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## Quantitative analysis of the fibrous microstructure of 3D collagen gels

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### 1. Introduction

Predicting and understanding cancer cell migration in the extracellular matrix is one of the most exciting and difficult problem in biophysics, biology or in medicine. For that purpose, 2D analyses of cell migration on collagen-coated substrates have been extensively carried out (Dembo and Wang 1999), but 3D studies represent a major technological and scientific challenge today. The first step is to characterize the collagen gels from morphological and mechanical standpoints. For example, Roeder et al. (2002) investigated how the collagen morphogenesis could be altered by the incubation temperature: some of the microstructural changes were detected such as fibril diameter or length observed using confocal reflection microscopy (CM), and related to the mechanical behavior of the collagen gels. However, the evolution of the microstructure (collagen volume fraction or fibril orientation) during mechanical testing of the gel still needs to be analyzed. Therefore, this study aims to process various synthetic collagen gels, and to analyze their 3D microstructure using confocal microscopy in order to determine precisely collagen volume fraction and fibril diameter for gels prepared at two different polymerization temperatures.

### 2. Methods

#### 2.1. Collagen gels preparation

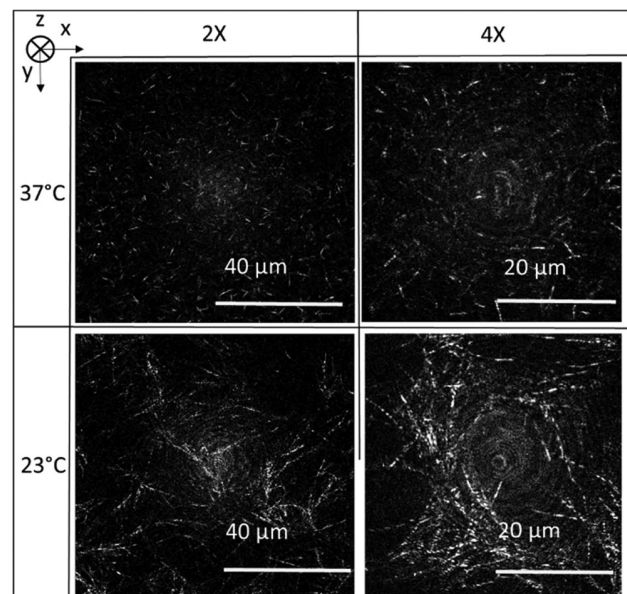
Collagen gels were prepared using type I collagen from rat tail collagen solution (Corning, USA) at a concentration of 2 mg/mL to mimic human ECM. To ensure the gelation process, the solution was homogenized at 4 °C by slow stirring to obtain a pH of 7.4. Two temperature conditions were used during the collagen network morphogenesis: 37 °C and 23 °C. These two conditions are prone to induce different microstructures (Yang 2009), therefore they present an interest for this study. The gels were incubated for 1 h in a humid chamber (approximately 99% humidity). Two samples, of 25  $\mu$ L each, were analyzed for each condition.

#### 2.2. Confocal microscopy

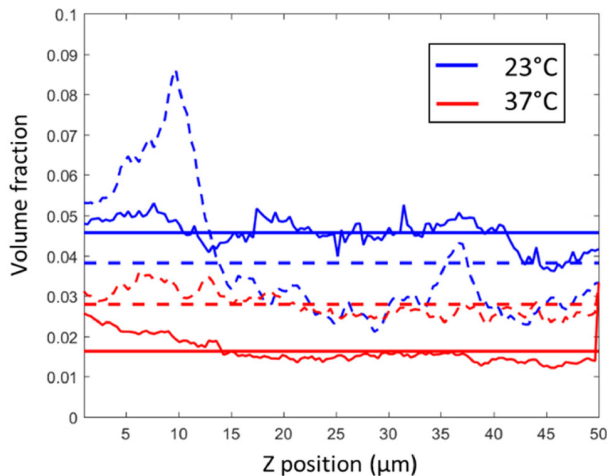
Confocal reflectance microscopy (Leica TCS SP8) was used to visualize the microstructure of the collagen gels with the 63X magnification objective (Iordan 2010). The 633 nm HeNe laser was used for reflectance. For each sample, two stacks of images were acquired at two different spatial resolutions (2X and 4X) to analyze the distribution of the volume fraction of collagen fibrils, i.e. with voxel sizes of  $0.18 \times 0.18 \times 0.36 \mu\text{m}^3$  and  $0.09 \times 0.09 \times 0.36 \mu\text{m}^3$  respectively.

#### 2.3. Image processing

The images obtained with the collagen gels were very noisy (Figure 1), and the standard segmentation tools (based on a fixed greyscale threshold) were not able to achieve proper fibril segmentation. Instead, we used the Trainable Weka Segmentation 3D plugin of Fiji, based on machine learning algorithms (Arganda-Carreras 2017). No pretreatment of the images was done before using Weka. By manually training the plugin to segment objects with various contrast and diameters, we could isolate the fibrils from the rest of the gels. We could then estimate the volume fraction of collagen fibril, obtained by dividing the number of voxels of the fibrous phase by the total number of voxels in the regions of interest. As a first rough approximation, we validated this value by comparing the diameter of the fibrils in the binary image and the CM image.



**Figure 1.** 2D in-plane slices of the collagen gels observed at two different scales and two gelation temperatures.



**Figure 2.**  $\varphi_m$  of the two samples calculated for each condition for each image of the stack ( $50\ \mu\text{m}$ ). the Dotted line presents the results for 4X, whereas the continuous line is for 2X. Finally, the straight line presents the mean over the volume (2X and 4X).

### 3. Results and discussion

#### 3.1. Visual morphological analysis

Figure 1 shows typical 2D slices obtained by using confocal microscopy, emphasizing rather well the multiscale architecture of the synthesized nanoscale collagen fibrils, assembling to form micron-scale fibril bundles (or fibers). We showed the grayscale images because observing the fibrils is easier for the eye than with the binary images. The top image shows a gel sample incubated at  $37^\circ\text{C}$  at two different scales. The bottom image shows the gels incubated at  $23^\circ\text{C}$ . The collagen gel polymerized at  $37^\circ\text{C}$  contain short and individual fibrils, homogeneously distributed in the gel. At  $23^\circ\text{C}$ , collagen fibrils are longer and thicker. These results are in agreement with the literature (Yang 2009). The typical diameters of fibrils were measured around  $371 \pm 53\ \text{nm}$  and  $488 \pm 57\ \text{nm}$  for  $37^\circ\text{C}$  and  $23^\circ\text{C}$  (mean + std), which is coherent with the literature data (Roeder 2002).

#### 3.2. Quantification of volume fraction

Figure 2 shows the evolution of the mean volume fraction of collagen fibrils ( $\varphi_m$ ) along the thickness of the gel, for the two imaged samples at two different scales, i.e. with two different ROIs:  $\varphi_m$  is higher for the gels polymerized at  $23^\circ\text{C}$ , whatever the ROI. Note that variations obtained around the mean values at either  $23^\circ\text{C}$  or  $37^\circ\text{C}$  are small.

In addition, we observe a difference between the observation at 2X and 4X for each condition. For example, at  $37^\circ\text{C}$ ,  $\varphi_m$  is close to 0.016 at 2X, while it

is 0.028 at 4X. There are two hypotheses to explain this effect. First, the segmentation itself may be the source of the problem. The image quality at 2X is not the same as at 4X, so the trainable Weka segmentation method could have an effect. Indeed, the major limitation of this technique comes from the user, as the thinner and lighter fibrils are lost in the training. The second hypothesis is that the volume fraction distribution is highly heterogeneous. Such a heterogeneous spatial distribution could have an impact on the mechanical properties of the gels and requires a deeper study of the volume fraction distribution at different scales, and the use of more relevant structural descriptors.

### 4. Conclusions

In this study, we prepared collagen gels at two different polymerization temperatures and proposed a method to analyze their 3D microstructure through image processing techniques. The results show significant differences in microstructure. At  $23^\circ\text{C}$ , the collagen gels have longer and thicker fibrils with a high fibril content. At  $37^\circ\text{C}$ , the fibril bundles are thinner, shorter and exhibit a lower fibril content. We also observed an improved fibril distribution at a smaller scale and need further analysis to determine the size of the Representative Elementary Volume (REV) to later analyze the macroscopic mechanical properties. Work is currently on-going to complete such analyses and probe other microstructural observables such as length distribution and 3D orientation of fibrils. Next step will be to subject these fibrous media to various physiological loadings and to study the strain-induced variations of all these 3D microstructural descriptors.

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