Histological evaluation of osteogenesis of 3D-printed poly-lactic-co-glycolic acid (PLGA) scaffolds in a rabbit model

2009 Biomed. Mater. 4 021001

(http://iopscience.iop.org/1748-605X/4/2/021001)

Biomed. Mater. **4** (2009) 021001 (7pp) doi:10.1088/1748-6041/4/2/021001

COMMUNICATION

Histological evaluation of osteogenesis of 3D-printed poly-lactic-co-glycolic acid (PLGA) scaffolds in a rabbit model

Zigang Ge[1,2], Xianfeng Tian[1], Boon Chin Heng[1], Victor Fan[1], Jin Fei Yeo[1] and Tong Cao[1,3]

[1] Stem Cell Laboratory, Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, National University of Singapore, 5 Lower Kent Ridge Road, Singapore 119074
[2] Department of Biomedical Engineering, College of Engineering, Peking University, Beijing 100871, People's Republic of China

E-mail: omscaot@nus.edu.sg

Received 17 November 2008
Accepted for publication 11 December 2008
Published 11 February 2009
Online at stacks.iop.org/BMM/4/021001

Abstract
Utilizing a suitable combination of lactide and glycolide in a copolymer would optimize the degradation rate of a scaffold upon implantation *in situ*. Moreover, 3D printing technology enables customizing the shape of the scaffold to biometric data from CT and MRI scans. A previous *in vitro* study has shown that novel 3D-printed poly-lactic-co-glycolic acid (PLGA) scaffolds had good biocompatibility and mechanical properties comparable with human cancellous bone, while they could support proliferation and osteogenic differentiation of osteoblasts. Based on the previous study, this study evaluated PLGA scaffolds for bone regeneration within a rabbit model. The scaffolds were implanted at two sites on the same animal, within the periosteum and within bi-cortical bone defects on the iliac crest. Subsequently, the efficacy of bone regeneration within the implanted scaffolds was evaluated at 4, 12 and 24 weeks post-surgery through histological analysis. In both the intra-periosteum and iliac bone defect models, the implanted scaffolds facilitated new bone tissue formation and maturation over the time course of 24 weeks, even though there was initially observed to be little tissue ingrowth within the scaffolds at 4 weeks post-surgery. Hence, the 3D-printed porous PLGA scaffolds investigated in this study displayed good biocompatibility and are osteoconductive in both the intra-periosteum and iliac bone defect models.

(Some figures in this article are in colour only in the electronic version)

Introduction

Bone regeneration can be enhanced through implantation of biocompatible and biodegradable scaffolds on which newly formed bone is deposited through creeping substitution from adjacent living bone [1]. Many materials, polymers or ceramics, natural or synthesized, have been adopted to make porous scaffolds for bone regeneration, while each of them has its own merits and drawbacks. Besides components of the scaffolds, fabrication technology also plays an influential role in the performance of an individual scaffold [2]. The majority of scaffolds are fabricated by various techniques developed during the last decade, which include solvent casting, membrane lamination, phase separation, freeze drying, polymerization and gas foaming [3]. Although such traditional fabrication methods are technically simple and often compatible with other techniques, there is a limitation in

[3] Author to whom any correspondence should be addressed.

the 3D shapes that can be obtained, which makes it difficult to customize scaffolds with biometric data from patients [4]. Rapid prototyping (RP) or solid free-form fabrication (SFF) represents a new fabrication technology that can make polymeric scaffolds with sophisticated 3D structures, which can easily be customized to biodata provided by CT and MRI scans of patients. This is achieved through the addition of material layers and particulates in a controlled mode, as specified by a computer program.

3D printing is one of several rapid prototyping techniques that have seen increasing usage in the tissue engineering field, ever since it was first developed at Massachusetts Institute of Technology [5]. After spreading a layer of fresh powders over a platform, a print head deposits the binder solution onto the powder bed. Once the 2D layer profile is printed, another fresh layer of powder is laid down, and the whole process is repeated. The resolution limitation, removal of unbound powder within the porous structure and the use of organic solvent binders are the major drawbacks of this technique. Both ceramic [6] and polymeric [7] scaffolds fabricated with 3D printing have achieved good progress for bone tissue engineering by limiting the potential drawbacks while keeping technological novelty.

Based on the results from the previous *in vitro* study, the 3D-printed PLGA scaffolds could provide fairly good mechanical support while having the potential to promote *in vivo* osteogenesis [8]. Hence in the current study, histology of the PLGA scaffolds within a bone defect model and an intra-periosteum model was evaluated temporally, while the intra-periosteum model was reported as a competent *in vivo* bone bioreactor [9]. To exclusively focus on the structure and materials of the scaffold, the cells and hydroxyapatite additives were not included in this study.

Materials and methods

Scaffold fabrication

PLGA scaffolds were fabricated as described previously [8]. Briefly, raw PLGA (Purasorb, L-lactide/glycolide, 85:15, Purac, the Netherlands) and polyvinyl acetate (PVA, Nippon Gohsei, Japan) granules were ground into 100 μm particles and mixed with a binder before being processed in a 3D printer (Zprinter® 310 PLUS, Z Corporation, USA). A solvent mixture of ethanol, acetone and de-ionized (DI) water was used as the bonding agent. PVA particles were leached with an ultrasonic cleaner and the remaining portion was annealed. Acquired PLGA scaffolds were cylinders (6 mm high and 6 mm diameter), with 50% porosity.

Surgical procedure

The use of live animals for this study was approved and supervised by the Institutional Animal Care and Usage Committee of the National University of Singapore. A total of 18 male New Zealand White rabbits weighing 3–3.5 kg with closed epiphyses were used for this study. The rabbits were given an intra-muscular injection of xylazine (8 mg kg^{-1}) and ketamine (40 mg kg^{-1}) followed by incubation. Two surgical procedures were performed on each rabbit. First, a mid-sagittal skin incision of 3 cm over the cranial vault was made and a full-thickness periosteal flap was raised to expose the calvaria. A PLGA scaffold was placed under the periosteal flap on the parietal bone. Secondly, a 2 cm skin incision was made at the super-anterior iliac crest and the periosteum was elevated. Subsequently, bi-cortical bone defects, 6 mm in diameter, were created in the iliac crest with a motor-driven surgical drill, and one scaffold was inserted into each bone defect within the iliac crest. Periosteum and subcutaneous tissue were closed layer by layer. Buprenorphine (Temgesic, 0.3 mg, 0.1 ml kg^{-1}; Schering-Plough, NJ) and cephalexin (Rilexine, 0.1 ml kg^{-1}; Virbac, Australia) were administered intra-muscularly to alleviate pain and prophylaxis.

Histology

Rabbits were sacrificed by the intravenous overdose of 1 ml/2.5 kg of pentobarbitone sodium individually at 4, 12 and 24 weeks post-surgery, so as to harvest the implanted scaffolds for histological analysis. Samples were fixed in 4% formalin, decalcified in 10% formic acid and subsequently embedded in paraffin and sectioned into 5 μm slices. The sections were then stained with hematoxylin and eosin for morphological observation.

Results

PLGA scaffolds

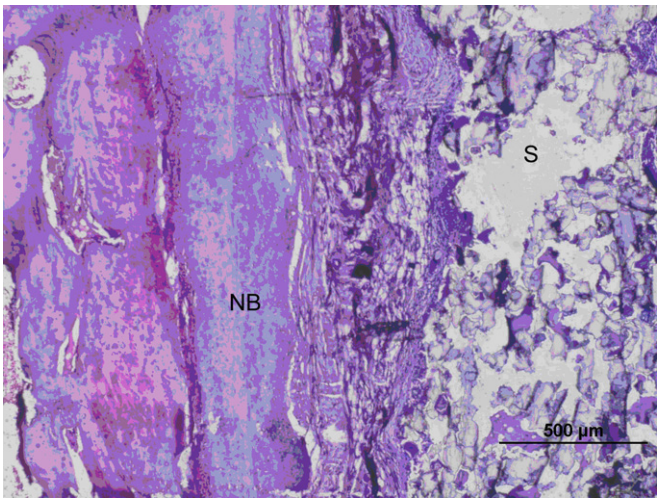
The fabricated PLGA scaffolds were white porous cylinders, 6 mm in diameter and 6 mm in height, with interconnected tunnels. The pore size is about 1 mm and porosity is around 50% of total volume. There are large numbers of micropores within macropore walls. The compressive strength of scaffolds was comparative with human cancellous bone [8].

Animal survival after surgery

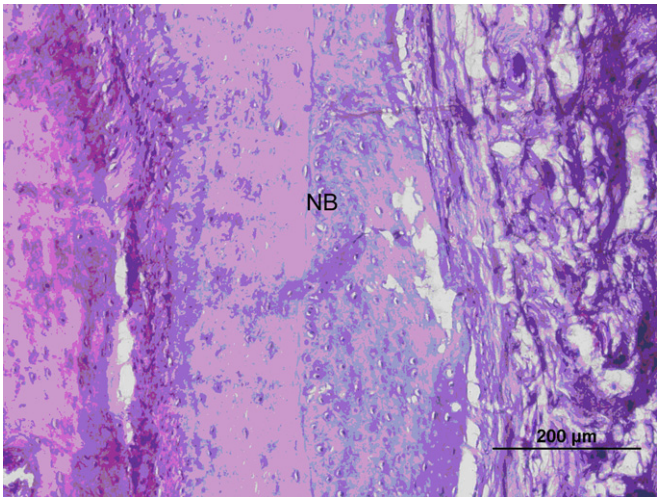
Of the 18 rabbits used in this study, two died during the course of the study. One died 3 days after the operation due to complications during surgery and another died due to a deep foot ulcer 4 months after the operation. Both rabbits were replaced. All other animals survived the course until pre-scheduled sacrifice.

Histology of scaffolds implanted within the periosteum

At 4 weeks post-operation, the PLGA scaffolds were observed to be integrated and fully encapsulated by dense connective tissues, with a little tissue ingrowth within the internal portion of the scaffold. The dense connective tissues were made up of layers of spindle fibroblasts, macrophage and extracellular matrix (ECM), with no lymphoid and plasma cells. There was a layer of newly formed bone between the original skull bone and the implanted PLGA scaffold, which was characterized by lightly stained immature bone matrix lacking small cavities (figure 1(*a*)). At high magnification, the newly formed bone appeared to be integrated with the skull bone directly but

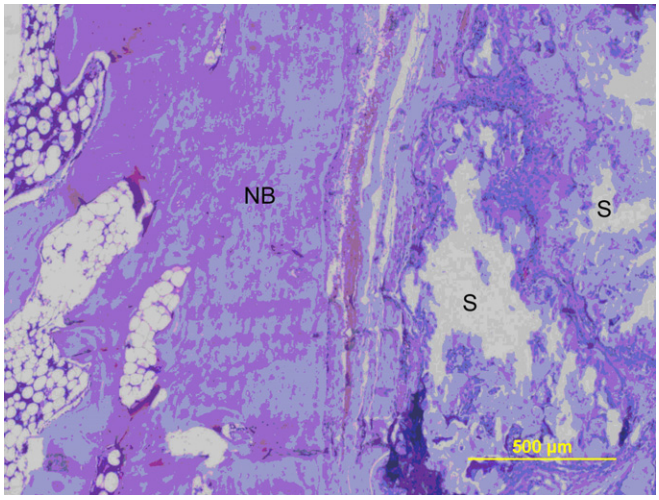


(*a*)

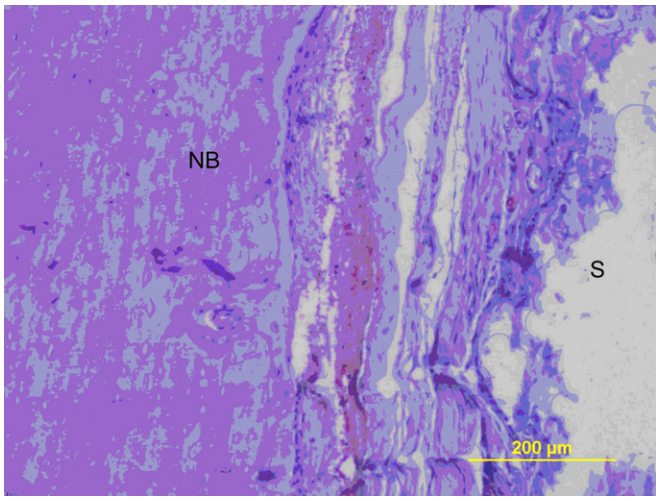


(*b*)

Figure 1. Histology of PLGA scaffolds implanted intra-periosteum of parietal bone at 4 weeks after operation: (*a*) 4× magnification; (*b*) 10× magnification. Note that S indicates scaffolds while NB indicates newly formed bone.



(*a*)



(*b*)

Figure 2. Histology of PLGA scaffolds implanted intra-periosteum of parietal bone at 12 weeks after operation: (*a*) 4× magnification; (*b*) 10× magnification. Note that S indicates scaffolds while NB indicates newly formed bone.

loosely, and was observed to become granular in the region surrounding the implanted PLGA scaffolds (figure 1(*b*)).

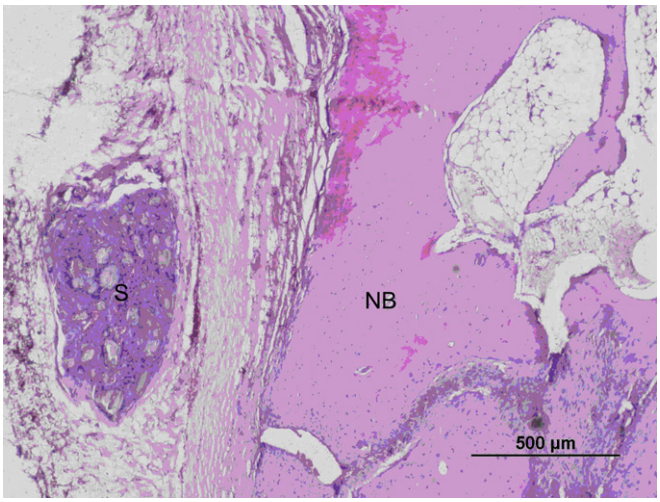
At 12 weeks post-operation, the implanted scaffolds lost their integrity and degraded into small slices, which were encapsulated by penetrating connective tissues. The newly formed bone appeared to be more mature, as compared to 4 weeks post-operation (figure 1), with densely stained and well-organized ECM (figure 2(*a*)). At higher magnification, it was visibly obvious that some connective tissues between skull and scaffolds were transforming into bone-like tissues that were densely stained with eosin (figure 2(*b*)).

At 24 weeks post-operation, the implanted scaffolds degraded further into even smaller pieces and were encapsulated by layers of more mature connective tissues (figure 3(*a*)). The layers of connective tissues facing parietal bone became even more mature as indicated by denser eosin staining (figure 3(*b*)). A visible boundary between newly formed bone-like tissue and native bone matrix existed at 4 weeks and subsequently disappeared. Thereafter, the newly formed bone-like tissues were well integrated with native bone tissues at 12 or 24 weeks post-operation, while they were distinguished by relatively weak eosin stain and poorly organized alignment of cells.

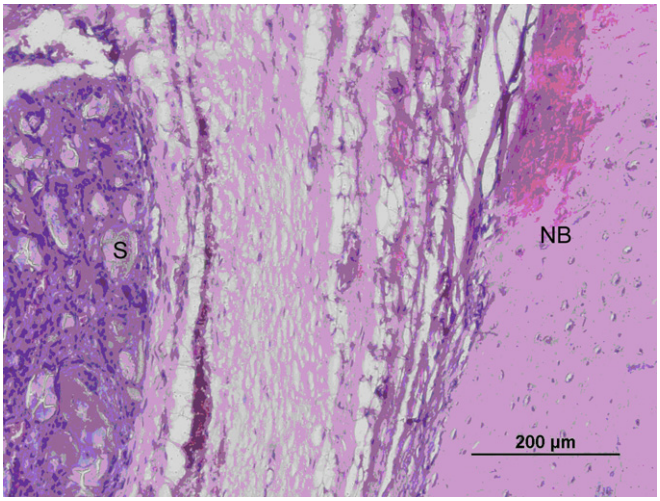
Low magnification of 12 weeks' histology of a skull model showed that new bone formed only along the old skull tissue, while the encapsulation tissue between the original bone and scaffolds was on the way to becoming bone-like tissues, which was supported by denser eosin staining and sparser cell density with time. Tissues penetrated into the scaffolds while the scaffolds degraded (figure 4).

Histology of scaffolds implanted within bone defects of the iliac crest

At 4 weeks post-operation, the PLGA scaffolds appeared to be well integrated into the surrounding tissue, but there was little tissue ingrowth within the internal portion of the scaffold. There were no dense connective tissues between the scaffolds



(*a*)



(*b*)

Figure 3. Histology of PLGA scaffolds implanted intra-periosteum of parietal bone at 24 weeks after operation: (*a*) 4× magnification; (*b*) 10× magnification. Note that S indicates scaffolds while NB indicates newly formed bone.

and adjacent bone, as was observed in the intra-periosteum model (figure 5(*a*)). At high magnification, the newly formed bone-like matrix was observed to have a higher cell-to-matrix ratio, was well organized, but had fewer cavities compared with the adjacent mature bone (figure 5(*b*)).

At 12 weeks post-operation, the PLGA scaffolds were degraded into small pieces that were well encapsulated by the newly formed bone-like matrix with the sparsely stained ECM (figure 6(*a*)). At high magnification, the newly formed bone-like matrix appeared to be tightly integrated with both the scaffold and mature bone tissue, and had a higher cell-to-matrix ratio with relatively sparse internal cavities (figure 6(*b*)).

At 24 weeks post-operation, the scaffolds were degraded even further while the newly formed bone layer between the scaffolds and the original bone tissue became even thinner than at 12 weeks. The newly formed bone integrated with both the original bone tissue and scaffolds even more tightly than at 4 and 12 weeks (figure 7(*a*)). At high magnification, the newly

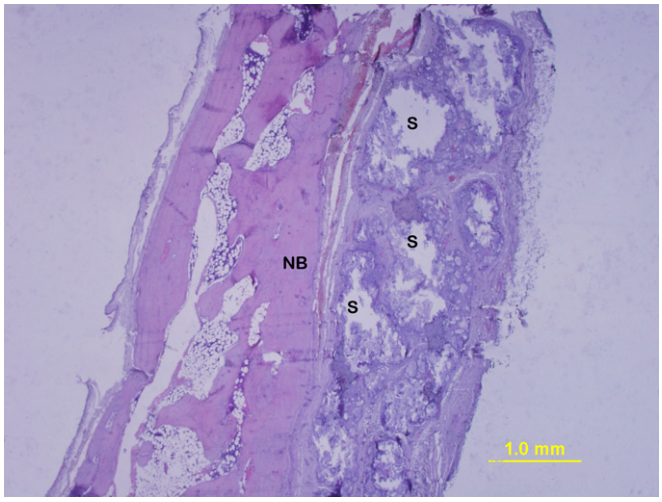
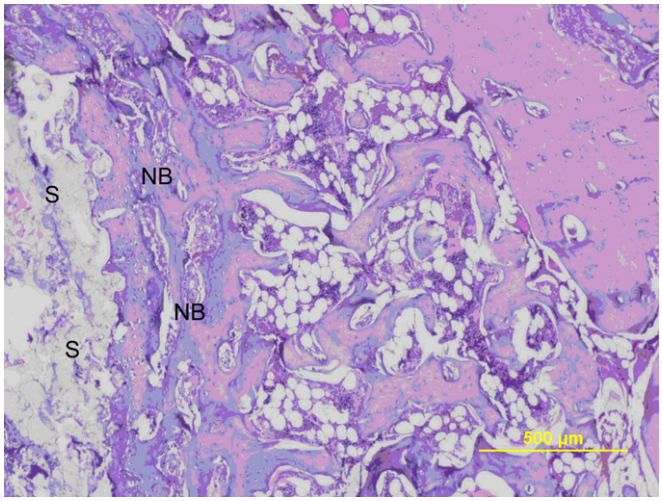
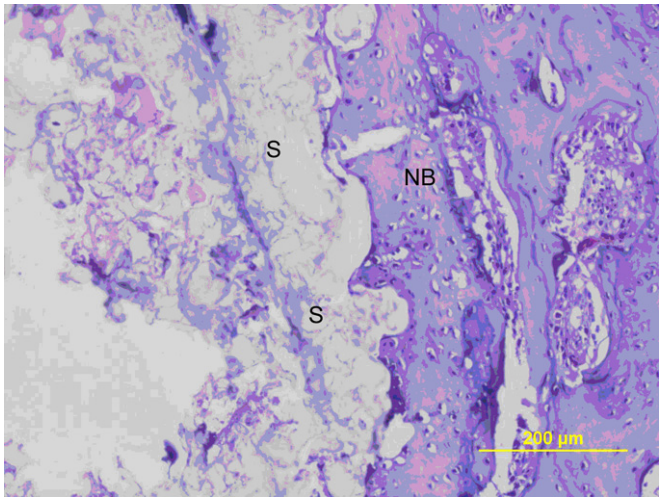


Figure 4. Low magnification view of 12 weeks' histology of the intra-periosteum model. Note that S indicates scaffolds while NB indicates newly formed bone.

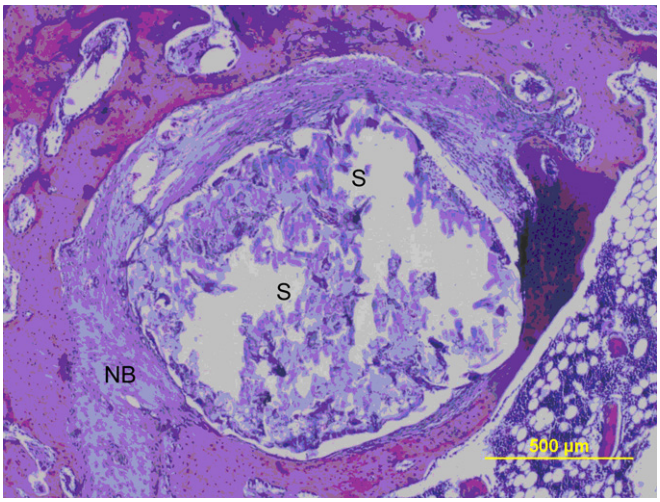


(*a*)

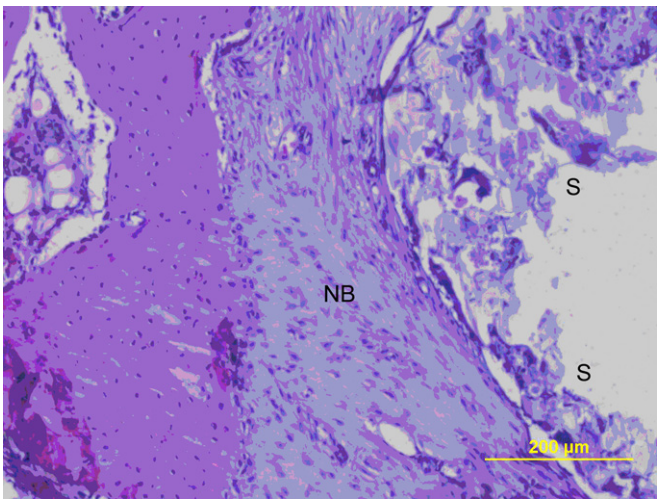


(*b*)

Figure 5. Histology of PLGA scaffolds implanted bone defect of ilium after operation: (*a*) 4× magnification; (*b*) 10× magnification. Note that S indicates scaffolds while NB indicates newly formed bone.

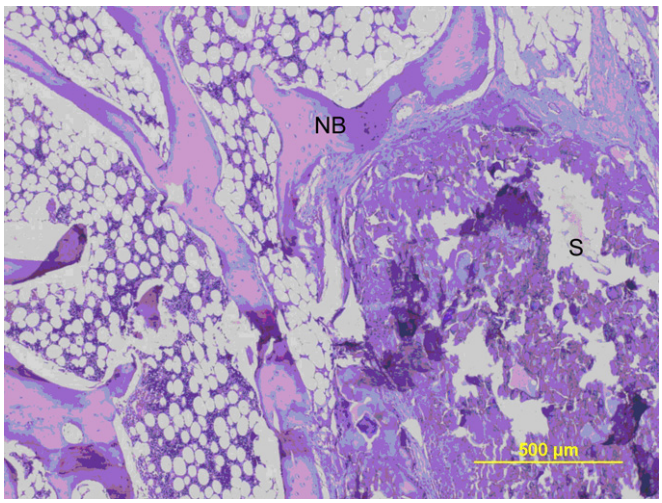


(*a*)

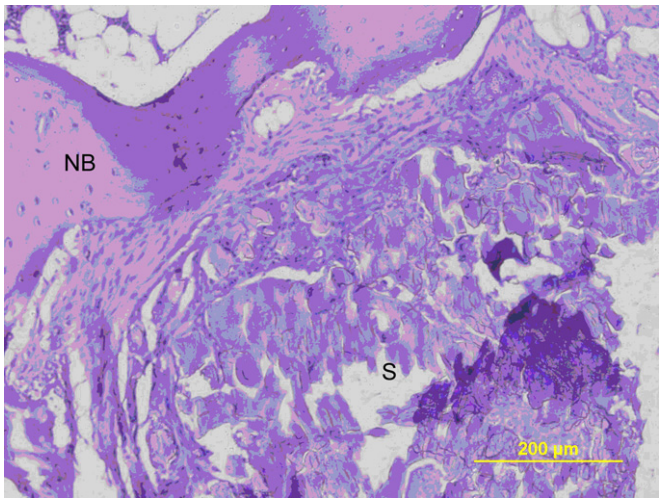


(*b*)

Figure 6. Histology of PLGA scaffolds implanted bone defect of ilium at 12 weeks after operation: (*a*) 4× magnification; (*b*) 10× magnification. Note that S indicates scaffolds while NB indicates newly formed bone.



(*a*)



(*b*)

Figure 7. Histology of PLGA scaffolds implanted bone defect of ilium at 24 weeks after operation: (*a*) 4× magnification; (*b*) 10× magnification. Note that S indicates scaffolds while NB indicates newly formed bone.

formed bone had a higher cell-to-matrix ratio than at 4 and 12 weeks (figure 7(*b*)).

The low magnification of 12 weeks' histology of the ilium model showed that a bone-like matrix penetrated into the central part of the scaffolds, while the scaffolds degraded into small pieces (figure 8).

Discussion

Polylactide, polyglycolide and their copolymers are the most commonly used biodegradable polymers in bone tissue engineering [10]. Compared with polyglycolide, polylactide has a much slower degradation rate and may cause sustained swelling and local foreign body reaction. PLGA, a copolymer of their monomeric constituents, can suppress these side effects by achieving controlled degradation through an optimal combination of polylactide and polyglycolide [11]. Though considered relatively safe for many years, the individual properties of PLGA scaffolds fabricated with various techniques should be examined carefully *in vivo*, particularly with regard to their degradation kinetics, since acidic degradation side-products can potentially damage newly formed bone nodules. Previous results from our research group [8] showed that current PLGA scaffolds are conducive for cellular osteogenesis *in vitro*. Nevertheless, all our previous *in vitro* experiments were performed within a time frame of a few weeks, during which there was little degradation of the PLGA scaffolds. Hence, there is a need to examine long-term *in vivo* biocompatibility, as well as its roles in osteogenesis upon implantation *in situ*. Porous polylactide scaffolds (BD Three Dimensional OPLA® Scaffold, Cat. 354614, BD Biosciences Discovery Labware, USA) were used in the current study as control, as they have the most similar chemical and morphological properties when compared with the porous 3D-printed PLGA scaffolds and also are one of a few commercial products available. The BD Three

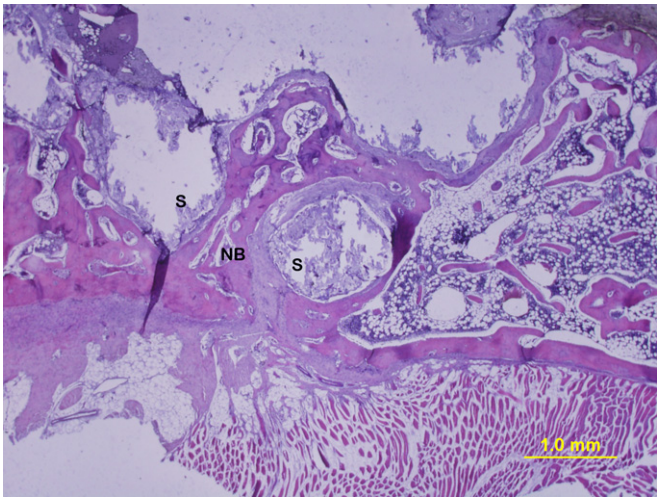


Figure 8. Low magnification view of 12 weeks' histology of the ilium model. Note that S indicates scaffolds while NB indicates newly formed bone.

Dimensional OPLA® Scaffolds were well used and can be regarded as a representative of mature PLA, PGA or PLGA scaffolds used in the tissue engineering field. However, all control scaffolds totally degraded within 4 weeks, with normal histology left in both models (pictures not shown). So there was no control result to show in the current study.

One major merit of 3D-printed scaffolds is that they can mimic any geometry and internal structures provided by patients through CT or MRI. Moreover, a larger pore size could possibly allow relatively slow degradation, and encourage more tissue penetration as well as nutrient exchange. Based on this consideration, a large pore size was adopted instead of imitation of the original bone structure. When implanted under the periosteum, the PLGA scaffolds incurred only a mild foreign body reaction after 4 weeks, and no immunological reaction was reported [9]. The capsules between the PLGA scaffolds and the newly formed bone became layered, well organized and heavily stained with eosin at 12 and 24 weeks, which indicated more extracellular matrix formation of bone-like tissues potentially. More importantly, implantation of the PLGA scaffolds induced reactive bone formation on the adjacent cortical bone, and these bones became mature with time. In the mean time, more tissues penetrated into the scaffolds with time when the scaffolds degraded into small pieces. The fate of these penetrating tissues is not clear. Though it was called the intra-periosteum model, the scaffolds were only partially covered by periosteum, due to a relatively large size of the current scaffolds. Some parts of the scaffolds were not encompassed by bone or bone-forming microenvironments (intra-periosteum), so they were likely not able to become bone-like tissue. In the previous study, intra-periosteum was delicately used as an *in vivo* bone bioreactor to form new bone, but the authors concluded that size did matter while only a tiny volume of scaffolds (1/580 of the current scaffolds used) could be successfully transformed to bone [9]. It is reasonable to conclude that parts of the scaffolds as well as some penetrating tissues inside could not become bone in the current 'partial intra-periosteum model' finally, while some other parts near the original bone tissue had a good chance to make it. Some minerals, such as nacre (pure calcium carbonate), have been reported to help establish a sophisticated regulatory system of *in vivo* biomineralization [12], but so far there has been no similar report for polymers. At this stage, it is difficult to conclude whether reactive bone formation was specifically from the PLGA scaffolds or not. After developing and evaluating the current form of PLGA as a basic supporting matrix, calcium components and growth factors could be evaluated as additives in future studies.

Periosteum plays an important role in osteogenesis. Although a study of cell sources in periosteal osteogenesis reported that osteoprogenitor cells in subperiosteal space originated exclusively from the bone surface, and not from periosteum [13], it is well accepted that orchestrated bone regeneration within subperiosteal space requires many fine-tuned factors as well as scaffolds serving as templates [9]. Subsequent intramembranous bone formation did not occur as reported, possibly due to the relatively large scaffolds used (580 times larger in volume) and broader elevation of periosteum, which probably disrupted homeostasis of the subperiosteum environment [9]. There was no new bone formation; instead the creation of a subperiosteal space using wider 92 mm incision resulted in the formation of fibroblastic scar tissue. It is hypothesized that because the biomechanical properties of the PLGA scaffolds matched those of human cancellous bone, this in turn could have contributed to *in vivo* osteogenesis, by providing a decisive induction niche for mesenchymal stem cells to differentiate into the osteogenic lineage [14]. However, there are insufficient data from this study to validate this hypothesis.

It is reasonable that histological results from the bone defect model of ilium are even better than those from the cranial model, as there was no thin layer of fibrous tissue surrounding the implanted scaffold [7]. In the ilium model, all penetrating tissues were well stained by eosin and were on the way to becoming bone-like tissues. Unlike the cranial model where the scaffolds were only partially encompassed by bone or periosteum, the scaffolds were fully indented with bone tissues. So it is reasonable that all parts of scaffolds were proven to support bone formation in the ilium model. This may indicate enhanced osteoconductivity. It is noteworthy that there are obvious differences in the anatomical locations of ilium and skull. Anatomically, ilium has more blood supply and osteoprogenitors, which lay the basis for bone regeneration.

When surrounded by or immersed in proper physical environments, the current scaffolds show good osteoconductivity, but may lack osteoinductivity in the current format. In our pilot study, the scaffolds were used to fill a 6 mm diameter mandible defect and left no sign of bone regeneration (data not shown); acquired data were in coincidence with previous published results [15]. In any case, both studies confirmed that porous channels can encourage tissue ingrowth within scaffolds.

Conclusion

Current 3D-printed porous PLGA scaffolds show good biocompatibility and are osteoconductive when implanted in proper locations, but may not be osteoinductive.

Acknowledgments

This study was partially supported by The Enterprise Challenge, Premier Minister Office (Singapore). The PLGA scaffolds were fabricated and provided by Bio-Scaffold International (Singapore).

References

[1] Groeneveld E H, van Den Bergh J P, Holzmann P, ten Bruggenkate C M, Tuinzing D B and Burger E H 1999 *J. Biomed. Mater. Res.* **48** 393
[2] Ge Z, Jin Z and Cao T 2008 *Biomed. Mater.* **3** 022001
[3] Cao T, Ho K H and Teoh S H 2003 *Tissue Eng.* **9** (Suppl 1) S103
[4] Rutkowski G E, Miller C A and Mallapragada S K 2002 *Methods of Tissue Engineering* ed A Atala and R P Lanza (San Diego: Academic)
[5] Sachs E M, Haggerty J S, Cima M J and Williams P A 1993 Three-dimensional printing techniques *US Patent* 5204055
[6] Seitz H, Rieder W, Irsen S, Leukers B and Tille C 2005 *J. Biomed. Mater. Res.* B **74** 782
[7] Dutta Roy T, Simon J L, Ricci J L, Rekow E D, Thompson V P and Parsons J R 2003 *J. Biomed. Mater. Res.* **67** 1228
[8] Ge Z G, Wang L S, Heng B C, Tan E P S and Cao T 2008 *J. Biomater. Appl.* Online, doi:10.1177/0885328208094301
[9] Stevens M M, Marini R P, Schaefer D, Aronson J, Langer R and Shastri V P 2005 *Proc. Natl. Acad. Sci. USA* **102** 11450
[10] Middleton J C and Tipton A J 2000 *Biomaterials* **21** 2335
[11] Bergsma J E, Rozema F R, Bos R R, Boering G, de Bruijn W C and Pennings A J 1995 *Biomaterials* **16** 267
[12] Westbroek P and Marin F 1998 *Nature* **392** 861
[13] Sasano Y, Kamakura S, Homma H, Suzuki O, Mizoguchi I and Kagayama M 1999 *Anat. Rec.* A **256** 1
[14] Engler A J, Sen S, Sweeney H L and Discher D E 2006 *Cell* **126** 677
[15] Ren T, Ren J, Jia X and Pan K 2005 *J. Biomed. Mater. Res.* **74** 562