S, Susanto E, Nurcombe V, Hutmacher DW, Cool SM. Sustained release and osteogenic potential of heparan sulfate-doped fibrin glue scaffolds within a rat cranial model. J. Mol. Histol. 2007; **38**: 425-433.

19. van Tienen TG, Heijkants RG, Buma P, de Groot JH, Pennings AJ, Veth RP. Tissue ingrowth and degradation of two biodegradable porous polymers with different porosities and pore sizes. Biomaterials. 2002; **23**(8): 1731-8.
20. Fisher JP, Vehof JW, Dean D, van der Waerden JP, Holland TA, Mikos AG, et al. Soft and hard tissue response to photocrosslinked poly(propylene fumarate) scaffolds in a rabbit model. J Biomed Mater Res. 2002; **59**(3): 547-56.