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3D Imaging of Tissue Integration with Porous Biomaterials

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Abstract

Porous biomaterials designed to support cellular infiltration and tissue formation play a critical role in implant fixation and engineered tissue repair. The purpose of this Leading Opinion Paper is to advocate the use of high resolution 3D imaging techniques as a tool to quantify extracellular matrix formation and vascular ingrowth within porous biomaterials and objectively compare different strategies for functional tissue regeneration. An initial over-reliance on qualitative evaluation methods may have contributed to the false perception that developing effective tissue engineering technologies would be relatively straightforward. Moreover, the lack of comparative studies with quantitative metrics in challenging pre-clinical models has made it difficult to determine which of the many available strategies to invest in or use clinically for companies and clinicians, respectively. This paper will specifically illustrate the use of microcomputed tomography (micro-CT) imaging with and without contrast agents to nondestructively quantify the formation of bone, cartilage, and vasculature within porous biomaterials.

1. Introduction

Porous biomaterials have long been used to enhance implant fixation and more recently to engineer the formation of three-dimensional (3D) tissues *in vitro* and *in vivo*. Biodegradable scaffolds composed of various polymers or ceramics serve as templates for tissue repair and regeneration. Scaffolds ranging from soft injectable hydrogels to load-bearing, rapid-prototyped porous structures are typically designed to conform to the external geometry of a tissue defect and facilitate the synthesis of new extracellular matrix (ECM) with the goal of restoring tissue function. The identification of biomaterial scaffolds optimized for tissue regeneration is an exceptionally challenging endeavor given the broad spectrum of compositional and architectural design variables and the complexity of tissue-biomaterial interactions. Moreover, biomaterial scaffolds are increasingly being used as delivery vehicles for biologics including cells, genes, peptides, and growth factors. Efficient methodologies that provide quantitative assessment of biomaterial scaffold integration with newly-formed tissues are thus essential to objectively compare the efficacy of different functional regeneration strategies.

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Histology continues to be the standard method for evaluation of host tissue reaction to biomaterials and ECM formation within the interconnected pores of scaffolds. The combination of exceptional in-plane resolution and cellular detail provided by histology is unparalleled, and histomorphometric analysis can be used to estimate cell numbers and tissue ingrowth. Histological analysis can, for example, readily identify the recruitment of macrophages and other cells associated with an inflammatory response to implanted biomaterials. Histological sections can also be stained with appropriate polychromatic dyes to identify multiple tissue types or viewed under polarized microscopy to assess ECM organization.

While histological methods offer unique capabilities, assessment of biomaterial-tissue interfaces is often difficult due to physical and chemical mismatches that may result in distortion artifacts or tissue separation during sectioning. Moreover, histologic processing is time-consuming and the resulting 2D sections provide an incomplete and potentially misleading representation of 3D tissue formation within porous scaffolds. Histological analysis therefore does not provide an efficient means of quantifying 3D tissue formation within multiple biomaterial samples and is thus not ideal for statistical comparison among experimental groups.

Imaging techniques are serving an increasingly important role in the rigorous characterization of biomaterial properties and function. Sophisticated 2D imaging technologies have been developed to complement histological evaluation and probe complex biological events occurring at the interface between tissues and biomaterials [3–5]. However, there is a clear need for high resolution 3D imaging technologies that reveal the spatial distribution of tissues forming within porous biomaterials *in vitro* and *in vivo*. Moreover, for regeneration of vascularized tissues such as bone or muscle, the ability to quantify 3D vascular ingrowth would be tremendously valuable, particularly for studies exploring the potential to enhance regeneration via therapeutic angiogenesis strategies [6].

The resolution required for analyzing tissue synthesis and vascular ingrowth within porous biomaterials is in the range of 1–30 microns. The imaging modality that has been most extensively applied for this purpose, particularly for bone tissue engineering studies, is microcomputed tomography (micro-CT). Micro-CT provides rapid reconstruction of high resolution 3D images and quantitative volumetric analysis of x-ray attenuating materials or tissues. First developed in the early 1980's, micro-CT imaging combined with morphometric analysis algorithms is currently the gold standard for quantifying 3D changes in the volume and morphology of trabecular and cortical bone associated with skeletal fragility.

Evaluation of mineralized matrix synthesis within porous biomaterials is a natural extension of micro-CT imaging, and recently developed *in vivo* scanners provide the opportunity for longitudinal monitoring of bone formation within biomaterial scaffolds post-implantation. Through the use of selected contrast agents, the boundaries of quantitative micro-CT analysis can also be expanded beyond mineralized tissues to include soft tissues such as cartilage and blood vessels. In addition to detailed 3D morphology, local voxel attenuation can provide another level of information regarding the type or density of local tissue synthesized. Micro-CT imaging is therefore a powerful tool widely available to biomaterials scientists and tissue engineers in many research settings for quantifying the volume, density, and distribution of tissues and blood vessels formed within 3D porous biomaterials.

2. Mineralized matrix synthesis

Micro-CT analysis has been used for many years to quantify trabecular bone morphology. It is not surprising, therefore, that several groups have used micro-CT scanners to assess mineralized matrix formation within a variety of porous biomaterial scaffolds *in vitro* and *in*

vivo [7–9]. Bone ingrowth throughout scaffolds may be limited by several factors, including incomplete pore interconnectivity, suboptimal degradation characteristics, and poor mass transport due to the initial absence of vascularization [10]. Micro-CT imaging can be used for the dual purposes of first quantifying the baseline architecture of the porous scaffolds (e.g. pore size, interconnectivity, anisotropy) and then relating those parameters to the amount and distribution of mineralized matrix formation [1,2,11,12]. Such studies can be performed *in vitro* within bioreactor systems or *in vivo* following implantation at ectopic sites or into critically-sized bone defects.

Image-based quantification of mineralized matrix volume requires accurate segmentation of mineralized tissue from other materials or tissues within the scanned region [11]. Selection of one or more global thresholds for x-ray attenuation is the simplest and most efficient method of segmentation. This approach works well for detection of mineralized ECM formed within low attenuating materials such as polymeric scaffolds. However, more sophisticated segmentation algorithms may be needed to separate multiple materials with overlapping density distributions [13]. For example, it is difficult to isolate small volumes of bone formed on the surface of hydroxyapatite or other bioceramic material scaffolds. In some cases, manual contouring of 2D image slices can be used to separate spatially distinct materials within a scan volume.

Another important issue to consider is that the density of newly-formed mineralized matrix is typically substantially lower and more variable than that of mature lamellar bone. Quantification of mineralized matrix volume can therefore be highly sensitive to the chosen threshold value. The selection of an appropriate threshold value is often a compromise between not over-estimating the volume of relatively larger structures and not missing smaller or less mineralized regions. A common approach to address this issue is to compare 2D grayscale and thresholded regions visually side by side and then maintain a consistent selected threshold value for all groups within a given experiment. For new applications, it is advisable to evaluate the sensitivity of the results to threshold value and compare thresholded image slices to registered histological sections. For applications involving highly variable attenuation levels or very thin structures, it may be informative to report data at more than one threshold value [14].

Taking advantage of the nondestructive capability of micro-CT imaging, repeated scanning can be used to monitor cell-mediated mineralization rate within constructs over time in static culture or as a function of flow rate and time in perfusion bioreactors [8,15]. The distribution of mineralized matrix formation can be determined by analyzing different volumes of interest. For example, static culture of cell-seeded constructs in osteogenic media creates a mineralized shell around the periphery due to mass transport limitations and the absence of a vascular supply *in vitro*. By analyzing a core volume at the center of constructs, it can be demonstrated that dynamic culture systems that enhance mass transport not only increase the amount of mineralized matrix synthesis but also produce a more homogenous distribution throughout constructs of clinically relevant size (Figure 1). 3D bioreactor systems in concert with micro-CT imaging can be used to objectively compare the mineralizing capacity of different osteogenic cell sources or assess cell-scaffold interactions prior to *in vivo* testing.

For implantation studies, micro-CT can be applied either to analyze retrieved samples or to scan defect regions *in vivo* using new generation live animal scanners [11,16–21]. Because micro-CT imaging is nondestructive, retrieved samples remain available for histological evaluation or biomechanical testing. The volume of bone ingrowth into polymeric scaffolds implanted into bone defects correlates well with functional integration strength as measured by post-mortem biomechanical testing (unpublished data) [18]. Noninvasive *in vivo* scanning allows time course and rate of mineralization data to be collected [2]. For fractures or segmental

bone defects, non-metallic fixation devices must typically be used to avoid artifacts in the reconstructed 3D micro-CT images. Although repeated exposure to ionizing radiation can be a concern, scanning at lower resolution, in a limited region, and at a reduced frequency over the course of an experiment can minimize this potentially confounding factor [22].

3. 3D vascular ingrowth

Revascularization is a critical issue in tissue engineering, and regenerative strategies increasingly incorporate angiogenic growth factors or vascular progenitor cells [23–27]. The infiltration of scaffolds with vascular structures can be visualized and quantified using 3D micro-CT analysis combined with a perfused contrast agent. Radiodense *in vivo* contrast agents provide the opportunity for longitudinal analysis and have been applied for studies of organ or tumor vascularization [28,29]. However, *in vivo* micro-CT imaging has not been used previously to evaluate vascular ingrowth into biomaterial scaffolds likely due to difficulties associated with contrast level, resolution, segmentation, or limited blood pool residence time. Repeated *in vivo* scanning at high resolutions also requires consideration of the potential effects of ionizing radiation on cellular activity during the repair process [22]. Although 3D *in vivo* vascular imaging remains a challenge, vascular ingrowth into 3D scaffolds can readily be evaluated postmortem using a perfused contrast agent that polymerizes within the vessels, creating a stable radiodense cast [30].

Using a murine hind limb ischemia model, Duvall *et al.* recently demonstrated the utility of contrast-enhanced micro-CT analysis to quantify 3D vascular network morphologic parameters, including vessel volume, thickness, number, connectivity, and degree of anisotropy [31,32]. Briefly, the technique involves perfusion of a radiodense silicone rubber contrast agent containing lead chromate (Microfil MV-122, Flow Tech; Carver, MA) through the vasculature immediately following euthanasia. The technique requires some experience or practice and has primarily been implemented in small animal models. To facilitate segmentation of the vascular structures, samples may be demineralized prior to micro-CT scanning. Incomplete perfusion is readily apparent by visual inspection since the yellow-colored contrast agent in vascularized muscles and organs is absent. Artifacts can also result from perfusing at too high of a pressure, resulting in leakage of the contrast agent from the vessels and producing large bulbous structures in the 3D images that are clearly not blood vessels.

The effects of image resolution and threshold value on detectable vascular structures and corresponding morphology parameters have previously been assessed and directly compared to transmitted light microscopy [31]. Collateral growth of arteriole-sized vessels primarily responsible for reperfusion following ischemic injury in the mouse can be evaluated using approximately 30 μm voxel scans. High density capillary beds can typically be detected at this level, but individual capillaries can not be resolved. At the cost of increased scan time, smaller voxel sizes can be used to resolve smaller vessels associated with angiogenesis. The limits of scanning refinement are also a function of sample size and configuration of the x-ray source and detector. Importantly, micro-CT based measures of vascular growth following ischemic injury correlate well with laser Doppler imaging assessment of limb perfusion and functional testing of muscle function [32].

The micro-CT vascular imaging technique has recently been applied to study heart morphogenesis and vascular growth associated with ischemic injuries, fracture healing, skeletal development, and engineered bone repair [21,24,30–36]. Rai *et al.*, for example, showed that platelet rich plasma delivery within polycaprolactone/tricalcium phosphate composite scaffolds enhanced early revascularization of 8 mm rat segmental defects and improved the rate of long-term fusion across the gap region [21]. The protocol for this study

was approved by the Georgia Tech Institutional Animal Care and Use Committee. We have used the same model and methods to evaluate interactions among co-delivered osteoinductive and angiogenic recombinant human growth factors, including BMP-2, TGF- β 3, and VEGF (Figure 2). Mooney and co-workers have also employed the vascular imaging technique to demonstrate that biomaterial-mediated release of vascular endothelial growth factor within scaffolds accelerates recovery of tissue ischemia in localized regions [36]. Nondestructive contrast enhanced micro-CT imaging is thus a useful method to measure vascular ingrowth within 3D porous scaffolds and thereby quantitatively evaluate and compare therapeutic angiogenesis strategies for promotion of tissue repair and regeneration.

4. Cartilaginous tissue formation

Although magnetic resonance imaging based methods have had a significant impact on clinical joint imaging, a high resolution 3D imaging technique to assess joint changes in small animal osteoarthritis models or cartilage matrix synthesis within biomaterials has previously been lacking. Palmer *et al.* recently introduced a technique to detect and quantify proteoglycan (PG) content by imaging the equilibrium partitioning of an ionic contrast agent via micro-CT (EPIC- μ CT) [37]. The principle of the technique relies on a negatively charged contrast agent (Hexabrix 320, Mallinckrodt, Hazelwood, MO) equilibrating within cartilaginous tissues at concentrations inversely proportional to the local concentration of negatively-charged sulfated glycosaminoglycans (sGAGs). Regions of low PG content should thus have relatively higher x-ray attenuation, while increasing PG levels should result in a reduction in contrast agent concentration and image voxel density.

The ability of EPIC- μ CT to nondestructively monitor changes in cartilage composition over time was validated in a bovine articular cartilage explant degradation model by showing a strong linear correlation between voxel attenuation and biochemical measurement of sGAG content [37]. Variations in the spatial distribution of voxel density indicated initial depletion of PGs from the deep zone after four days of exposure to interleukin-1 in culture and progressing throughout the explants by day 10. A proof of concept analysis of an intact rabbit femur indicated that contrast-enhanced cartilage regions could be segmented from subchondral bone, providing a detailed thickness map of the articular cartilage [37]. Subsequent studies have validated the ability of the EPIC- μ CT technique to quantify changes in the morphology and composition of articular cartilage in a rat knee arthritis model (unpublished data).

Finally, a preliminary study has shown that this technique can be used to monitor the synthesis of cartilage extracellular matrix within biomaterials. Bovine chondrocytes were seeded within hydrogel constructs in chondrogenic media conditions for 16 days. The constructs were equilibrated in Hexabrix and scanned by micro-CT every four days. The contrast agent was then desorbed and culture of the constructs continued. As expected, the average micro-CT attenuation level within the constructs decreased over time, consistent with an increase in cartilage matrix content (Figure 3A and 3B). The EPIC- μ CT technique can therefore be used to quantitatively and noninvasively monitor cartilaginous tissue matrix accumulation within biomaterial scaffolds over time in culture. Collectively, these studies also suggest that contrast-enhanced micro-CT imaging can be an exceptionally valuable addition to small animal studies of joint development, degeneration, and regeneration.

5. Conclusions

Nondestructive 3D imaging techniques such as micro-CT are increasingly providing a powerful set of quantitative tools to aid in the development and evaluation of porous biomaterials and new approaches to engineering tissues and organs. Quantitative imaging can reduce the cost and improve the efficiency of *in vitro* experiments. For *in vivo* studies, imaging methods can

provide quantitative metrics to objectively compare different approaches, complement histological and biomechanical analyses, and maximize the data collected from each animal. A key advantage of micro-CT imaging is that it provides adequate resolution to detect tissue synthesis and vascular ingrowth into the pores of biomaterial scaffolds. Moreover, most micro-CT scanners are integrated with software that provides quantitative morphometric analysis of thresholded and reconstructed 3D images. Finally, contrast agents may be used to overcome one of the limitations of micro-CT imaging and analyze non-mineralized tissues such as blood vessels and cartilage. Future developments include micro-CT scanners with nearly micron level resolution and novel contrast agents that target specific tissue components, cells, or biological processes.

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Fig. 1.

Micro-CT image (A) of a polycaprolactone (PCL) scaffold (5 mm diameter $\times$ 9 mm length) created using fused deposition modeling to create a defined porous architecture with 85% porosity. Micro-CT image (B) showing mineralized matrix synthesized within a PCL scaffold by amniotic fluid derived stem cells following 15 weeks of exposure to osteogenic differentiation media under dynamic culture conditions. In this experiment, type I collagen was lyophilized within the PCL scaffold to enhance cell seeding efficiency and six million stem cells were seeded per scaffold. Different volumes of interest may be defined for morphometric analysis to quantify the distribution of mineralized matrix. Whereas static culture conditions create a shell of mineralization isolated to the scaffold periphery, dynamic flow conditions provided by bioreactor systems produce a more homogenous distribution of mineralized matrix production throughout the construct (C).

Fig. 2.

Micro-CT image (A) of a 70% L-lactide and 30% DL-lactide co-polymer (PLDL) with oriented porosity created using a fiber-coating and porogen decomposition method [1]. Sustained release of co-delivered recombinant human growth factors (combinations of BMP-2, TGF- β 3, and VEGF) is achieved by polymerizing RGD alginate hydrogel within the PLDL scaffold pores prior to implantation into segmental bone defects [2]. Micro-CT images of vascular (B) and bone (C) ingrowth several weeks after implantation. Bone ingrowth can be quantified noninvasively using in vivo micro-CT scanners, while the vascular imaging technique must be done post-mortem.

Fig. 3.

Hydrogel constructs containing bovine chondrocytes were equilibrated with contrast agent and scanned using micro-CT every four days until day 16. The contrast agent (Hexabrix) is approved for clinical use in humans and does not affect cell function (unpublished data). Following each scan, therefore, the contrast agent could be desorbed and construct culture continued. The average attenuation within the constructs decreased over time consistent with the accumulation of cartilaginous extracellular matrix.