Micro-CT: a powerful tool for the characterization and evaluation of the bone formatting capacity of calcium phosphate – stem cells tissue engineering constructs

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Aims

Ageing is irrevocably related to the degeneration of tissue and organs. Diseases and accidents often result in tissue or organ malfunction. Their replacement by artificial implants, donor tissue, or organs, holds very important restrictions. A new, more advanced biological approach seems to be the only long-term solution. An important contribution to this approach can be given by tissue engineering (TE) \cite{1}. Bone TE specifically, topic of this study, is a multidisciplinary field of science focusing on healing large bone defects.

One of the most promising bone TE approaches is the use of stem cells in combination with biomaterials (= scaffolds) and signalling molecules in order to create a 3D environment for bone tissue formation. For the evaluation of the bone forming capacity of specific stem cell – biomaterial combinations, both \textit{in vitro} biomaterial screening/selection and \textit{in vivo} assessment are required. In general, for the evaluation of \textit{in vivo} experiments, the implanted cell-scaffold combination together with the newly formed tissue are explanted after a defined period and investigated by histomorphometry to identify and quantify the newly formed tissue \cite{2-5}. However, this procedure is destructive, time consuming, labour intensive, and provides only discrete 2D information. The latter is intrinsically related to the use of consecutive 2D histological sections with a significant interspace. Therefore, the quantification of the newly formed bone volume lacks information and cannot be referred to as real 3D information.

As frequently reported in literature, microfocus X-ray computed tomography (micro-CT) shows a potential for partial replacement of, or complementary use with histomorphometry for the visualization and/or quantification of the newly formed bone tissue in and around bone TE scaffolds \cite{6-13}. Additionally, the scaffold characteristics prior to implantation and after explantation can be quantified.

In this study, for combinations of human periosteal derived cells (hPDCs) with clinical calcium phosphate (CaP) scaffolds, micro-CT was used for in-depth and 3D initial material and explant characterisation. Based on this information, a correlation between specific material properties and the \textit{in vivo} bone formation has been developed.

Materials and methods

Five different commercially available orthopaedic 3D matrices composed of calcium phosphate (CaP) particles in an open collagen network (NuOss\textsuperscript{TM}, CopiOs\textsuperscript{TM}, Bio-Oss\textsuperscript{®}, Collagraft\textsuperscript{™} and Vitoss\textsuperscript{®}) were selected. Each scaffold was scanned using high-resolution micro-CT on a Skyscan 1172 system [Skyscan NV, Kontich, Belgium] at an isotropic voxel...
size of (4.5 μm)³. Since each biomaterial had a similar composition, for all materials a source voltage and current of 60kV and 167 μA respectively and a filter of 0.5mm Al were applied. Using a rotation step of 0.3° over a total of 180° resulted in 640 radiographic images. After reconstruction using dedicated software [NRecon, Skyscan NV, Kontich, Belgium], a total of about 900 greyscale axial micro-CT images per sample were generated. Manual, but consistent global segmentation of the CaP within each implant was carried out based on the greyscale histogram to allow quantification of parameters such as average grain size, surface area, volume fraction and specific surface area using CTAn [Skyscan NV, Kontich, Belgium].

One million hPDCs in suspension were applied to the upper surface of each scaffold. To allow cell attachment, the seeded scaffolds were incubated overnight at 37°C. After incubation, the constructs were directly implanted subcutaneously in the back at the cervical region of NMRI-nu/nu mice. After 8 weeks of implantation, the implants were collected. Each explant was fixed in 4% formaldehyde, scanned by high-resolution micro-CT, decalcified in EDTA/PBS for 2 weeks, paraffin embedded and processed for histology. Fluorescence histomorphometry, as described by Martin et al. [14], was performed on tissue sections from each implant. Micro-CT image analysis was used to quantify the volume of newly formed bone in three dimensions by segmenting the newly formed mineralised tissues from the CaP grains in each material. Additionally, the bone interconnectivity was assessed, together with a spatial localisation of the bone formed within the scaffold structure. The same acquisition parameters as for the implanted biomaterials were used. For segmentation, again a manually selected, but consistent global threshold value was selected for each biomaterial. This time, the choice of threshold value was confirmed by visual comparison to the corresponding histological sections.

Results and discussion

- **Morphological quantification of the scaffold characteristics**

  When considering the volume of CaP in relation to the entire scaffold, CopiOs™ contained significantly less than any other material (approximately 10 fold less than NuOss™ and Collagraft™; and 6 fold less than Bio-Oss® and Vitoss®), as shown in Figure 1A. Both NuOss™ and Collagraft™ contained approximately 20% CaP while Bio-Oss® and Vitoss® contained significantly lower levels at approximately 12%. Each material was distinctly different to NuOss when comparing the average grain size with Bio-Oss® containing on average the largest grains and CopiOs™ the smallest grains (Figure 1B). When analyzing the surface area of CaP within each material both Bio-Oss® and CopiOs™ contained significantly less than any other material, which was approximately 2 fold less than NuOss™ and Vitoss® (Figure 1C). The specific surface area however was significantly higher for CopiOs™ and Vitoss® compared to the other materials (Figure 1D).

- **Quantification of the newly formed bone volume**

  In the micro-CT images of the explants of NuOss™, Bio-Oss®, and Collagraft™ bone spicules could be clearly seen (white arrows; Figures 3A). Also, some explants of Vitoss® showed some bone spicules, however this was not seen in all explants. No bone formation was observed within the CopiOs™ material. Quantification of bone by micro-CT analysis (Figure 2C) revealed that NuOss™ implants contained the highest percentage of newly formed bone at 11.40% ± 1.48%, which was significantly higher than Bio-Oss® at 4.99% ± 0.82%, Collagraft™ at 3.04% ± 0.30%, and Vitoss® at 1.32% ± 0.14% (p<0.001). Bio-Oss® contained significantly more bone than Collagraft™ (p<0.001) and Vitoss® (p<0.05), and Collagraft™ contained significantly more bone than Vitoss® (p<0.001). The volume of new formed bone was also calculated by histomorphometric analysis of the explants, which corresponded well (R² = 0.971) to the micro-CT data.
Figure 1: High-resolution micro-CT was used to analyse the internal structure of the materials with respect to (A) volume fraction of the CaP grains (B) average grain size (C) surface area of CaP (D) specific surface area of the CaP grains. Statistical significance is compared to NuOss™ unless otherwise indicated. *: p < 0.05, **: p < 0.01, ***: p < 0.001.

The available surface area is of importance for the bone forming capacity of the scaffold materials as it has previously been shown that CaP granules act as a local anchorage site for human periosteal cells [15], hence directly influence in vivo tissue formation. Additionally the scaffold materials with a low (Vitoss® and Collagraft™), medium (Bio-Oss®) and high (NuOss™) bone forming capacity can be discriminated mainly based on surface area, average grain size and volume fraction of CaP. Each of these parameters has previously been shown to have an effect on bone formation.

- **Assessment of bone interconnectivity and the spatial localisation of the bone formed within the scaffold structure**

Figure 2B shows on a typical cross-sectional micro-CT image of a NuOss™ explant two interconnected bone spicules when analysed in three dimensions (indicated by the blue and yellow areas). For most of the explants of the different scaffold materials highly interconnected bone spicules were found. In order to locate the bone formed within the scaffold structure, the volume fraction of the CaP grains (Figure 3B) and the corresponding volume of newly formed bone (Figure 3A) were calculated within confined cylinders containing only the core, the middle and the edge region of the explant as indicated in figure 3D. The trendlines in both figures 3A and 3B show that there is a clear link between the volume fraction of the CaP grains and the volume of newly formed bone.
Figure 2: (A) Typical high-resolution micro-CT images of NuOss™, Bio-Oss®, Collagraft™, CopiOs™ and Vitoss® implants seeded with one million cells eight weeks after implantation (isotropic voxel size = (4.5µm)³). Bone spicules are indicated with white arrows. The example shown in (B) displays 2 interconnected bone spicules when analysed in three dimensions (indicated by the blue and yellow areas). (C) Quantification of the newly formed bone volume in comparison to total implant volume allowed a bone volume fraction calculation to be carried out (n=4: duplicate implants in two mice). Statistical significance: *: p < 0.05, **: p < 0.01, ***: p < 0.001. Scale Bar = 1mm.

Figure 3: For the different scaffold types (A) the micro-CT-based volume fraction of the newly formed bone distributed over the core, middle and edge of the scaffold and (B) the micro-CT based volume fraction of the CaP particles distributed over the core, middle and edge of the scaffold, showing the same distribution as the volume fraction of newly formed bone. (C) Typical cross-sectional micro-CT images of a NuOss™ explant, indicating how the core, middle and edge region are defined (scale bar = 1 mm).
Conclusion

After validation, micro-CT image analysis enables an in-depth 3D and quantitative characterization of the material properties of CaP scaffolds and their in vivo bone formation capacity in combination with hPDCs. The results from the micro-CT image analysis have been used to link specific scaffold properties to the scaffold’s bone forming capacity, which can provide a rationale for initial scaffold selection and/or design based on the materials characteristics.

References