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MICRO-CT IN TISSUE ENGINEERING SCAFFOLDS DESIGNED FOR BONE REGENERATION: PRINCIPLES AND APPLICATION

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Micro-CT (micro-computed tomography) is a modern preclinical imaging method allowing non-destructive visualizations and structure analysis yielding at a resolution of a few micrometres. Tissue engineering scaffolds are a promising treatment for bone defects. Micro-CT application for the evaluation of tissue engineering scaffolds for bone surgery and traumatology application is increasing, which comprises in vitro, in vivo and ex vivo studies. Micro-CT itself is not able to replace conventional approaches completely, such as scanning electron microscopy (SEM) or histological examination, but it may offer important benefits regarding non-destructive approach, direct 3D model structure analysis, and visualization and time efficacy. The overview presented herein focuses on a micro-CT application in the field of tissue engineering scaffolds aimed at bone regeneration.

THEORETICAL AND DISCUSSION

Tissue engineering scaffolds and bone regeneration

The application of biomaterials in the form of scaffolds is considered as a promising method for treatment of bone defects resulting from pathological conditions (e.g. trauma, tumour, inflammation) [1]. The current gold-standard method is autologous bone grafting [2, 3]. However, it is limited by the bone defect volume and requires second operating field, which may increase morbidity and the probability of associated complications [4]. The possibility of using artificially produced biomaterials with similar effect on bone healing and predictable outcomes would be advantageous. Scaffolds for bone regeneration are usually porous and biodegradable. Scaffolds must provide temporary three-dimensional support for new bone formation with a desirable biodegradation profile. Various methods for preparation exist (e.g. freeze-drying, solvent casting, electrospinning, rapid prototyping) leading to different 3D structure [5, 6]. Scaffolds are frequently based on natural or synthetic polymers (e.g. collagen, polylactide) in combination with hydroxyapatite particles [7]. Collagen is frequently cross-linked to improve its chemical and mechanical properties [8, 9]. Such scaffolds should present appropriate chemical, biological and mechanical properties that are necessary to promote normal cellular behaviour and function [10]. A major challenge remains in terms of designing an ideal bone graft that mimics the features of natural bone, both in terms of the main composition and hierarchical microstructure [11]. Biologically active substances can be incorporated into scaffold structure to enhance its effect (e.g. BPM – bone morphometric proteins; [12]) and cell seeding (e.g. mesenchymal stem cells) can be performed prior to implantation [13].

Scaffold structure – its importance and methods of analysis

Three-dimensional scaffold structure is considered to have major influence on scaffold-tissue interaction, especially when considering porosity arrangement. 3D structure is usually very complex and complicated across scales. Prevalence of open pores with a high degree of interconnectivity and convenient pore sizes substantially influences cell migration, vascularisation,

fluid flow, mechanical properties, and available surface [14]. Pore sizes ranging from 100 to 300 μm were found to be the most efficient for new bone formation [15, 16]. However, pore size values are dependent on the method of measurement (our unpublished results) and no consent in this respect exists. Scaffold structure is usually evaluated in dry state, which may not be accurate, as implantation into the bone defect inevitably leads to scaffold hydration, which can change the three-dimensional structure due to hydrophilic compounds.

Scaffold structure analysis is achievable by numerous methods. Total porosity can be assessed e.g. by using gravimetry. Open porosity can be measured by liquid displacement or mercury intrusion [17, 18]. In some scaffolds, the application of these methods may alter its structure. Liquid displacement may be influenced by scaffold swelling due to hydrophilic compounds. A common approach is scanning electron microscopy (SEM) image analysis, which allows the measurement of various structural parameters (e.g. pore diameters, area, shape, wall and struts thickness). SEM image analysis offers precise visualization, is readily available and affordable. However, there are many drawbacks. Based on sectioning, this approach is destructive, time consuming, orientation dependant and limited to two-dimensions. 3D structure evaluation is achievable by using the stereology method based on structure assumptions. Evaluation of pore size based on a 2D section may not be accurate because the designation of scaffold walls is often very unclear (it may not be evident, whether the structure is directly on or below the section plane). Differentiating between closed and open pores from SEM images is unreachable, and alternative methods (e.g. mercury intrusion porositometry) may alter the scaffold structure, as mentioned above.

Micro-CT analysis of scaffold structure

Basic principles

Micro-CT (microtomography) is an X-ray preclinical imaging method, which enables both 2D and 3D structure analysis and visualization [19, 20]. Important benefits are non-destructivity, time efficacy and direct 3D model analysis. Isotropic pixel or voxel size in modern micro-CT devices is below 1 μm, but continuous improvement of resolution in micro-CT devices has to be considered, especially when evaluating previous studies. Its employment in the field of tissue engineering is still increasing, especially in the structure characterization of biomaterials. However, its availability is still limited because of its high acquisition price.

Specimens are scanned in micro-CT, usually in air or in a plastic tube, placed on a microstage inside a device. Appropriate specimen size differs according to the micro-CT device. In *ex vivo* desktop micro-CT designed for material testing the usual specimen size is 0.1 - 10 cm^3, while in *in vivo* devices, specimen size can be larger, enabling live mice or rats can be subjected to scanning. Scanning parameters comprise of resolution (pixel size), camera binning, source, current, filter, rotation step, 180° or 360° rotation etc. Image acquisition leads to a dataset of projection images, which are reconstructed into a 2D cross-section greyscale image dataset (usually based on a modified Feldkamp algorithm; Figure 1). Image data can be subjected to further modifications (e.g. creating regions or volumes of interest) and image processing (e.g. noise reduction, morphological operations, binary operations). Visualizations may be achieved using 2D virtual sections or 3D visualizations created by volume or surface rendering. Computed tomography generally leads to the presence of specific artifacts in image data, such as ring artifacts, beam hardening or misalignment and the presence of image noise, which may negatively influence visualizations and analyses [21].

Micro-CT analysis

Prior to analysis, image data must be converted from greyscale to black and white pixels (binarization), so the object (white colour) and background (black colour) are clearly differentiated. This process may significantly influence the results of the following analysis. The influence depends on the structure of the analysed object (simple or complex), material composition, homogeneity and image data quality (e.g. image noise, artifacts). The influence of image binarization on the structural parameters was evaluated for bone tissue [22, 23]. However, the binarization effect in scaffolds is yet to be sufficiently studied, and is complicated by the overlapping X-ray density of its materials, thin structures and partial volume effect [24, 25].

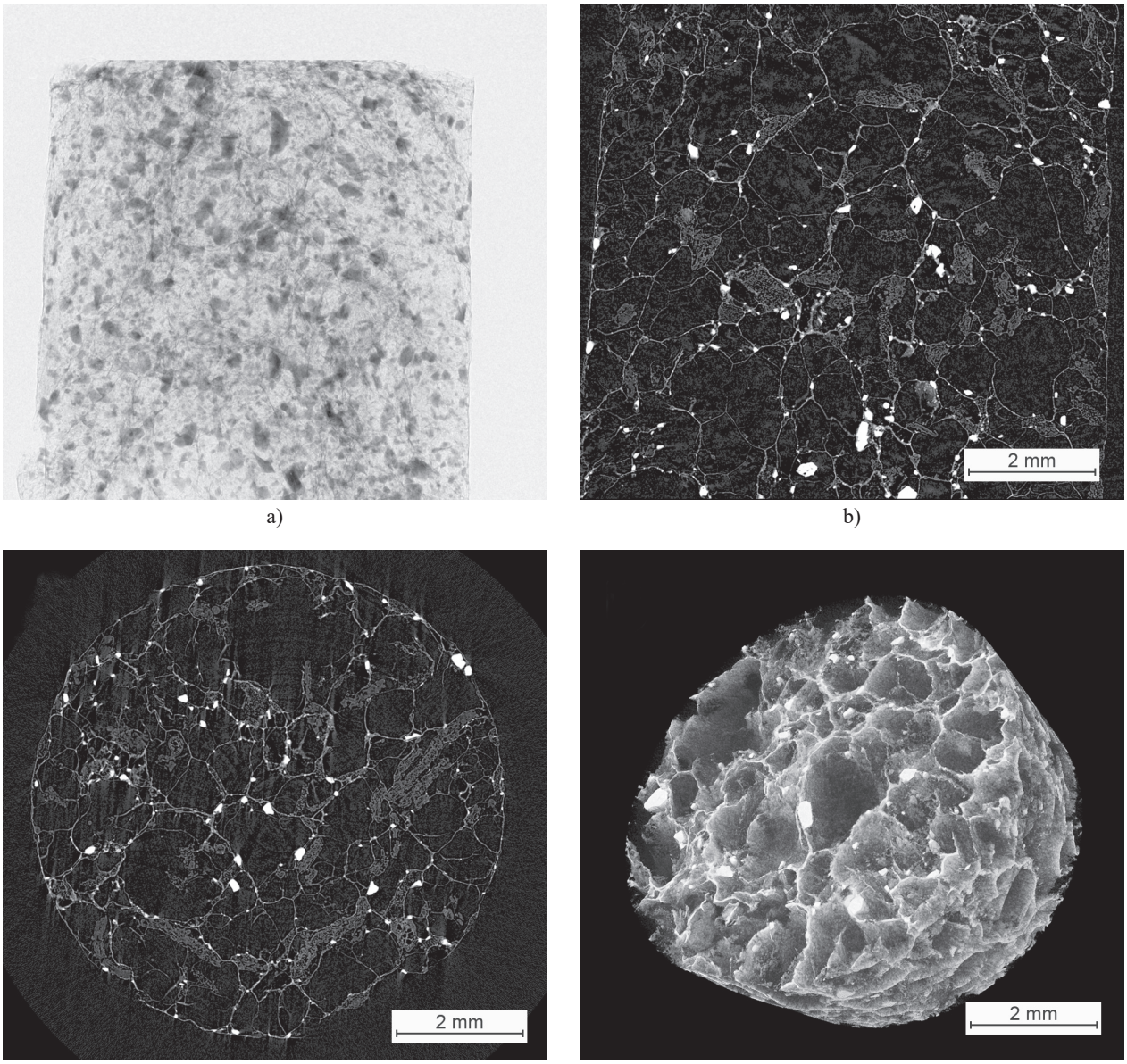
Micro-CT enables the evaluation of many structural 2D and 3D parameters. Micro-CT offers 2D analysis based on individual sections (slices) or direct 3D model analysis, which is orientation independent. Basic parameters are e.g. specimen volume, surface, intersection surface, number of objects and porosity (open and closed pores). Volume and surface parameters are measured using a marching-cubes algorithm. Structure thickness and separation is calculated based on a medial axis computation and sphere-fitting algorithm, which offers new insight into structure evaluation in 3D, and may be applied for pore size measurement [26, 27, 28]. Many other parameters may be obtained as a result of 3D analysis e.g. anisotropy, Euler number, fractal index and Structure Model Index [20, 26]. However, certain parameters provided by micro-CT analytical software must be used with caution, since their interpretation is not straightforward in such complex structures. Image processing and analysis can be partially automated leading to better time efficacy, and a reduction of subjectivity in evaluation. Micro-CT visualizations prior to histological examination may improve sectioning orientation thus

improving its outcomes. Together with its advantages, micro-CT presents important drawbacks: limitation by resolution (even though modern devices offer pixel or voxel size below 1 μm, it is still substantially less than e.g. in SEM) and high-dependency on image data and its processing prior to analysis.

Micro-CT application: *in vitro, in vivo, ex vivo*

In vitro

Tissue engineering scaffold structure is usually studied in dry state and micro-CT may be a complementary method to conventional analysis [15, 17, 29]. The fabrication process and its modifications can be evaluated and thus optimized with regard to appropriate structural parameters (e.g. total porosity, open porosity, pore size). Based on micro-CT results, the most prospective types of fabricated scaffolds can be selected for further evaluation. Structural changes related to hydration are considered very important, but their assessment is difficult to reach. Micro-CT is able to provide such analysis. However, only a few micro-CT studies focus on the evaluation of both hydrated and dry-state scaffolds to date [30, 31]. There are technical difficulties resulting from scanning hydrated scaffolds, e.g. insufficient X-ray contrast between scaffold matrix and solution or motion artifacts caused by gravitation, specimen rotation and swelling. X-ray contrast has to be enhanced by contrast agents (e.g. phosphotungstic acid or iodine solution;



a) b) c) d)

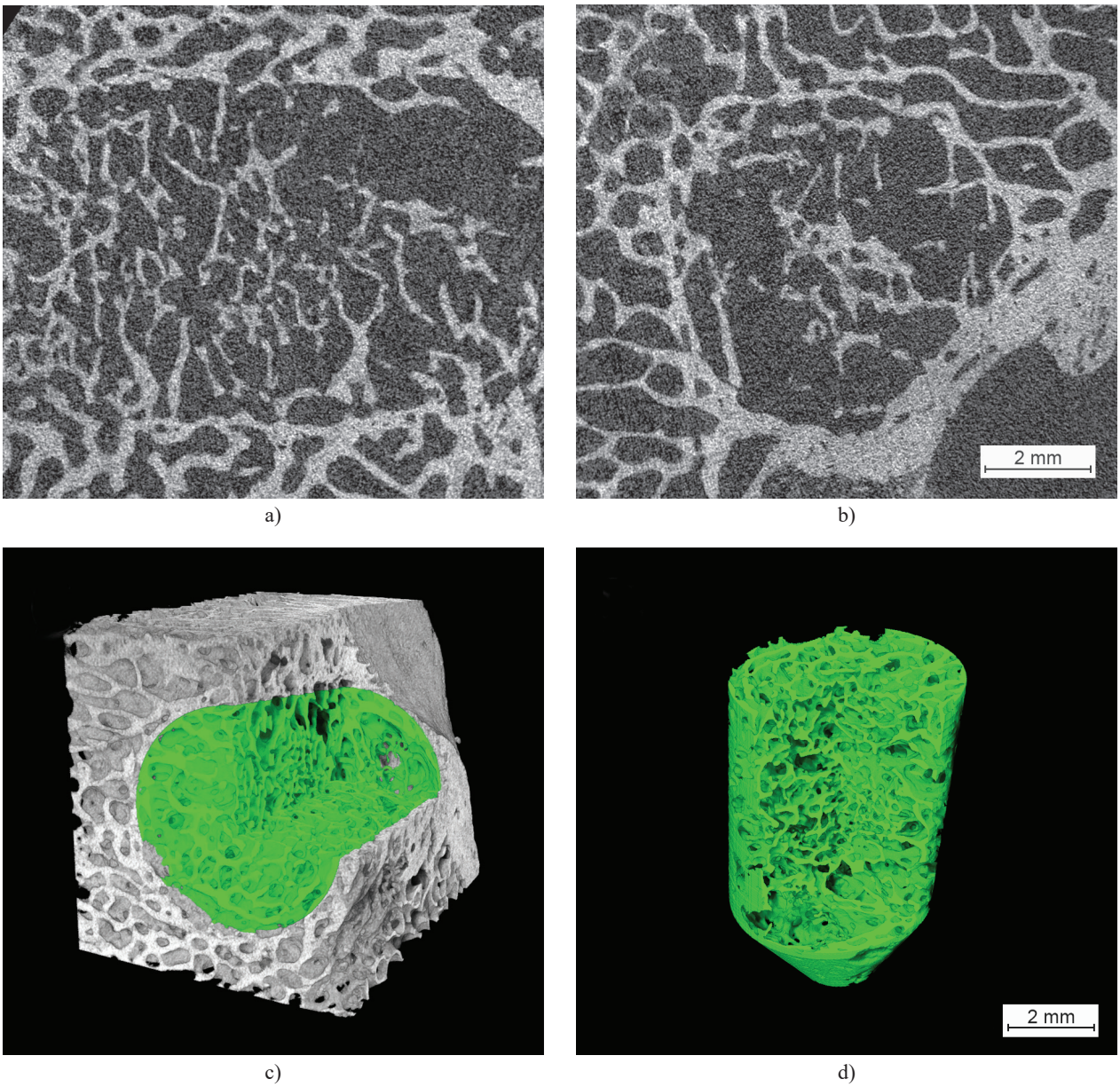
Figure 1. Illustration of micro-CT scanning of collagen-based scaffold using desktop micro-CT SkyScan 1272 with 4 μm pixel size (Bruker, Belgium): a) 2D projection image, b, c) reconstructed 2D cross-section images d) 3D visualization.

[32]). However, its influence on scaffold properties remains relatively unknown, and therefore may lead to alterations.

Cell seeding (e.g. by mesenchymal stem cells) prior to implantation may be performed in order to improve the effect on bone healing and regeneration [13, 9, 33]. Assessment of the presence and distribution of seeded cells in the whole scaffold in 3D would be advantageous, otherwise it is limited to specimen sectioning. Only a few studies have focused on cell visualization [34]. Cells have to be X-ray contrasted to provide appropriate contrast. However, this is not standard application since the resolution of even modern devices is insufficient (or nearly insufficient) regarding cell size in 3D, and cell to scaffold X-ray contrast. Nanocomputed tomography (nano-CT), a new developing field of preclinical imaging, may introduce cells and scaffold scanning as a common procedure.

In vivo and *ex vivo*

Micro-CT can be employed for evaluation of implantation of tissue engineering scaffold into bone defect. There are two basic approaches: *in vivo* and *ex vivo* [35]. The first uses an *in vivo* micro-CT device capable of scanning of live animals (usually mice or rats), which



a) b)

c) d)

Figure 2. Demonstration of micro-CT 2D and 3D visualizations of bone defect treated by autologous bone graft. Images from preliminary ex vivo study: a, b) 2D cross sections: former bone defect is evident, but new bone tissue formation is clearly observed, c) volume of former bone defect is depicted with new bone tissue d) segmented new bone tissue, which may be subjected to 3D analysis.

are anesthetized and their vital functions are controlled. This method is well established for studying metabolic diseases influencing bone tissue (e.g. osteoporosis), fracture healing, bone tissue engineering or tumour bone metastases [36, 37, 38, 39]. The evaluation of the same specimen at a different time enables both the precise observation of newly generated bone tissue and scaffold material degradation. This leads to a higher efficiency of such experiments based on an increasing number of measurements. Moreover, the number animals experimented on can be reduced, which is advantageous from the ethical point of view and leads to financial cost reduction. Repeated scanning of the same animal does not result in the alteration of bone structure and its healing processes [40, 41]. *In vivo* scans are much faster (minutes) compared to *ex vivo* scans (hours). However, resolution is lower compared to *ex vivo* devices that may negatively influence the evaluation of thin bone structures and in particular scaffold structure. Since micro-CT detects only X-ray dense objects, differentiation between new bone and scaffold structure is based mainly on subjective assessment of visualized structure and may not be evident. The detection of less mineralized bone and osteoid is not sufficient, so verification by histological examination is appropriate. *Ex vivo* micro-CT analysis combines benefits of non-destructive high-resolution analysis and scaffold-tissue interactions (Figure 2). Specimens are harvested after the euthanization of the experimental animal in a defined time interval after implantation. Volume of interest (VOI) is defined in selected specimen (usually in the place of former bone defect), so effect of different treatment approaches may be compared (e.g. autologous bone graft versus tissue engineering scaffold implantation). Visualization of VOI in 3D and virtual 2D sections in any selected plane helps understanding to bone regeneration and scaffold degradation process. Volume of new bone, its structure, homogeneity and spatial distribution (e.g. peripheral versus central regions) is considered.

CONCLUSION

Micro-CT is an important preclinical imaging method for structure analysis and visualization. Its application in the field of tissue engineering is notably increasing. Promising bone defect treatments comprise of tissue-engineering scaffolds. Their 3D structure is of great importance and micro-CT can be employed in different settings (*in vitro, in vivo* and *ex vivo*) for its evaluation. Major advantages are non-destructivity, direct 3D model analysis and time efficiency. In cases following histological examinations, micro-CT improves the orientation of specimen sectioning. The major disadvantage of micro-CT is a high dependency on image data processing, which is generally not standardized. The overview presented herein focuses on micro-CT application in bone engineering scaffolds.

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