Michael de Wild*, Simon Zimmermann, Marcel Obrecht and Michel Dard

Marker for the pre-clinical development of bone substitute materials

X-ray opaque histology cages for bone substitute testing

Abstract: Thin mechanically stable Ti-cages have been developed for the *in-vivo* application as X-ray and histology markers for the optimized evaluation of pre-clinical performance of bone graft materials. A metallic frame defines the region of interest during histological investigations and supports the identification of the defect site. This standardization of the procedure enhances the quality of pre-clinical experiments. Different models of thin metallic frameworks were designed and produced out of titanium by additive manufacturing (Selective Laser Melting). The productibility, the mechanical stability, the handling and suitability of several frame geometries were tested during surgery in artificial and in *ex-vivo* bone before a series of cages was preclinically investigated in the female Göttingen minipigs model. With our novel approach, a flexible process was established that can be adapted to the requirements of any specific animal model and bone graft testing.

Keywords: Histology, marker, titanium, preclinical study, X-ray opaque.

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1 Introduction

There is an increasing interest in bone substitute materials such as synthetic BoneCeramic™ [1], Max-Resorb™ [2], Nanobone™ [3], BoneWool™ [4] or chronOS™ [5], the xenograft Bio-Oss™ [6] or natural Algipore™ [7] in order to overcome bone defects after tumour resection or for regenerative treatments of bone with reduced healing time. The pre-clinical assessment with histologic analysis in animal models is a necessary research step during product development and finally for registration and will play an increasing role in the future. In order to study the *in-vivo* response of bone substitute biomaterials, the bone graft material often is filled into surgically created bony defects, e.g. in the mandible of minipigs or in the tibia of sheep [8-10]. After a predefined healing time, the bones are collected and processed for histological analyses to quantify ingrown bone volume, angiogenesis and bone structure. It is often problematic to find the exact position of the original defect by biological landmarks only. In case of degradable materials like polylactides or ceramic β-TCP, resorption of the biomaterial is studied. The investigation of these degradable or X-ray transparent materials is particularly challenging because it becomes difficult to localize the correct implantation site after some months of material degradation and tissue remodelling. The definition of the cutting planes in histologic specimens, the specification of the defect volume and the quantification of bone parameters then becomes imprecise. For quality aspects, it is important to simplify and optimize this experimental procedure, e.g. by using standardized histology and additional markers to locate the implantation site. Currently several titanium reference pins are occasionally placed into the bone around the implant site area, which is time consuming, expensive and still accuracy is very limited.

In this study, we developed and tested X-ray opaque histology markers for pre-clinical performance evaluation of bone substitute materials. Our approach is to place a fine metallic frame prior to filling the defect site with the biomaterial. In X-rays images as well as in histologic analysis it will then be straightforward to identify the implant site, even in cases of resorption at the interface of the defect.

As the defect size and shape is individual in every animal model, we propose to use the *Additive Manufacturing* technique *Selective Laser Melting* (SLM) to generate the subtle frames out of biocompatible titanium. This process

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allows producing metallic meshes adapted to the specific animal model with structural features below 300 µm. Thereby the object is virtually designed and then produced layer-by-layer by scanning a laser on a powder bed of titanium particles.

During this study, the cages have been investigated in Synbone™ [11], *ex-vivo* and *in-vivo*. The cage system was investigated regarding geometry, X-ray visibility, suitability for implantation and biocompatibility. First, the geometry, the mechanical stability against buckling during insertion into synthetic bone material and the X-ray visibility of the cages were studied. In order to examine the surgical handling, trial implantations of frames were done in a cadaveric pig jawbone with a standard bone graft material. Finally, *in-vivo* experiments were performed in the mandible of female Göttingen minipigs to test feasibility for histological use.

2 Material and methods

2.1 Sample design

Different types of cylindrical cages (diameter ø8 mm and height h = 8 mm) were designed with a CAD software (Solidworks 2013, Dassault, France). A first simple design consists of rings with four connecting trusses, see Figure **1**A.

a)

b)

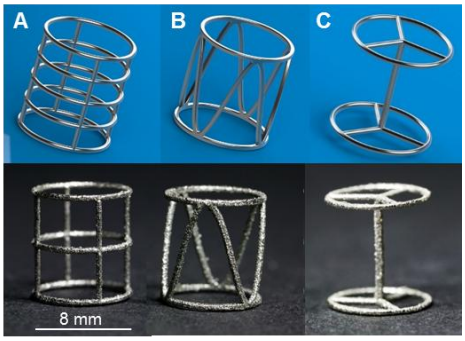

Figure 1: a) Virtual models and b) photos of different frame geometries A, B and C with strut thickness 0.4 mm.

Several different strut thicknesses were constructed: 0.2 mm, 0.4 mm, and 0.6 mm, see Figure 3. The second design consists of a sinusoidal lateral termination with circular closures on the cylinder top and bottom, see Figure **1**B. In horizontal histological sections, the height can be determined by the separation of the undulated struts. The third cage type consists merely of a central axis that is held in place by an orthogonal top and bottom ring, see Figure 1C. This minimal model without a surface shell is assumed to have marginal interaction with both the bone and the biomaterials under investigation.

2.2 Sample production

The titanium cages were produced by a SLM Realizer 250^{HT} system using a 200 W infrared fiber laser. The implants were detached from the support structures, see Figure 2, sandblasted with Al_2O_3 (MS-EKRA80A, 5 bar on a PEENMATIC 620S, Iepco AG), cleaned with 4% Deconex® 15PF (Beiersdorf), three times in ultrapure water (resistivity 18.2 MΩ·cm), oxygen RF plasma treated (PDC-32G Harrick, 99.9995% pure oxygen, Carbagas) and γ-sterilized with 25 kGy.

2.3 X-ray visibility and

The x-ray imaging performance was tested with the C-arm (Ziehm Vision FD Vario 3D, 43 kV) and the µCT system (SkySkan 1172, SkySkan NV, 100 kV).

2.4 Mechanical testing

The compressive mechanical strength was determined by a testing machine (Z100 Zwick GmbH & Co). The cages were placed between a guided pressure unit in order to avoid shear forces. The maximal force *F* before buckling was determined.

2.5 *In-vivo* testing

The handling and performance of these titanium reference cages was tested *in-vivo* in the mandible of six female Göttingen minipigs, aged 14 to 16 months with an average body weight of 35 kg. The animals were kept in boxes and were fed a restricted soft diet to control their weight gain. The implantation duration was of 2 months. Every animal received three implants one side of the mandible and another three implants on the contralateral side. All the surgical procedures were performed under anaesthesia, in aseptic conditions in an animal operating suite. Sedation was achieved by a combination of 50 mg/mL ketamine and 5mg/mL midazolam by means of intramuscular injections in the neck region. Local anaesthesia was provided by means of an infiltrative injection with 1.8 mL of 20 mg/mL xylocaine, and 12.5 mg/mL adrenalin per hemimandible. Under full narcosis, extractions of the premolars and first molars were conducted on both sides of the mandible. Three months later, a one-stage surgical approach was used to place the cages

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following the standard implantation protocol. A mid-crestal incision was performed and full-thickness buccal and lingual flaps were raised. The crest of the ridge was then flattened to allow for more accurate implant placement. The cage upper part was positioned at the level of the bone crest. After complete filling of the internal part of the cages with BoneCeramics™ [1], the flaps were sutured to provide non-submerged healing without any additional treatment.

After a duration of 2 months, the minipigs were sacrificed for histologic and histomorphometric evaluation of the mandible tissues. The minipig mandibles were sectioned into hemimandibles and were stored for fixation by immersion in 10% buffered formalin solution at room temperature for 3 weeks. Once the hemimandibles were ready for histologic examination, radiographs of the specimens were taken to identify the position of the sites. The samples were then dehydrated in alcohol of increasing concentration, cleared in xylene and embedded in polymethylmethacrylate resin. The hemimandibles were sectioned by a microcutting and grinding technique resulting in histologic sections reaching a thickness of 30 µm. The histologic sections were observed using a microscope.

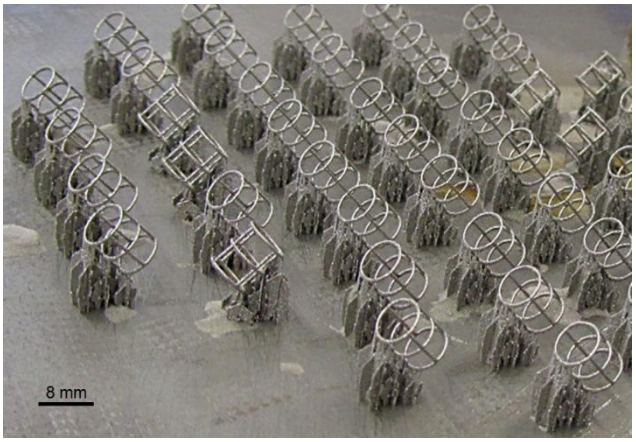

Figure 2: Ti cages connected to the SLM-platform via support structures.

3 Results

3.1 Sample production

All designed titanium implants were realized by SLM, see Figure 1. However, the samples with the thinnest 0.2 mm struts broke during the detachment from the support structures while the 0.6 mm links were too massy, see Figure 3.

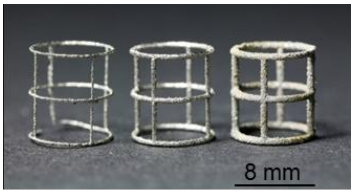

Figure 3: Trial cages with strut thickness 0.2 mm, 0.4 mm and 0.6 mm.

3.2 X-ray visibility

The x-ray visibility in radiographs (2D) and with µCT (3D) was assured even for the thinnest struts (0.2 mm), see Figure 4 in Synbone™ and Figure 7AB in tissue. The marker can be exactly positioned in the simulated bony defect under X-ray observation.

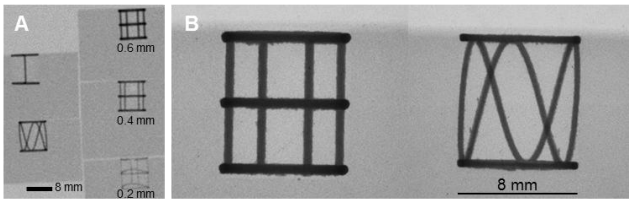

Figure 4: X-ray contrast in A) medical C-arm tomography and B) µCT.

3.3 Mechanical testing

As expected, the strut diameter as well as the cage design have a significant influence to the mechanical strength. In uniaxial compression tests, the mechanical stability of type A cage with strut diameter 0.3 mm was verified to be high enough for a correct surgical handling, see Figure 5.

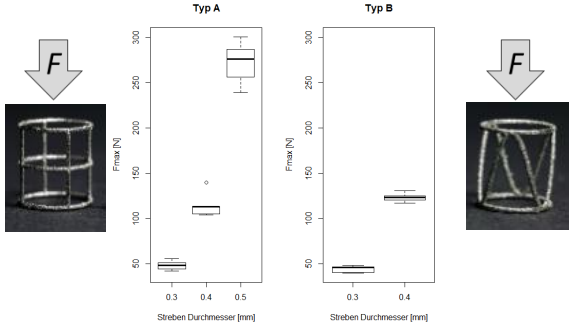

Figure 5: Design-dependent mechanical stability.

3.4 *Ex-vivo* results

The mechanical stability of the 0.3 mm thick cages proofed to be sufficient for placement into the predrilled cavities in

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porcine jawbones, see Figure 6A. The bone granules is straightforwardly filled into the cage (Figure 6B). Finally, the metallic struts of the cage are clearly visible in the histological section of the *ex-vivo* sample, see Figure 6C. Six identification marks are representing the outlines of the original bone defect.

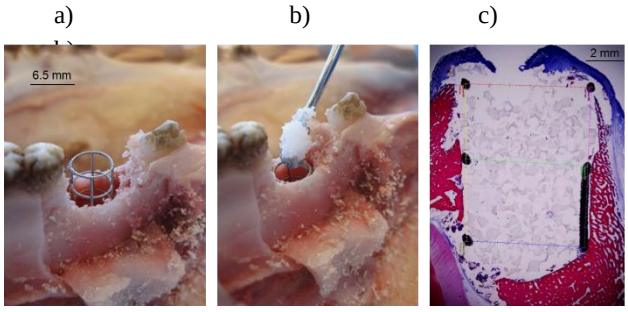

Figure 6: Ex-vivo placement of the 0.3 mm thick cage (h =8 mm, ø6.5 mm): a) Insertion into predrilled hole, b) filling with bone substitute material, c) Histological section showing old bone (pink), soft tissue (blue), bone substitute (grey) and reference points of the cages (black).

3.5 *In-vivo* results

The animals were sacrificed 6 weeks post-op and the samples were retrieved for histological preparation. The cages helped to define the cutting plane for histological sections, see Figure **7**. The old bone (pink) can be distinguished from the new bone (purple) that was growing in the vicinity of the bone substitute (grey). With help of the cage profile, it is easy to observe the original defect margin and set a region of interest in histomorphometric analysis. No adverse reactions were detected from the SLM Ti cage, as was expected from earlier studies [12].

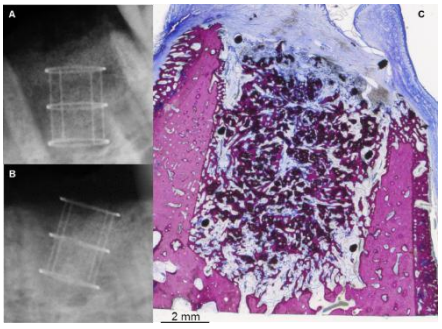

Figure 7: 0.3 mm thick Ti cage (h =8 mm, ø6.5 mm): a) Mesio-distal X-ray at implantation, b) after sacrifice, c) histological section in bucco-lingual orientation showing old bone (pink), new bone (purple), soft tissue (blue) and reference points of the Ti cages (black).

4 Conclusion

We have developed and tested X-ray opaque histology markers for pre-clinical studies in order to facilitate the evaluation and interpretation of the *in-vivo* performance of degradable bone substitute materials. By inserting the newly developed fine titanium frames, a consistent localization of the implantation site is possible both during surgery, during sample processing and histomorphometric evaluation. Subjective variables are eliminated by standardizing the procedure and circumscribing the original periphery of the defect.

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References

[1] De Lange GL et al. A histomorphometric and micro-computed tomography study of bone regeneration in the maxillary sinus comparing biphasic calcium phosphate and deproteinized cancellous bovine bone in a human split-mouth model, Oral Surg Oral Med Oral Pathol Oral Radiol. 2014 117(1):8-22.

[2] Jelusic D et al. Monophasic ß-TCP vs. biphasic HA/ß-TCP in two-stage sinus floor augmentation procedures - a prospective randomized clinical trial, Clin Oral Implants Res. 2016.

[3] Sadeghi R et al. Histologic and histomorphometric evaluation of bone regeneration using nanocrystalline hydroxyapatite and human freeze-dried bone graft: An experimental study in rabbit J Orofac Orthop. 2017.

[4] Schneider OD et al. Biocompatibility and Bone Formation of Flexible, Cotton Wool-like PLGA/Calcium Phosphate Nanocomposites in Sheep, Open Orthop J. 2011 16;5:63-71.

[5] Dos Santos Pereira R et al. Maxillary Sinus Elevation Surgery with ChronOS and Autogenous Bone Graft: Analysis of Histometric and Volumetric Changes, Int J Periodontics Restorative Dent. 2016 36(6):885-892.

[6] van Houdt CI et al. The performance of CPC/PLGA and Bio-Oss for bone regeneration in healthy and osteoporotic rats, J Biomed Mater Res B Appl Biomater. 2016.

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[7] Schulz MC et al. Oral Surg Oral Med Oral Pathol Oral Radiol. 2012 114(5 Suppl):S160-6.
[8] Jensen SS et al. J Biomed Mater Res B Appl Biomater. 2009 90(1):171-81.
[9] Buser D et al. Clin Oral Implants Res 1998 9 (3): 137–150.
[10] Becker S et al. Spine 2006 31(1):11–17.
[11] http://www.synbone.ch
[12] de Wild M et al. Bone regeneration by the osteoconductivity of porous titanium implants manufactured by selective laser melting: A histological and µCT study in the rabbit, Tissue Engineering Part A, 19 (23-24):2645-54 (2013).

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