3D morphological characterization of polymeric microcarriers for stem cell expansion

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Aims

Tissue Engineering (TE) is an interdisciplinary field aiming to provide solutions for the regeneration of organs and tissues. Many typical TE processes make use of stem cells due to their pluripotent behavior and their self-renewal capacities. The latter allows scientists to minimize the number of stem cells harvested from a donor and expand them at large scale to reach a desired amount of cells for a given therapy. In the field of large scale stem cell expansion, micro-carriers are commonly used. These are typically degradable porous or non-porous beads on which cells are seeded and expanded during culture in a spinner flask. However, one of the remaining challenges related to the use of micro-carriers for cell expansion is the limited information on their morphological characteristics provided by the manufacturers. This information is, however, crucial to improve process parameters for cell expansion, such as the cell seeding density, culture time, etc. For instance, the available surface area, on which the cells can attach, is an important property influencing the initial cell seeding density, and is highly dependent on the micro-carriers morphology (porosity, pore size, roughness, etc.), but it is rarely provided by the manufacturers. Therefore, it has been highlighted how useful a database of 3D morphological characteristics of different micro-carriers could be. X-ray microfocus computed tomography (microCT) could provide a solution, as it allows for non-invasive analysis of the 3D morphology of porous materials. However, most of the micro-carriers we currently use are polymeric and they should be characterized in wet state to obtain the proper morphometric characteristics. Since there is no or negligible image contrast difference between the micro-carriers and the surrounding liquid when they are in wet state, in this study we propose the use of contrast-enhanced X-ray computed tomography (CE-CT), combined with detailed image processing and analysis, for 3D morphological characterization of polymeric micro-carriers. We will present the results for one specific type of porous micro-carrier, namely CultiSpher S, by which we highlight the added value of CE-CT combined with morphological quantification for in-depth characterization of polymeric micro-carriers.

Methods

Micro-carriers

CultiSpher STM (HyCloneTM) micro-carriers were used. These micro-carriers are based on gelatin derived from collagen to enhance cell attachment and proliferation. From the manufacturer and providers websites, the particles are claimed to be in the 130 – 380 µm diameter range (255 µm on average) in wet state. By assuming spheres with diameters in this range and taking into account the average porosity of the micro-carriers found in the current study, this information can be extrapolated to get an approximation of the average volume of the particles of around 0.068 mm³. The micro-carriers have macro-pores to increase the attachment surface of the cells, yet no information on the pore size or porosity is provided by the manufacturers.
**Contrast-enhanced CT**

In order to enhance the contrast of the micro-carriers, two different contrast agents were used. The commonly used and commercially available phosphotungstic acid (PTA) was compared to an in-house developed hafnium-substituted polyoxometalate compound (referred to as Hf-POM). For PTA, a concentration of 2.5% in phosphate buffered saline (PBS) was used, while for the Hf-POM a concentration of 3.5% in PBS was applied. Two staining times (30 minutes and 7 days) were compared to get a better understanding of the diffusion dynamics of these compounds through the micro-carriers.

The samples were scanned, in wet state, on a Nanotom S [GE] at 2µm voxel size, 75 kV, 0.1mm Al filter, 500ms exposure time, 2400 images, fast scan mode (20 min. scan time).

**Morphological assessment**

A global threshold using Otsu’s automatic thresholding was applied to binarize the full dataset. In order to separate the micro-carriers from each other, regions of interest (ROIs) were drawn manually around each individual micro-carrier using CTAn (Bruker MicroCT). From these ROIs, each micro-carrier was represented as an individual binary dataset. On each of these datasets, a convex envelope around the micro-carrier was calculated using a 2D+ approach, written in Matlab. More specific, convex envelopes were computed along the three axes for all the slices covering the micro-carrier, and they were combined with a Boolean inclusive ‘OR’ operation.

Using the 3D convex envelope and with CTAn (Bruker MicroCT), 3D analysis of the micro-carrier volume, the open pore volume (pores in the micro-carrier, accessible from the outside), the closed pore volume (pores trapped inside the micro-carriers) and the porosity were calculated. From the envelope volume of each micro-carrier, the particle size was computed assuming a sphere with the same volume. This parameter is useful for comparison with the information provided by the manufacturer.

Using the ROI shrink-wrap function in CTAn with increasing stretch over holes (from 0 to 20 voxels), the throat size of the pores was calculated, which is an important parameter to determine the percentage of open internal pores that is accessible to the cells to expand. 3D renderings of the micro-carriers were done using CTVox (Bruker MicroCT).

Finally, by convoluting a 1 pixel wide cubic structural element over the micro-carrier datasets from which the closed pores were removed, the pixels that were part of the inner surface of the micro-carrier were identified (i.e. the pixels in contact with the outside void in their direct neighborhood). This way, a 1 pixel thick layer can be generated for each micro-carrier, corresponding to the surface area of the micro-carrier.

**Results**

**Contrast agent staining**

Figure 1 shows typical reconstructed cross-sections of the CultiSpher S micro-carriers, stained with the different contrast agents, and for different staining times. It is clear that there is a difference in diffusion dynamics between the two contrast agents. Indeed, the grayscale profile through PTA-stained micro-carriers showed that after 30 minutes of staining time, only the outer surface of the micro-carriers was stained. Even after 7 days of staining, PTA did not fully penetrate the micro-carriers. In contrary, Hf-POM showed much faster diffusion throughout the micro-carriers, as only 30 minutes of staining was enough to have a homogeneous staining throughout the micro-carriers.
Figure 1: Typical reconstructed cross-sections of CultiSphere S micro-carriers after staining using the two contrast agents (PTA and Hf-POM) and two staining times (30 minutes and 7 days). The plot in each image corresponds to the grayscale profile along the blue line across a typically stained micro-carrier, generated using DataViewer (Bruker MicroCT).

**Morphometrical characterizations**

Figure 2 shows 3D renderings of a CE-CT dataset of 128 micro-carriers stained for 7 days with Hf-POM. Different grey-scales were assigned for each individual micro-carrier.

Figure 2: 3D visualization of CultiSpher-S (Sigma Aldrich ©) micro-carriers from CE-CT scans (7 days Hf-POM staining). The micro-carriers (left) were manually segmented and assigned random grayscale values (right).

The different structures of interest, computed as explained above, are shown on Figure 3, on one typical micro-carrier that was stained for 7 days with the Hf-POM. This figure qualitatively highlights the added value of 3D visualization of the micro-carriers, as their core structure can be deeply understood.

Figure 3: 3D visualization of one typical CultiSpher-S and the different structures of interest. From left to right: the micro-carrier, the convex envelope of the micro-carrier, the open pores inside the micro-carrier (in dark transparent shade), the open pore network inside the micro-carrier and the opened surface area.
Figure 4 presents the CE-CT based morphometric properties of 128 Hf-POM stained micro-carriers, along with the information provided by the manufacturer. The morphometric characteristics were highly variable amongst one dataset, indicating that one micro-carrier is not the other in terms of shape, size and pore structure. However, the porosity ratio and the estimated particle size seemed to be less variable than the other parameters; the lower variation of the latter being inherently due to its estimation method with a cubic root smoothing the variation from the computed volumes. The micro-carrier volume and convex envelope volume showed a strong correlation, indicating a certain amount of consistency amongst the micro-carriers in terms of porosity, which was confirmed by the effective calculations.

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<th>Bead Volume [mm³]</th>
<th>Envelope vol. [mm³]</th>
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<td>HfPOM 30m</td>
<td>HfPOM 7d</td>
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<th>Particle size [mm]</th>
<th>Open pore vol. [mm³]</th>
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<td>HfPOM 7d</td>
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<th>Porosity [J]</th>
<th>Bead surface area [mm³]</th>
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<td>HfPOM 30m</td>
<td>HfPOM 7d</td>
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Figure 4: Average volume (top left), convex envelope volume (top middle), particle size (top right), open pore volume (bottom right), porosity (bottom middle) and surface area (bottom right) of the micro-carriers (Hf-POM stained samples, 30 min and 7 days staining), along with the provided manufacturer data (MD) or
extrapolated data (ex.). The error bars show the standard deviations among all the micro-carriers (n = 128) of one dataset.

The statistical distribution of each morphological property can be analyzed based on histograms. These are shown in Figure 5. The histogram distributions give a deeper understanding on the homogeneity of those properties amongst the micro-carriers. Briefly, we can see that the porosity and the particle size showed normal distributions, whereas the other properties presented more skewed distributions.

The results of the throat size analysis are shown in Figure 6 for one micro-carrier of the Hf POM, 7 days staining sample. Around 2.1% of the pores had a throat size between 0 and 4 µm, 7.3% had a throat size between 4 and 8 µm, 10.4% had a throat size between 8 and 12 µm and around 80% had a throat size about 12 µm. Therefore, given the size of the cells that are expanded with these micro-carriers, one can estimate on average which percentage of the pores is effectively accessible by the cells.

Figure 5: Histogram distributions of the volume (top left), convex envelope volume (top middle), particle size (top right), open pore volume (bottom right), porosity (bottom middle) and surface area (bottom right) of the micro-carriers (Hf-POM stained sample, 7 days staining).

Figure 6: Throat size analysis showing the percentage of pores with a throat size below 4 µm, between 4 and 8 µm, between 8 and 12 µm and above 12 µm.

Conclusion

Using CE-CT combined with 3D image processing and analysis to characterize the morphometric properties of CultiSpher-S micro-carriers, we were able to highlight the added value of this technique for full morphological screening of polymeric micro-carriers to be used for stem cell expansion. Our novel approach allowed to generate much more information on
the 3D morphometric properties of micro-carriers than is currently available. This information is important to improve the cell expansion process using micro-carriers. We would like to highlight that the detailed morphological quantification method developed in this study can be applied to any type of polymeric micro-carrier, and is not limited to the microCT device we used. Future steps consist of developing an automatic micro-carrier separation algorithm and validating it against the manually separated data presented in this study. Moreover, other types of micro-carriers will be screened and characterized to further update the morphometric database of available micro-carriers.

References: