The necessity of X-ray computed tomography to reveal the hierarchical structure of materials

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Abstract

Two recent developements of μCT to reveal the hierarchical structure of materials are presented. In the first case study, the determination of the permeability of a non-crimp carbon textile reinforcement using simulations of the fluid dynamics with µCT based voxel models is revealed. The voxel models are constructed from the μCT images of the material samples using a statistical algorithm for image segmentation, based on the Gaussian mixture model. The method is architecture independent and allows studying the variability of the permeability for different unit cells of the textile. In the second case study, the use of contrast agents for the visualization and quantification of soft biological tissues is described. More specifically, contrast-enhanced μCT (CE-CT) facilitates the distinction between bone from cartilage, bone marrow and blood vessels in implanted biomaterials that were seeded with osteoprogenitor cells, to be screened as potential therapy for the healing of large bone defects.

Keywords: hierarchical structure, materials, X-ray computed tomography

1 Introduction

X-ray computed tomography is a very important tool for doctors, material scientists, geologists, biologists, civil engineers, bioengineers, dentists, etc., all dealing with materials of which the fine internal structure or the changes within the material are of utmost importance to understand the behaviour of the material or to have insights in the processes going on. X-ray CT is now well accepted in those disciplines as well as submicron or nano tomography facilities. In this paper details are given of two recent case studies, obtained at the Department of Materials Engineering, to reveal the hierarchical structure of materials (textiles and biological tissues) for behaviour studies or modelling purpose or materials optimization. A high resolution X-ray nanoCT system is used having a nanofocal spot X-ray tube, which differs from conventional medical CT scanning and microCT in its ability to resolve details as small as a few hundreds of nanometers in size.

1.1 Case study 1: Permeability of a non-crimp carbon textile reinforcement

Fabrication of fibre reinforced composites by (liquid) composite moulding involves impregnation of the dry reinforcement with a low viscosity resin, which is injected into the mould cavity. In the process of impregnation, the liquid resin flows through the system of channels inside the reinforcement, which can be considered as a porous medium. The flow of a liquid through a porous medium is described by the Darcy’s law, which states a dependence of the flow velocity on the permeability of the medium, the viscosity of the liquid and the applied pressure gradient. The permeability of the composite reinforcement is determined by the size and shape of the flow channels inside it; it is usually anisotropic and can be described by a second order tensor. Permeability is an important parameter of the technological process, which determines the quality of the impregnation and the duration of the production cycle. For the simplest and ideal case of unidirectional arrangement of fibres, analytical models have been developed, which allow to calculate the longitudinal and transversal permeability as a function of the fibre radius and volume fraction. The structure of textile fabrics is more complex and includes two levels – the channel network between the yarns, and the intra-yarn channels, where the liquid can flow in the space between fibres. The intra-yarn structure can be considered as a unidirectional arrangement of fibres; its permeability can be estimated from analytical models with fair accuracy. The level of yarns, however, is more complex; its contribution to the permeability of the preform is determined by the parameters of the preform: weave type, fibre volume fraction, yarn linear density, sett and crimp. Calculation of the permeability of composite preforms can be done by numerical methods, through the flow simulations, but it requires a detailed description of the geometry of the flow channels.

This paper presents the results on the determination of the permeability of a non-crimp carbon textile reinforcement based on μCT images. The computation of the permeability is done using simulations of the fluid dynamics with voxel models, constructed from the μCT images of the material samples. The voxel models are constructed using a statistical algorithm for image segmentation, based on the Gaussian mixture model, which belongs to the class of supervised classification. This method does not require calibration with experimental data due to the fact that it is based on the quantities, extracted from a μCT image, which reflect local physical properties of the material. The results are validated with experimental data. The validated calculation of the
permeability allows studying its variability for different unit cells of the textile, extracted from an image of the same sample.

1.2 Case study 2: Contrast-enhanced µCT (CE-CT)

Currently, tissue engineering (TE) is still facing challenges with respect to the quantity and quality of its products. Advanced 3D imaging is one of the enabling technologies that are of increasing importance in the field of TE in order to assess and obtain TE products of high quality. Indeed, TE constructs (scaffolds with cells and/or growth factors) are 3D structures with complex spatial heterogeneity, meaning that traditional 2D imaging techniques are insufficient to characterize them or assess their quality.

Organs and biological tissues have a complex heterogeneous 3D structure. Thus, measurements of these tissues made in 2D, such as histomorphometry, only partially reveal the full 3D structure and interconnections between different tissues. Innovations in imaging techniques are fundamental to fully understand the complex 3D tissue structures. Currently, the standard technique for evaluating organs and biological tissues is histological sectioning. It has a high discriminative power, both on tissue and cellular level. However, it shows limited potential for quantifying 3D properties of the tissues as it is destructive and costly in terms of time and resources. Most importantly, in standard settings, it only allows the assessment of the tissue distribution in 2D, with loss of information due to a restricted sectioning orientation and with limited depth resolution.

X-ray microfocus computed tomography (micro-CT) has frequently been applied as 3D quantitative imaging technique to assess mineralized skeletal tissues [1-3]. However, soft tissue contrast is inherently poor. Phase contrast imaging, in most cases only available by synchrotron radiation, could be a solution because of its high attainable spatial and contrast resolution. However, routine access to synchrotron facilities is limited. Therefore, a recent shift in µCT imaging focuses on the use of X-ray opaque contrast agents for visualizing soft tissues, such as cartilage [4-9], blood vessels [10-12] and connective tissues [1, 13]. For cartilage, containing negatively charged glycosaminoglycans, specific ionic and anionic contrast agents have been developed [4-9]. For other soft tissues, however, there are currently no tissue-specific X-ray opaque contrast agents. Using trial-and-error, several groups have assessed different chemicals to stain these tissues with varying degrees of success, but without in-depth validation or sufficient knowledge of the tissue-specific binding mechanisms [14, 15].

In this paper, a significantly improved imaging technique is presented that can provide spatiotemporal information on the dynamic biological processes during skeletal disease development or treatment, and 3D tissue formation, namely contrast-enhanced µCT (CE-CT). It combines µCT, with a high attainable spatial resolution, with contrast agents. This combination allows 3D quantitative multi-tissue imaging, i.e. using one imaging modality for visualizing multiple tissues.

To validate the potency of CE-CT as a virtual histology tool for skeletal tissues, this study focuses on bone tissue engineering (TE), a multidisciplinary field of science focusing on healing large bone defects. It typically involves a 3D carrier material (scaffold) with cells, soluble signaling molecules or their combination (i.e. TE construct), to support bone forming processes.

2 Materials and Methods

2.1 Case study 1: Permeability of a non-crimp carbon textile reinforcement

- **Materials**

The material used in the study is a non-crimp carbon/epoxy composite from Saertex (540 g/m², +45/-45, French stitch). The manufactured test plate had a thickness of 4.0 mm and a resulting fibre volume fraction of 45.5%. The data on the permeability of the studied NCF reinforcement is presented in [21]: at a fibre volume fraction of 50.8% the saturated permeability was measured as 0.5×10⁻⁴ mm² (in the 45° direction to the production direction). Based on a linear fit of the fibre volume fraction – log (permeability) dependencies presented in [22] for non-crimp fabrics, similar to the one studied here, the permeability at fibre volume fraction of 45.5% can be estimated as (1…2)×10⁻⁴ mm². Three samples of different size were cut from the plate and scanned with a Nanotom S system [GE Measurement and Control Solutions, Germany]. Parameters of the tomographic acquisition are given in Table 1. The total number of unit cells in the scanned samples is 27.

Table 1. Parameters of the tomographic imaging.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Voxel size</td>
<td>2.25 μm</td>
</tr>
<tr>
<td>Number of projections</td>
<td>1800</td>
</tr>
<tr>
<td>Exposure</td>
<td>0.9 sec</td>
</tr>
<tr>
<td>Averaging</td>
<td>8</td>
</tr>
<tr>
<td>Skip</td>
<td>1</td>
</tr>
<tr>
<td>Voltage</td>
<td>50 kV</td>
</tr>
</tbody>
</table>

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- Construction of voxel models and permeability computation

In order to construct voxel models, the micro-CT images of the material samples were segmented using a method proposed in [23] (Fig. 1). The segmentation is based on two feature variables calculated from the image: the average grey value and the structural anisotropy. The structural anisotropy is calculated based on the structure tensor [24]. Based on the two variables, a statistical Gaussian mixture model is constructed, which consists of a set of two-dimensional Gaussian distributions for each component of the model (fluid/solid). The voxel model is constructed through classifying each voxel of the model to one of the components, based on the maximum probability, where the probabilities are calculated from the statistical model and the values of the feature variables at each voxel. Permeability calculations were performed with FlowTex software [25], developed at KU Leuven in collaboration with the Institute for Numerical Simulation at the University of Bonn.

![Sample images](image_url)

Figure 1: Micro-CT images of the carbon/epoxy samples, cross-sections orthogonal to the sample rotation axis in the micro-CT scanner.

In the context of the present study, segmentation is a problem of finding in the image a set of non-overlapping domains, corresponding to the components of the voxel model, which are solid and fluid phases. The task of segmentation therefore involves classification of each voxel of the model into a finite set of classes. This classification was done using two feature variables: average grey value and structural anisotropy. Denoting the grey value distribution in the image as \( I(p) \), the average grey value is calculated as:

\[
g(p) = \int_W I(p + r) dr
\]

where \( W \) is the integration window, and \( dr = dx_1 dx_2 dx_3 \). Structural anisotropy is defined as follows:

\[
\beta(p) = 1 - \frac{\lambda_1}{\lambda_3}
\]

where \( \lambda_1 \leq \lambda_2 \leq \lambda_3 \) are the eigenvalues of structure tensor:

\[
S_{ij}(p) = \int_W \frac{\partial I(p + r)}{\partial x_i} \frac{\partial I(p + r)}{\partial x_j} dr
\]
Here vector \( \mathbf{p} \) defines the position of the centre of the integration window in the global coordinate system of the image. Vector \( \mathbf{r} \) is the relative position of a pixel of the image inside the integration window. In the present study the integration window had a size of 17 x 17 pixels.

The segmentation is performed by constructing a statistical model for the \( g \) and \( \beta \) distributions in the form of a mixture of bivariate Gaussian distributions. In order to construct the model, small regions of interest (ROI) were selected in the image, which contained a single component of the material. The feature variables \( g \) and \( \beta \) were calculated inside the selected ROI on a regular grid, with the density of the grid chosen so that the total number of points was sufficiently large (>1000). The obtained points in i-th ROI were fitted with a bivariate Gaussian distribution \( N_i(\mathbf{\mu}, \Sigma) \), where \( \mathbf{\mu} \) and \( \Sigma \) are the mean and the variance of the distribution. Parameters of the distributions were calculated using maximum-likelihood estimation:

\[
\mathbf{\mu} = \frac{1}{N} \sum \mathbf{X}
\]

\[
\Sigma = \frac{1}{N-1} \sum (\mathbf{X} - \mathbf{\mu})(\mathbf{X} - \mathbf{\mu})^T
\]

where \( \mathbf{X} = [g, \beta]^T \). Note that the mean \( \mathbf{\mu} \) is defined on the basis of the variable \( g \), which is obtained as an average over integration window, whereas the \( \mathbf{\mu} \) itself is an average over a selected ROI. In general, orthogonal yarn systems in micro-CT images have slightly different distributions of feature variables (evidence for this was found in regard to the structural anisotropy). Due to this possible difference, two separate Gaussian distributions were created for the yarns with orthogonal primary orientations. The Gaussian mixture model therefore contained three components: matrix (fluid phase), and two components for the fibre bundles (solid phase). The segmentation of the image was done by computing for each spatial point in the image the probabilities \( P_i \) for this point to belong to each of the material components:

\[
P_i(\mathbf{X}) = \frac{1}{\sqrt{(2\pi)^d|\Sigma|}} \exp \left[ -\frac{1}{2} (\mathbf{X} - \mathbf{\mu}_i)^T \Sigma^{-1} (\mathbf{X} - \mathbf{\mu}_i) \right]
\]

The decision on how to classify each point was made on the basis of maximum probability:

\[
\mathcal{C}(\mathbf{X}) = \arg\max_i P_i(\mathbf{X})
\]

Here \( \mathcal{C}(\mathbf{X}) \) denotes the classification function, which maps the feature variable values \( \mathbf{X} \) into a finite set of material components, i.e. \( \mathcal{C}(\mathbf{X}) \rightarrow \{ \text{matrix, bundle1, bundle2} \} \).

The Gaussian mixture models created for the purpose of segmentation must contain statistical distributions that are well separated from each other, where the reliable classification decision is required. In the studied case a sufficient distance is required between matrix component and the components of the bundles. Separation between the two types of bundles is not required as they both represent the same phase of the model (solid phase). In addition, it may be useful to check the stability of the distribution parameters to the choice of ROI. In order to make this evaluation, the measure of the distance between Gaussian distributions \( N_i(\mathbf{\mu}_1, \Sigma_1) \) and \( N_2(\mathbf{\mu}_2, \Sigma_2) \) known as Bhattacharya distance was used, which is defined as follows:

\[
D = \frac{1}{8} (\mathbf{\mu}_1 - \mathbf{\mu}_2)^T \Sigma^{-1}(\mathbf{\mu}_1 - \mathbf{\mu}_2) + \frac{1}{2} \ln \left( \frac{|\Sigma|}{\sqrt{|\Sigma_1||\Sigma_2|}} \right)
\]

where

\[
\Sigma = \frac{\Sigma_1 + \Sigma_2}{2}
\]
2.2 Case study 2: Contrast-enhanced µCT (CE-CT)

- Tissue engineering explants

Animal experiments were performed in accordance with the Belgian legislation and were approved by the Ethical Committee of the Faculty of Biomedical Sciences of the University of Leuven. The tissue formation of TE constructs based on the combination of a calcium phosphate (CaP)/collagen composite scaffold, a growth factor and human periosteal derived cells (hPDCs) has been evaluated at 4 and 6 weeks post implantation in an ectopic nude mouse model using CE-CT.

- Contrast-enhanced nanofocus computed tomography (CE-CT)

With X-ray nanoCT a complete 3D set of images is acquired to visualize the internal architecture of a sample at the micron/submicron level in a non-destructive way. The nanoCT system applied was a Phoenix NanoTom S [GE Measurement and Control Solutions, Germany] with a 180 kV / 15 W high-performance nanofocus X-ray tube. Because of the relatively high X-ray attenuation of the stained constructs, a diamond-coated tungsten target was applied. The system was operated at a voltage of 65 kV and a current of 162 µA. To reduce beam hardening during the acquisition, a 1 mm filter of aluminum was used. The exposure time was 500 ms, and 2400 images were acquired over 360°. The fast scan mode (frame averaging = 1; image skip = 0) was used resulting in a scanning time of 20 minutes. The isotropic voxel size was (2.5 µm)³. During reconstruction [Datos|x, GE Measurement and Control Solutions, Germany], a beam hardening correction of 7 and a Gaussian filter of 6 was applied.

After a fixation step in paraformaldehyde, all samples were imaged without contrast agent. Hexabrix® 320 [Guerbet Nederland B.V., The Netherlands] was then used as contrast agent for a second scan. Finally, after washing overnight, all samples were immersed in phosphotungstic acid (PTA) and scanned a third time.

Hexabrix® 320 is an injectable solution of ioxaglate meglumine 39.3% and ioxaglate sodium 19.6%. Ioxaglate is a radio-opaque negatively charged ionic iodated dimer, which will be locally repulsed by the negative fixed charge density of the cartilage that results from the glycosaminoglycans (sGAG) in the cartilage. A solution of 20% Hexabrix® in phosphate buffered saline (PBS) was used. The incubation time was 1 hour.

PTA is a heteropolyacid containing tungsten, which makes it radio-opaque. It binds to fibrin, collagen, and fibers of connective tissues. A PTA concentration of 2.5 wt% in PBS was applied, and the samples were put in the contrast solution for 12 hours for sufficient diffusion of the contrast agent throughout the explants.

- Histology

After CE-CT imaging, the samples were decalcified in a 0.5M EDTA/PBS solution and embedded in paraffin. Sections of 6µm were stained with haematoxylin and eosin (H&E) for bone and bone marrow, and Toluidine Blue (TB) for the sGAG containing tissues.
- 3D image processing and visualization

Both Mimics® [Materialise, Belgium] and Avizo® Fire [FEI Visualization Sciences Group] were used for 3D image processing and visualization of the different CE-CT datasets. The histological sections were matched with the corresponding CE-CT images using DataViewer [Bruker MicroCT, Belgium] and an in-house developed MatLab tool [26]. Also for the co-registration of the multi-tissue CE-CT datasets DataViewer [Bruker MicroCT, Belgium] was used.

3 Results and discussion

3.1 Case study 1: Permeability of a non-crimp carbon textile reinforcement

The calculated permeability for all unit cells in the three studied samples is shown in Fig. 3. The permeability varies quite significantly across unit cells, in the range of \((0.5 \ldots 3.5) \times 10^{-4} \text{ mm}^2\), which is however in a good agreement with the experimental data \((1.0 \ldots 2.0 \times 10^{-4} \text{ mm}^2)\). Fig. 3 shows average values over the unit cells in the samples. Analysis of the correlation of permeability with the solid volume fraction in the unit cell models showed a significant negative correlation, i.e. permeability is lower with a higher solid volume fraction. Table 2 provides a summary of the results on permeability calculations and the correlation with the solid volume fraction in the models.

Figure 3: Permeability of the unit cells in the studied samples.
Table 2. Average permeability and correlation with solid volume fraction.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Permeability Ky, mm$^2$ Mean</th>
<th>STD</th>
<th>Permeability Kx, mm$^2$ Mean</th>
<th>STD</th>
<th>Pearson’s correlation with solid volume fraction Ky</th>
<th>Kx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample #1</td>
<td>7.23E-05</td>
<td>-</td>
<td>1.43E-04</td>
<td>-</td>
<td>-0.70</td>
<td>0.83</td>
</tr>
<tr>
<td>Sample #2</td>
<td>1.26E-04</td>
<td>4.60E-05</td>
<td>2.08E-04</td>
<td>8.23E-05</td>
<td>-0.93</td>
<td>-0.93</td>
</tr>
<tr>
<td>Sample #3</td>
<td>1.11E-04</td>
<td>2.72E-05</td>
<td>7.06E-05</td>
<td>3.90E-05</td>
<td>-0.39</td>
<td>-0.89</td>
</tr>
</tbody>
</table>

The computation of the permeability using voxel models depends on a correct definition of the phases in the model, which is the result of the segmentation procedure. The presented segmentation method is based on the two quantities – average grey value and structural anisotropy – which reflect local physical properties of the material. The average grey value reflects X-ray attenuation of the material, which is at a certain energy level proportional to its density and atomic weight (averaged). The structural anisotropy reflects local structural properties of the material and allows making a distinction between the matrix, which is structurally isotropic, and the reinforcement, which is structurally anisotropic due to the presence of fibers with a particular primary orientation. The construction of a Gaussian mixture model on the basis of the selected subset of data is known as supervised classification. In the case of micro-CT images of composite materials, the application of statistical methods is necessitated by the noise, always present in the CT image, and the variability of the material’s microstructure, which make all the derived quantities inherently non-deterministic. Compared to the existing approaches for the modelling of composite reinforcements on the basis of experimental data, the presented method does not require any significant manual effort for the data extraction to create a geometrical model. The amount of efforts needed is constant for each dataset, and not proportional to the size of the dataset.

Three samples of the non-crimp carbon epoxy composite were modelled, with the total number of unit cells modelled being 27. The results showed a good agreement with the experimental data. The variability of the permeability across unit cells is quite significant, in the range of (0.5…3.5)×10$^{-4}$ mm$^2$. The observed difference in standard deviation of the predicted permeability between samples #2 and #3 might be a result of a different number of unit cells studied in these samples. In the presence of a spatial auto-correlation in the local geometry of the preform (such as yarn trajectory), a larger volume of the material will result in a larger dispersion of the predicted properties. This corresponds to the observed difference, as a larger sample #2 shows a higher standard deviation of permeability compared to a smaller sample #3. The computed permeability shows a significant correlation with the solid volume fraction in a unit cell. The good agreement of the modelling results with the experimental data indicates that the segmentation procedure provides the phase domain boundaries that are close to the true ones.

3.2 Case study 2: Contrast-enhanced µCT (CE-CT)

CE-CT allows virtual 3D histology of sGAG-containing tissues in TE explants.

By comparing with TB staining (Fig. 4B – yellow circles), it is shown that Hexabrix® highlights sGAG-containing tissues (Fig. 4A– white circles) such as cartilage and non-mineralized osteoid. This is a confirmation of previous work, where it was shown that Hexabrix® even allows quantitative measurements of the sGAG content [4].

TB staining is a colorimetric histological stain, which could also allow quantitative measurements of the sGAG content. However, if there is no proper control within the same section, this can be challenging. Additionally, quantitative measurements are usually done on a global scale, for the full section. For these reasons, a one-to-one correlation between the grey-scales in the CE-CT image and the color in the TB-stained section is difficult. Segmentation of the CE-CT images will therefore be a challenge to be tackled in future work, and thus currently no quantitative measurements of the sGAG content can be made. Fig. 4C, shows an indicative 3D visualization of the sGAG-containing tissue together with the mineralized tissue and the scaffold remnants in a TE explant.
CE-CT allows virtual 3D histology of bone marrow and its blood vessels

H&E staining confirmed that PTA allows a grey-scale difference between bone and mineralized osteoid in the CE-CT images (Fig. 5A-B – black arrow), depending on the mineralization degree of the osteoid. The less mineralized the tissue is, the more the PTA stains the tissue, resulting in a lighter grey-scale. Additionally, comparing to H&E, it was proven that PTA stains bone marrow (Fig. 5A-B – dark grey arrow), but does not stain fat cells and large blood vessels (Fig. 5C-D – white and black arrows respectively). The fat cells and blood vessels have a similar grey-level, but with a connected component analysis, the 3D blood vessel network in the bone marrow compartment can be extracted (Fig. 5E). This provides a better insight in the blood vessel interconnectivity in the bone marrow compartment of the TE explants.

To conclude, CE-CT is a multi-tissue 3D imaging technique that can be used for virtual histology as it can reveal the 3D structure of different skeletal tissues (i.e. bone, cartilage, osteoid, bone marrow, fat cells and blood vessels) in explanted TE constructs. Since it can provide additional information to standard histomorphometry, with a spatiotemporal dimension, CE-CT could provide novel insights in the dynamic biological processes during tissue formation.
Figure 5. (A) A typical cross-sectional CE-CT image (using PTA staining – white arrow shows bone, dark grey arrow indicates bone marrow and the black arrows indicate osteoid) of a TE explant (after 6 weeks of ectopic implantation in a nude mouse model) and (B) the corresponding histological section (H&E staining – white arrow is bone, dark grey arrow is bone marrow and black arrow indicates osteoid). (C, D) A zoom of (A, B), where the black arrows indicate blood vessels and the white arrows indicate fat cells. (D) A 3D representation of the blood vessel network (in red) in the bone marrow. In grey-scale, a cross-section through the explants is shown. Note that the grey-scales are inverted compared to (A) and (C).

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