Nano to Macro-Scale Remodeling of Functional Tissue-Engineered Bone

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Keywords: composite scaffold; polycaprolactone; bone material characterization; tissue engineering; additive manufacturing
The drive to develop bone grafts for the regeneration of large gaps in the skeletal structure, whilst circumventing the need to use permanent implants, has led to a major research thrust towards developing biodegradable scaffolds for bone tissue engineering. Forerunners, Langer and Vacanti led the birth of the tissue engineering field with their seminal 1993 Science paper which remains one of the most influential and cited works in the field of regenerative medicine. [1] The application of the principles of biology and engineering towards development of functional grafts for diseased or traumatized tissue has seen laboratories worldwide forging impressive multi-disciplinary teams to focus on restoring, maintaining or improving the function of a wide range of human tissues. [2-6] Unfortunately, the promise of tissue engineering which was so vibrant a decade ago has so far failed to deliver the anticipated results, from a clinical perspective. One reason is the virtual absence of long-term in-vivo studies which are tantamount to robust data collection associated with taking tissue engineering therapies from bench to bedside. [7-9]

The basic concept fundamental to bone tissue engineering is to combine a scaffold with living cells, decellularized extracellular matrix, and/or recombinantly produced growth factors such as bone morphogenetic protein (BMP), platelet derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) to form a “tissue engineering construct” (TEC) which aims to promote the repair and/or regeneration of tissues. [9, 10]

A well engineered scaffold for bone tissue engineering combines inspired design, technical innovation and precise craftsmanship. Original thinking in the field endorsed scaffold degradation to occur as soon new tissue started to form. [6, 8] In contrast, we emphasize the importance of the scaffolds remaining intact as newly formed tissue matures within the porous and fully interconnected scaffold architecture and that the onset of degradation should only occur after the regenerated tissue has remodeled at least once in the
natural remodeling cycle. The scaffold plays a significant role in tissue development by providing a spatial and temporal support for cells to attach and migrate whilst enabling cell differentiation and stimulating production of different ECM components in this microenvironment. The design of these scaffolds also needs to consider physico-chemical properties, morphology and degradation kinetics. External size and shape of the construct are also of importance, particularly if the construct is customized for an individual patient. Most importantly, clinically successful bone constructs should stimulate and support both the onset and the continuance of bone in-growth as well as subsequent remodeling and maturation by providing optimal stiffness and external and internal geometrical shapes. Therefore, scaffolds must provide sufficient initial mechanical strength and stiffness to substitute for the loss of mechanical function of the bone. Hence, mechanical properties in the lower range of cancellous bone are sufficient for low-load-bearing locations such as the skull and mid-face whereas for high-load-bearing bones such as the tibia and femur, additional internal or external fixation is needed.

Continuous cell and tissue remodeling is important for achieving stable biomechanical conditions which are essential for angiogenesis and subsequently vascularization at the host site; a process depicted schematically in Figure 1. Part (a) shows a scanning electron microscopy (SEM) image of a medical grade composite scaffold implanted into a pig cranial defect (b). Over 24 months the scaffold degrades slowly via surface erosion (in phosphate buffered saline solution (c)) with clear erosion of the scaffold struts shown using SEM; this process is depicted schematically in part (d). At the same time, the scaffold becomes gradually vascularised \textit{in vivo} via the migration and proliferation of endothelial progenitor cells through the scaffold-entrapped hematoma. Subsequently, osteoblastic
The degree of tissue turnover and maturation within the scaffold depends on the tissue itself (e.g. muscle 6-8 weeks, bone 12-18 months), and its host anatomy and physiology. In addition to these essentials of mechanics and geometry, a suitable construct will (i) possess a 3D and highly porous interconnected pore network with surface properties which are optimized for the attachment, migration, proliferation and differentiation of cell types of interest (depending on the targeted tissue) and enable flow transport of nutrients and metabolic waste, and (ii) be biocompatible and biodegradable with a controllable rate to compliment cell/tissue growth and maturation. [11]

It is absolutely essential to understand and control the different stages of the scaffold degradation process to achieve not only the initially successful tissue formation but also subsequent remodeling, and maturation at the defect site which occurs later. Initial thinking in the field of tissue engineering advocated that scaffolds should degrade and vanish at the same time as the tissue is growing. [6,8] Yet, tissue in-growth and maturation differs temporally from tissue to tissue and, furthermore, tissue in-growth does not necessarily equate to tissue maturation and remodeling, hence we should not consider a defect filled with immature tissue as being “regenerated”. For this reason, many scaffold-based strategies have failed in the past as the scaffold degradation was more rapid than tissue remodeling and/or maturation. [12,13]

The long-term characteristics of bone growth and remodeling within TECs are not well known. Warnke et al. introduced an innovative bone engineering concept into the clinic. A titanium mesh cage was filled with bone mineral blocks and infiltrated with 7 mg recombinant human bone morphogenetic protein 7 (BMP-7) and 20 mL of the patient's own
bone marrow. The transplant was then implanted into the latissimus dorsi muscle and 7 weeks later transplanted as a free bone-muscle flap to repair the mandibular defect. Postoperatively the patient had an improved degree of mastication and aesthetics, but unfortunately the graft failed from a long-term perspective. \[^9\] This highlights the imperative to study long-term bone regeneration and remodeling to gain optimum insight into the clinical success of such procedures, and this can only be achieved using the most sophisticated techniques. Here we use a suite of advanced analytical techniques to assess the material properties of the tissue-engineered bone generated during long-term \textit{in vivo} studies, proposing that the onset of degradation should only occur after the regenerated tissue within the scaffold has remodeled at least once in the natural remodeling cycle. This paradigm shift is particularly relevant for higher load bearing tissues, such as bone. Original hypotheses promoted scaffold degradation to onset immediately as new tissue starts to form. In contrast, we underline the importance of the scaffold remaining intact as the tissue matures in the scaffold pores with bulk degradation occurring later. We illustrate this process schematically in Figure 1 whereby the scaffold struts slowly degrade over time as the newly formed bone matures within the pores.

In the work described in this paper, we demonstrate that this rationale leads to structural and functional bone regeneration in a large critical-sized defect model. Bone regeneration is influenced by a complex interplay of biochemical and biomechanical processes at the tissue, cellular and molecular levels. \[^{14}\] This integrated hierarchical system cannot be fully understood using qualitative or intuitive approaches only, nor by focusing on single spatial or temporal considerations in isolation. \[^{15}\] Hence, here we report a long-term bone engineering study which supports the new paradigm which leads to superior bone regeneration within slowly degrading composite scaffolds. We detail the post explantation
analysis techniques which enable bone quantity and quality to be assessed on the macro, micro and nano-scale. To our knowledge, this is the first time such detailed examination of long-term regenerated bone has been undertaken using the comprehensive techniques presented here, which include micro-computed tomography (µCT), advanced mineralized hard-tissue resin histology, scanning electron microscopy and small angle x-ray scattering. This provides key insight for the first time into the cellular and extracellular matrix function and organization pertaining to long-term bone remodeling behavior within clinically relevant defect sites which are treated with a clinically proven tissue-engineered bone strategy.

Medical grade polycaprolactone-tricalcium phosphate scaffolds (mPCL-TCP) (4cm x 2 cm x 1 cm, volume of 8 cubic centimeters) were implanted into bilateral porcine critical-sized cranial defects, and 6 scaffolds were seeded intra-operatively with bone marrow stromal cells (BMSCs) and implanted for a duration of 2 years. Scaffolds and the surrounding tissue were explanted after 2 years and it was observed that extensive bone regeneration had occurred within the defect sites containing mPCL-TCP scaffolds both with and without BMSC addition (Figure 2). The scaffolds pores were filled with regenerated mineralized bone with extensive bone remodeling evident around scaffold struts (labeled s) and clear evidence of surface degradation of the composite scaffold (Figure 2d-g). The fully interconnected scaffold architecture was still evident within the defect sites after 2 years of implantation, albeit with a reduction in strut diameter owing to some surface degradation of the composite material and the pores were seen to be completely filled with tissue, mostly identified as mineralized bone.

Micro-computed tomography was performed to determine the bone volume fraction and bone mineral density and three-dimensional (3D) rendered images were generated to
demonstrate the extent of mineralization throughout the entire scaffold (Figure 2b). Histological assessment (Figure 2c-g) using von Kossa staining with Macneal’s tetrachrome counter stain highlights the black mineralized tissue reflecting near complete mineralization within the scaffold pores (Figure 2e,g). It can clearly be seen from histological sections that the scaffold has undergone surface erosion with scaffolds’ struts having decreased in diameter; however, as designed and predicted the overall scaffold morphology remained largely intact. The slow process of surface erosion and degradation of the scaffold struts is also evident along with remodeling taking place within the pores (Figure 2d,e). Goldner’s trichrome staining (Figure 2d,f) confirms bone maturation via positive staining for osteocytes (black arrows) embedded within the mineralized matrix and clear osteoid formation (actively mineralizing bone front) around the scaffold struts (stained red in Figure 2d,f) as well as osteoblasts (labeled ob) encompassing the struts (Figure 2g). There is notable osteoid formation around the scaffold struts demonstrating a tissue remodeling and maturation occurring as the scaffold gradually degrades via surface erosion enabling new bone to progressively replace the mPCL-TCP scaffold itself as it slowly erodes and its by-products are metabolized via the Krebs-cycle without causing any inflammation. We next quantitatively compared scaffolds implanted with and without BMSCs to study the effect of autologous cell implantation on bone regeneration. Scaffold groups without cells demonstrated bone regenerative capabilities, yet the quantity of new bone formed throughout the entire scaffold was significantly lower than those scaffolds loaded with BMSCs as depicted in Figure 3k. Fully mineralized tissue, from histology sections (von Kossa staining) were measured within the defect and values were reported as a percentage of bone volume per tissue volume (% BV/TV). Positive pixel areas were divided by total tissue available for growth (defect area minus the strut area of the scaffold) and revealed significantly higher
(p<0.05) mineralization (75%) for scaffolds implanted with BMSCs compared to scaffold alone (49%).

The µCT and histology trend corresponded for all samples containing adjacent cell and cell-free scaffolds (Figure 3 a-f) with bone regeneration occurring in all cases but statistically more bone was formed from the addition of the BMSC cells (Figure 3k). The calcium content of both the tissue-engineered bone and the native bone in adjoining regions of the skull was also visualized by environmental scanning electron microscopy (ESEM) in backscattered electron (BSE) mode, as shown in Figure 3, indicating that bone had been forming all around and in-between the scaffold struts (Figure 3g-j). The areas corresponding to native calvarial bone show some porosity surrounded by bone material often with a lower mineral content (Figure 3i). This indicated high remodeling activity, since lower mineral content usually means younger bone. Interestingly, the bone material surrounding the struts of the scaffold does not have a lower mineral density (Figure 3h, j). A possible explanation could be that the pores in the native calvaria gradually decrease by new bone formation at the inside of the pore, while tissue-engineered bone starts to grow on the surface of the struts and expands from there. As a consequence, the bone matrix around pore spaces in the skull is the youngest, while around the struts of the scaffold it is the oldest compared to the surroundings.

We utilized small angle x-ray scattering (SAXS) to determine thickness and orientation of the mineral particles in the newly generated bone tissue and compared this with the native, host bone. Areas of interest for SAXS analysis were chosen in the vicinity of pores and struts. Figure 3h-j shows a characteristic difference in the bone ultrastructure between normal and tissue-engineered bone. The mineral particles in native bone show no preferential orientation (as revealed by nearly spherical SAXS patterns, Figure 3i), in agreement with earlier observations that elongated mineral particles are mostly oriented within the plane of
the calvaria, but with no preferential alignment within this plane. In contrast to this, mineral particles are preferentially orientated along the circumference of the scaffold struts in tissue-engineered bone, as shown by the eccentricity of the SAXS pattern (Figure 3h, j). This is also very different from the earliest mineralized tissue found in the callus during fracture healing, which is much less organized (woven) with nearly isotropic fibril and mineral orientation. Indeed, it has been found in scaffold-free fracture healing that lamellar bone is deposited only once this disordered substrate is generated, which acts as an “endogenous scaffold”. Similar observations have also been made in deer antler, a bone which re-grows every year. Most interestingly, no traces of primary woven bone are found in the present study next to the scaffold struts, which seems to indicate that lamellar bone is forming directly on the internal surfaces of the scaffold thus short-circuiting the need for the intermediate framework.

Importantly this direct comparison shows significantly faster bone formation without alteration of the ultrastructural patterns, when the implanted scaffolds contain BMSCs, as revealed by µCT and histological staining (Figure 3a-f, k), as well as ESEM and SAXS analysis (Figure 3g-j) which demonstrated similar orientation of mineral particles around the scaffold struts for cell-containing and cell-free scaffolds, compared with native bone.

Slow degrading composite scaffolds seeded with BMSCs regenerated the entire defect and showed extensive bone remodeling inside the fully interconnected pore architecture. Osteoid formation around the scaffold struts and viable vascular network demonstrated a tissue remodeling and maturation occurring as the scaffold material gradually degraded via surface erosion. This enabled new bone to progressively replace the mPCL-TCP itself as it slowly degraded without the over-production of detrimental acidic by-products which may lead to inflammation. The cell-free scaffold group demonstrated bone regenerative
capabilities and good osseointegration into the host bone due to the osteoconductive properties of the mPCL-TCP surface. However, the quantity of new bone formed throughout the entire architecture was significantly lower than those scaffolds loaded with BMSC’s. Hence, our results demonstrate the principle challenges to clinical translation of bone engineering of clinically relevant defect regeneration need to be addressed by the combination of a well-designed composite scaffold with a cell source which has strong osteogenic potential.

In conclusion, we have spent the last decade translating bone tissue engineering concepts from the bench to the bedside. After a series of in vitro and small animal studies reviewed in detail by Woodruff and Hutmacher [11], we have designed and executed a long-term, pre-clinical study to regenerate clinically relevant critical-sized cranial defects and have successfully demonstrated not only extensive bone regeneration but also remodeling over a period of two years within these defects implanted with a scaffold/BMSC construct. The importance of long-term implantation studies followed by in depth analysis at different orders of magnitude, using sophisticated methods prove highly organized and functional regenerated bone is crucial to future development and optimization of TEC’s. These spatial and temporal considerations collectively enforce the vision which was so vivid a decade ago and remains a key target in regenerative medicine - to advance these promising tissue engineering therapies into the clinic.
Materials and Methods

BMSCs were attained from the same animals under general anesthesia using routine bone marrow extraction and cultured based on published protocols. On reaching 80% confluency, BMSC’s were treated with osteoinductive media for 4 weeks prior to seeding onto mPCL-TCP scaffolds using fibrin glue (Baxter) during cranial implantation. Scaffolds comprising medical grade ε-poly-caprolactone incorporating 20% β-tricalcium phosphate (mPCL–TCP) were produced by fused deposition modeling (FDM) as described previously (Osteopore International, Singapore; www.osteopore.com.sg). The structural parameters of the scaffolds, tailored by computer aided design, were 100% pore interconnectivity within a range of 350–450 μm size, 70% scaffold porosity, and a 0/90° lay down pattern. mPCL-TCP scaffolds were implanted into the defect sites either alone or in combination with BMSC cells which were immobilized inside the scaffold by using fibrin glue. Eight defect sites were implanted with TEC’s, eight defect sites were implanted with scaffold alone. Animals were euthanized after 2 years with an overdose of barbiturates.

Scaffolds and the entire surrounding skull were explanted and immediately fixed in 4% paraformaldehyde for 2 weeks and then transferred to 70% ethanol. For quantitative evaluation of bone formation, the bones were then scanned in a micro-computed tomography scanner (microCT 40, Scanco Medical, Brüttisellen, Switzerland) based on published protocols. After completion of the embedding process, the resin blocks were trimmed and cut in half, directly through the center of the scaffolds, to enable histology sections to be taken from the center outward based on published protocols. Resin sections were stained using Macneals/von kossa and Goldner’s Trichrome stains and were measured semi-
quantitatively using Bioquant Image Analysis® software (Nashville, TN, USA). Fully mineralized tissue was measured by applying a fixed threshold to select for the positive stain (black von Kossa staining) within the defect and calculating the positive pixel area per stain divided by the total area available for bone in growth.

Polished sample blocks with the embedded scaffold and the entire skull were investigated with an ESEM (FEI-Company, Oregon, USA) in low vacuum using backscattered electron (BSE) as described by Fratzl et al.\textsuperscript{16,23} The two-dimensional SAXS patterns were analyzed for mean mineral crystal thickness, T-parameter, and the degree of alignment of the mineral crystals, Rho-parameter, within each sample.
References


Figure 1. Schematic illustrating the degradation of a 3D scaffold over time. SEM shows surface erosion of scaffold struts over 24 months in vitro (a,c) with the process depicted schematically in (d) Scaffold implanted into a pig cranial defect (b) at t=0 and is immediately filled with a hematoma on implantation followed by vascularization and gradually new bone is formed within the scaffold (e). As the scaffold degrades over time there is increased bone remodeling within the implant site until eventually the scaffold pores are entirely filled with functional bone and vascularity. (Figure 1 partially adapted from Muschler et al. [15]).
Figure 2. mPCL-TCP scaffolds with and without BMSCs were implanted within a critical-sized porcine cranial defect for 2 years. After 2 years, the scaffolds and surrounding tissues were explanted. The mPCL-TCP struts were still clearly visible in the defect site with excellent integration (a). Samples were cut directly through the centre before being analyzed using µCT (b). Samples were then sectioned at 6µm for detailed histological analysis using von Kossa (c, e, g) and Goldner’s trichrome staining (d, f) to reveal extensive bone formation within the scaffolds with almost 100% of the pores filled with new bone. The scaffold struts (labeled s) dissolved during histological processing leaving clear evidence of new immature bone (nb) and osteoid which had formed during the process of scaffold surface erosion (red). Osteoblasts are clearly seen active in the osteoid tissue surrounding the scaffold struts, with osteocytes (labeled oc) embedded in mineralized matrix in the scaffold pores.
Figure 3. MicroCT, histology ESEM and SAXS results from an implantation of mPCL-TCP scaffold and mPCL-TCP scaffold plus BMSC cells into the same porcine model - with side by side critical sized defect created. (a) MicroCT results show good bone regeneration within both scaffolds, with a higher degree of mineralization in the scaffold plus cells implantation group compared with the scaffold alone. (b-f) Histological assessment shows similar bone quality in the scaffold site compared to the scaffold site plus cells. (g) ESEM, X-ray imaging and SAXS analysis demonstrate the same phenomenon observed in figure 3. (h, j) Bone formation in the tissue-engineered bone areas depicting a preferential orientation of mineral particles around the scaffold struts compared with the (i) native bone which does not orientate around local osteons. (k) Extent of mineralization within scaffolds implanted with and without MSCs using Bioquant Image Analysis to quantify of stained mineralized tissue sections revealed a higher degree of mineralization in the scaffold plus cells implantation group (75%) compared to scaffold alone (49%). n=6.