Electronic Supplementary Information

Tuneable Microfibrillar Collagen Structures within Dense Chitosan Hydrogels

Enguerran Devernois, Christophe Hélary, Jérôme Charliac, Gervaise Mosser, Thibaud Coradin

Detailed protocol for hydrogel synthesis

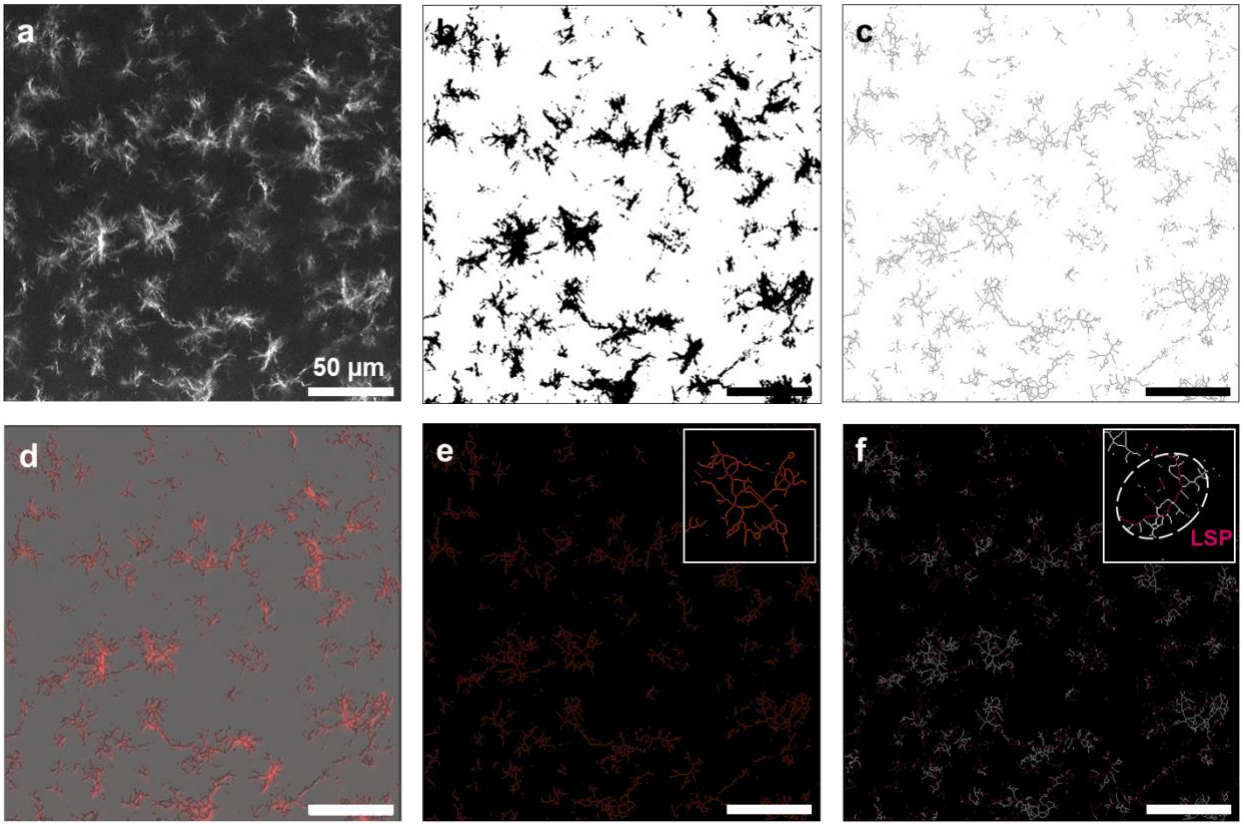
Type I collagen was extracted from rat-tail tendons and kept at 4°C and collagen concentration was determined by hydroxyproline titration. Concentrations usually varied between 3.5 $mg.mL^{-1}$ and 7 $mg.mL^{-1}$. To reach the desired collagen concentration (25 $mg.mL^{-1}$), controlled evaporation was performed on the solution. Briefly, solution was places in a sterile beaker, weighted and placed in a Biological Safety Cabinet. Acetic acid evaporation resulted in collagen concentration. Final concentration was calculated according to:

$$Ci * Vi = Cf * Vf$$

Ci : initial collagen solution, Vi : initial collagen solution volume, Cf : final collagen solution and Vf : final collagen solution volume.

Chitosan-collagen type I hydrogels (3 mL) at fixed final chitosan content (25 $mg.mL^{-1}$) and varying final collagen content (0-7.5 $mg.mL^{-1}$) were prepared. For this purpose, 2.7, 2.4 or 2.1 mL of a chitosan solution at 27.7, 31.0 or 35 $mg.mL^{-1}$ were mixed with 0.3, 0.6 or 0.9 mL of the 25 $mg.mL^{-1}$ of the collagen. Solutions were collected using a positive displacement pipet (Gibson, M1000E) and mixed in a 35 mm Petri Dish with an IKA Ultra-Turrax T25 Basic Disperser. Head speed was fixed at 6500 rpm and mixing time was 30 seconds. The vapour or gas process consisted in placing 3 mL of the chitosan/collagen in a Petri Dish and 40 mL of 1 M ammonium hydroxide solution in a 300 mL beaker for 24 h in a 5 L desiccator. The wet or liquid process consisted in immersing a Petri dish containing 3 mL of the chitosan/collagen solution in 40 mL of 1 M ammonium hydroxide solution placed in a 300 mL beaker for 1 h. After gelation, hydrogels were immersed in 30 mL of deionized water to remove the excess of ammonium hydroxide and acetate salts. Water was renewed every hour and pH at the gel surface were checked. When pH was neutral, hydrogels were taken out water and used for experiments.

Figure S1. Collagen fiber analysis using the Skeletonize plug-in. (a) Collagen fibers from SHG (b) Conversion into a binary image (c) Collagen fibers skeleton obtained from Skeletonize plug-in (d) Superposition of skeleton and native image (e) junctions (purple), branches (orange) (f) Longest shortest path (red). Scale bar : 50 µm



Skeletonize is an Image J plugin.[1] This tool is adapted to treat branched structures as skeleton can used to represent the shapes. To generation a skeleton from a Second Harmonic Generation images (a) it has to be converted in binary 8-bit images (b). Skeletonize plugin and then produces the object skeleton by converting the binary image into images in 1 pixel representation (c). The resulting skeleton possesses pixel value of 0 at the skeleton (black) and 255 pixels at the background (white). Native and skeletonized images can be superposed (d). Image J plugin AnalyzeSkeleton can draw information about the skeleton organization that is to say the connectivity in the object. In two dimensions, this plugin tags every pixel in the object and in each case counts a certain number of parameters including: junctions (purple), branches (orange), triple and quadruple-branching points, or information about branch lengths. Branches can be defined as the segment between two junctions. Triple and quadruple-point branches are junction that connect three or four branches (e).

The longest shortest path (red) is the shortest path between the two farthest junctions in the object (f). This can give information about the size of the object (red).

Figure S2. SEM image of 20% collagen-chitosan gels obtained by (a) vapour and (b) wet process (Scale bar: 2 μm.) TEM images of 20% collagen-chitosan gels obtained by (c) vapour and (d) wet process (Scale bar: 1 μm).

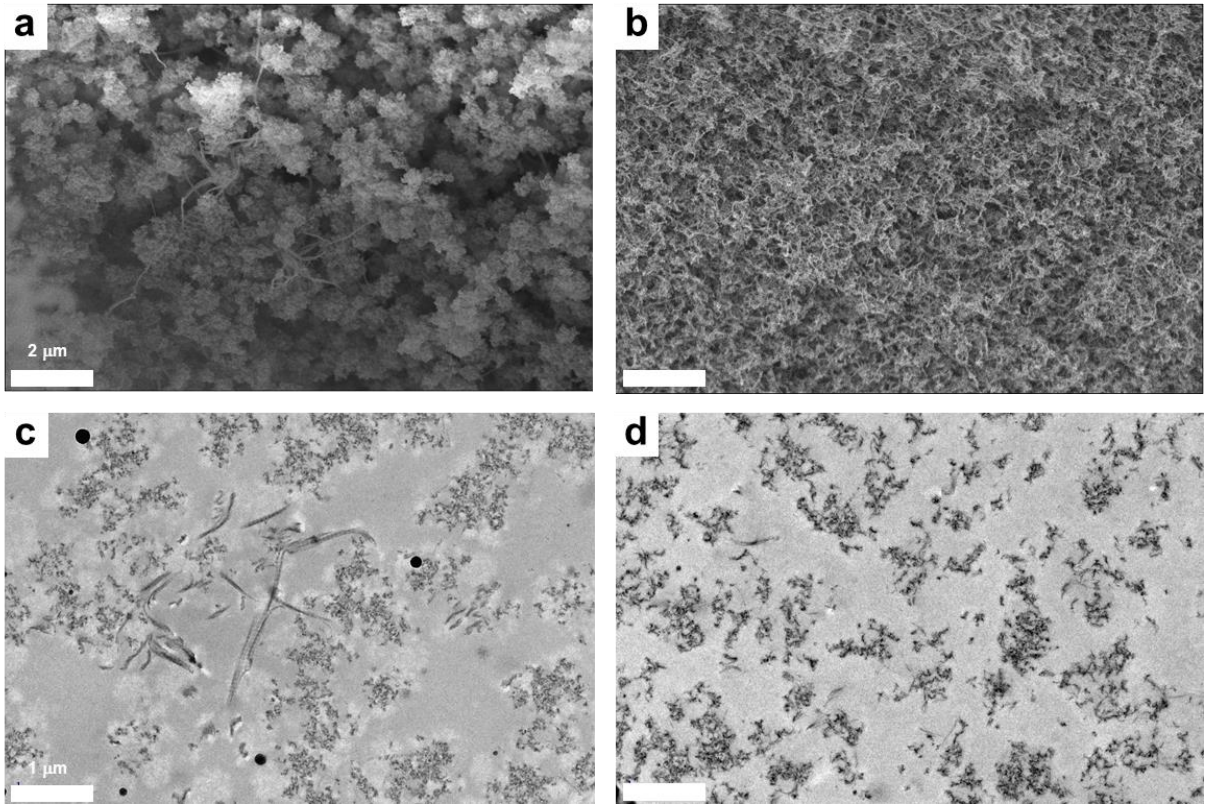


Figure S3. Compression curves for hydrogels obtained from (a) vapour and (b) wet process.

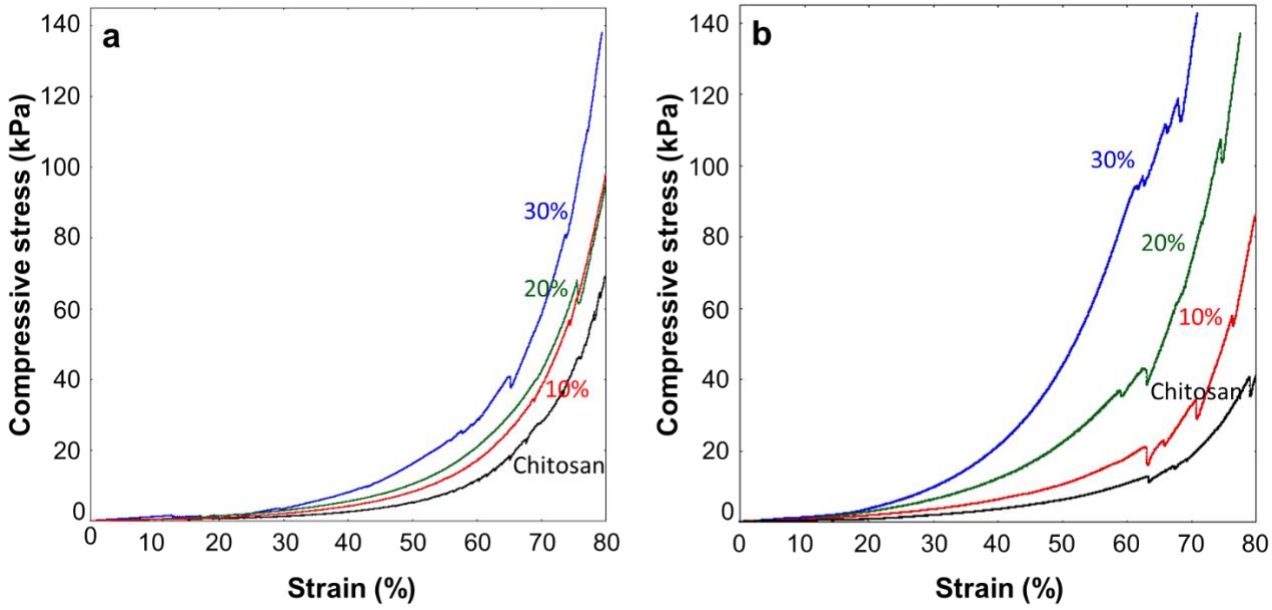


Figure S4. Cell nuclei stained with DAPI on (a,c) pure chitosan substrate (b,d) 10% collagen substrate, (a,b) obtained by wet and (c,d) vapour process. Scale bar : 100 μm

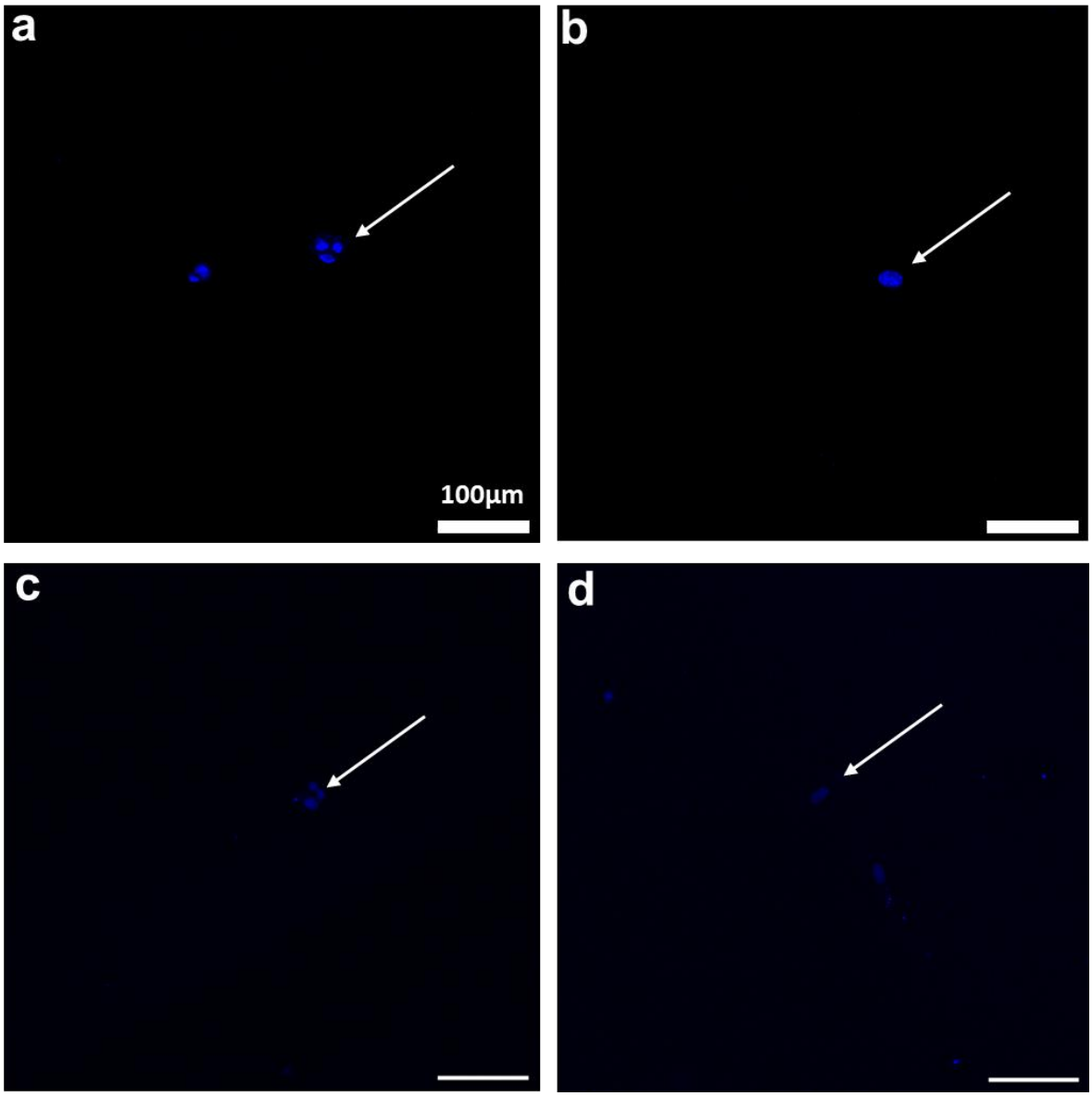


Figure S5. Cell count on hydrogels obtained using gaseous (a) and wet process (b). Cells nuclei were counted using ImageJ software. Cell counts were performed on 5 images for each sample.

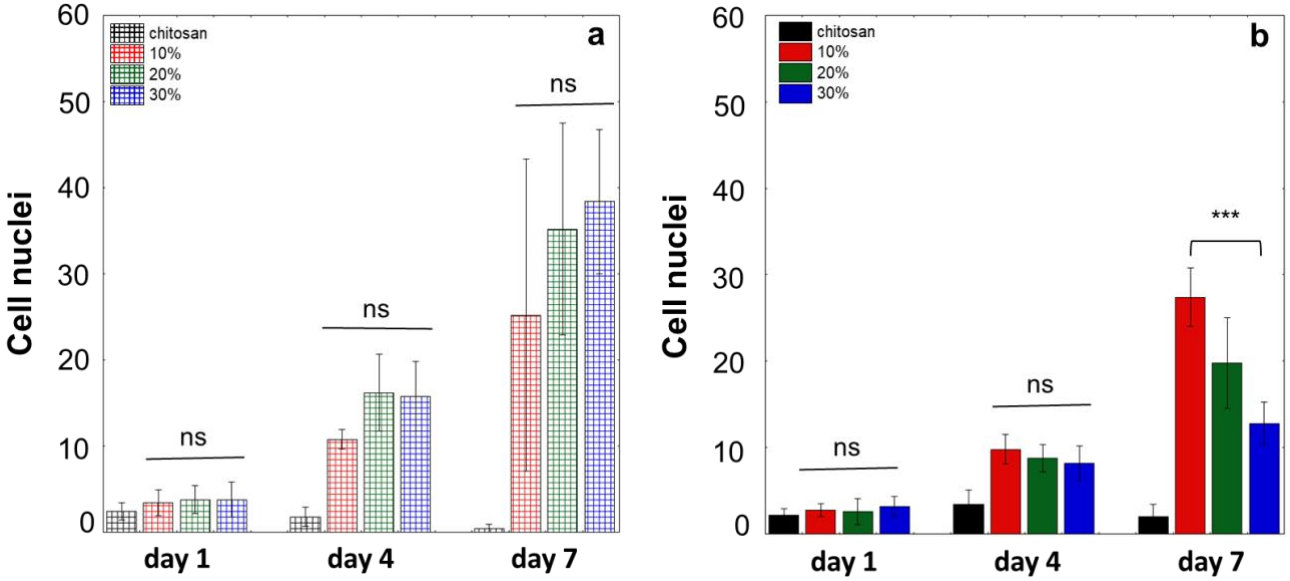
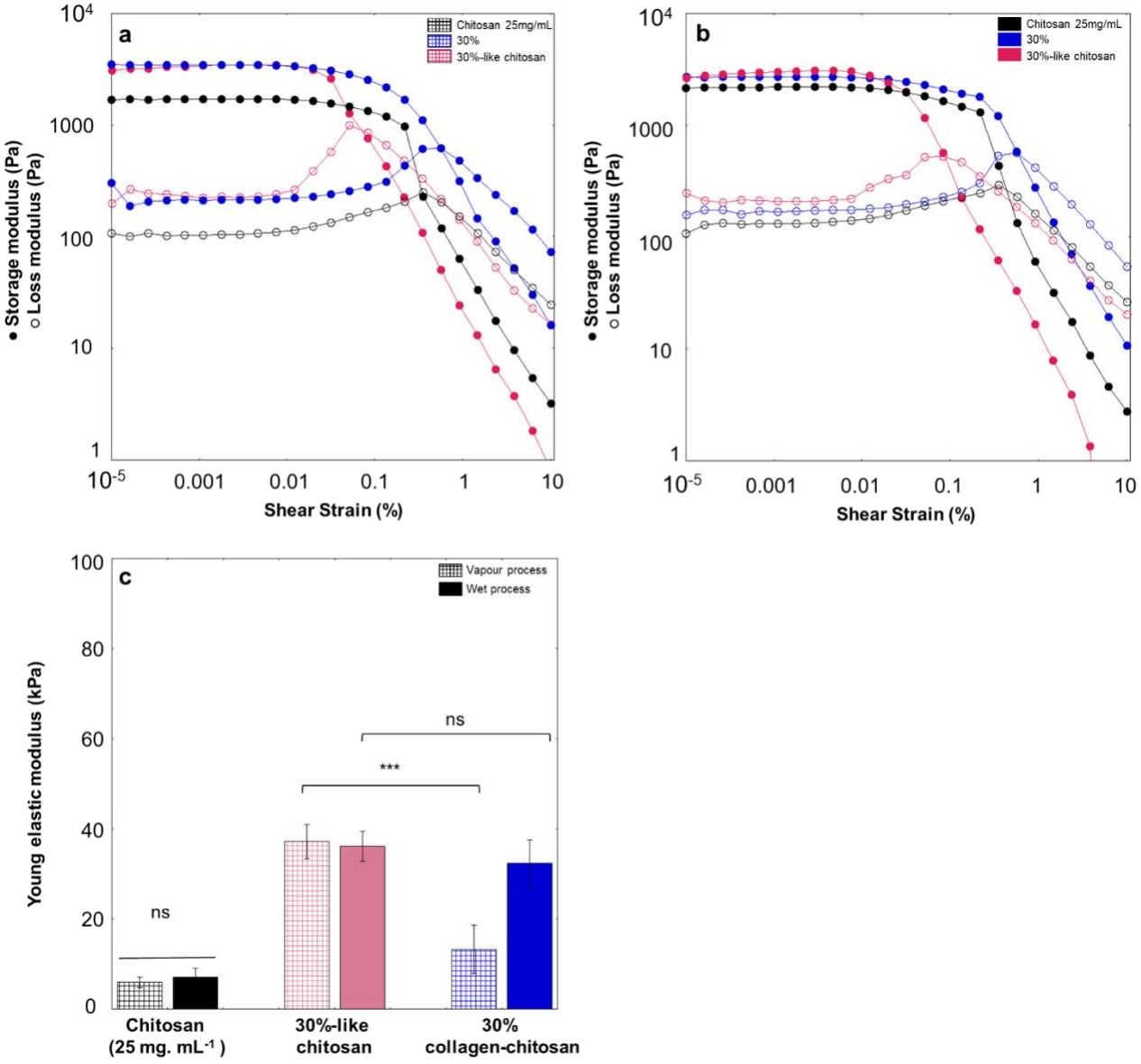


Figure S6. Rheological and mechanical properties of pure chitosan hydrogels of total polymer content similar to 30 % chitosan-collagen hydrogels**.** Large amplitude oscillatory sweeps on hydrogels obtained by (a) gaseous and (b) wet process. (c) Compressive modulus of hydrogels obtained by gaseous and wet process (n=5**).**



REFERENCES

(1) Arganda-Carreras, I.; Fernández-González, R.; Muñoz-Barrutia, A.; Ortiz-De-Solorzano, C. 3D Reconstruction of Histological Sections: Application to Mammary Gland Tissue. *Microsc. Res. Tech.* **2010**, *73* (11), 1019–1029. https://doi.org/10.1002/jemt.20829.