# *Going green and mimetic: new ECM-based hydrogel for meniscus regeneration using supercritical $CO_2$-assisted decellularization*

Chou I Ho*, Francisco A. P. Rodrigues, Mariana S. Reis, Clara Sousa, Viviana P. Ribeiro, Ana Leite Oliveira, João B. Costa*

Universidade Católica Portuguesa, CBQF - Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia., Rua Diogo Botelho 1327, 4169-005 Porto, Portugal. *Chou I Ho – s-chho@ucp.pt *João B. Costa – jpbcosta@ucp.pt

CATÓLICA ESCOLA SUPERIOR DE BIOTECNOLOGIA PORTO

## Introduction

**Meniscal injuries** (Fig. 1) affect approximately 66 to 70 per 100,000 individuals annually and are associated with an increased risk of osteoarthritis and other degenerative joint changes over time.

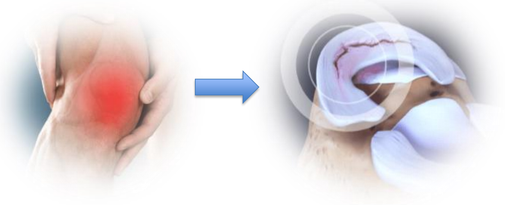


Fig 1. Meniscal injuries.

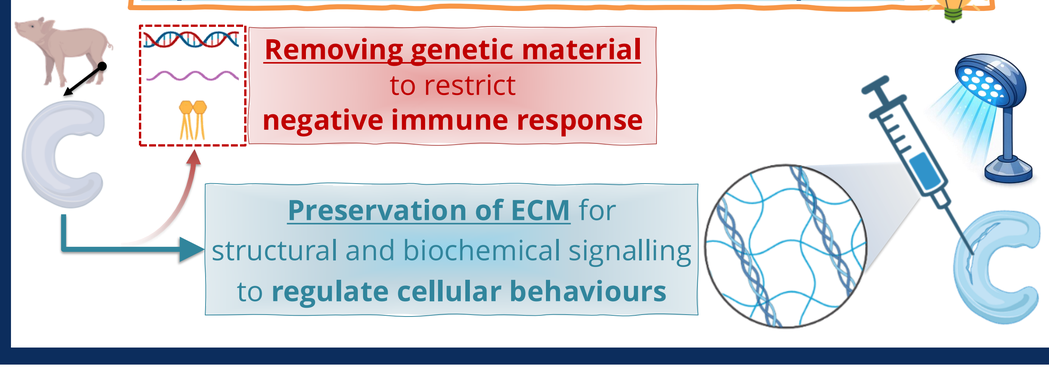
Although several clinical treatment strategies are available, they still present major limitations in restoring native meniscal function.

**LIMITATIONS**

- Altered knee joint biomechanics
- Limited donor availability
- Possible disease transmission
- Immunological complications
- Inability to promote intrinsic regeneration
- Lack of long-term functional restoration

## Objectives

To establish a **greener strategy** for producing a **biomimetic meniscus-derived decellularized extracellular matrix (dECM)-based hydrogel** via **supercritical carbon dioxide ($scCO_2$)-assisted process**.




## Methods

### Cyclic protocol for Decellularization (Fig. 2):

- **Number of cycles:** 9
- **Physical methods employed during each cycle**: freeze-thaw, direct ultrasonication, orbital shaking, and vortex
- **Solutions used**: ultrapure water, phosphate-buffered saline (PBS)
- **Protocol duration:** 5 days

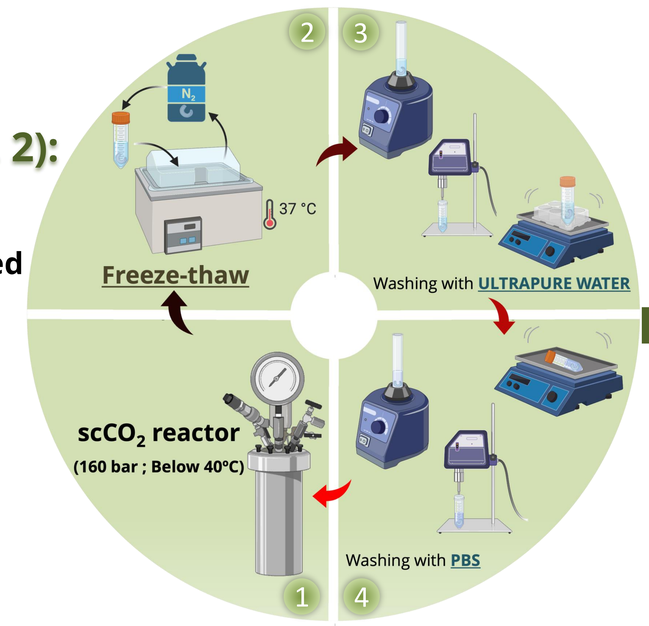
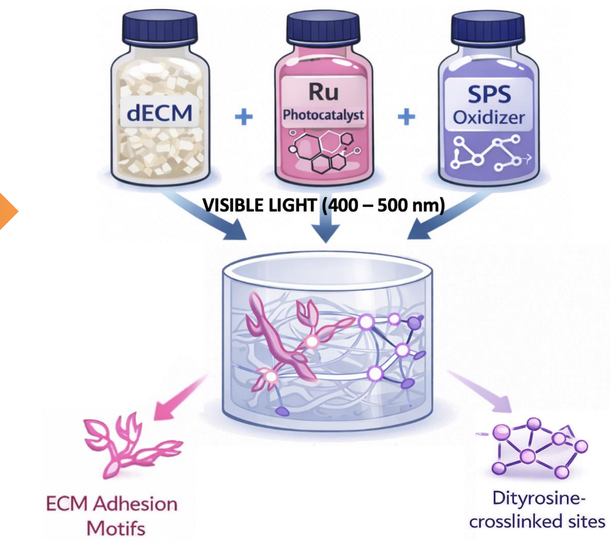



Fig 2. Diagram of procedures in each cycle with four steps.

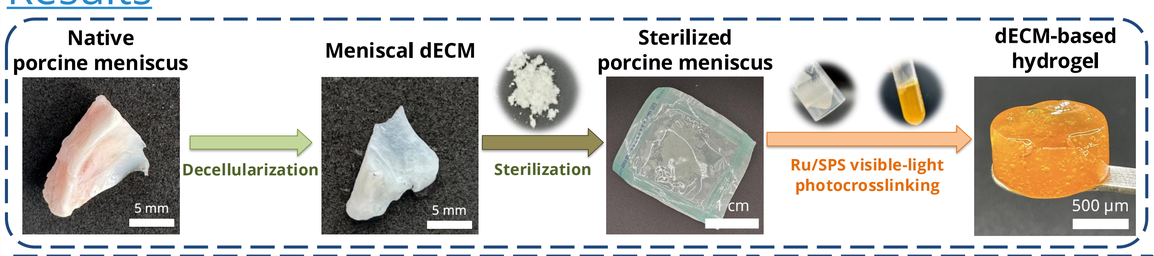
### Sterilization:

A **$scCO_2$-based sterilization protocol** was performed using a mixture of **NovaKill™** as co-solvent [1].

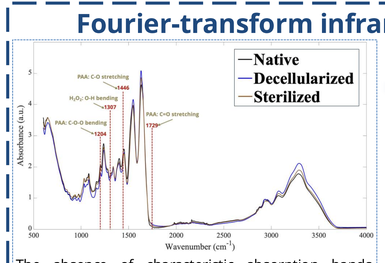
### Ru/SPS visible-light photocrosslinked dECM-based hydrogel production



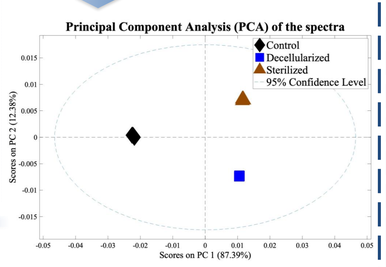

## Results

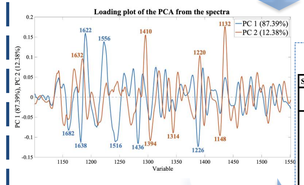



### Fourier-transform infrared spectroscopy (FTIR)




The absence of characteristic absorption bands highlighted in the spectra suggests **the effective removal of residual NovaKill™** from the matrix [2].



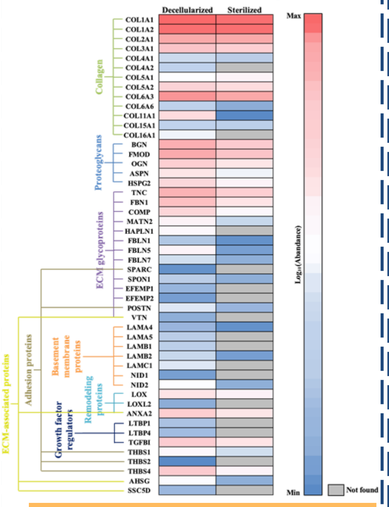

**PCA band-assignment tables**

| Side of PC1 | Peak position ($cm^{-1}$) | Vibrational assignment | Biochemical contribution |
|---|---|---|---|
| Positive | 1622 | Amide I (C=O stretching) | Collagen-rich protein matrix |
| | 1556 | Amide II band | Collagen |
| Negative | 1682 | Amide I (C=O stretching) | Collagen-associated protein structure |
| | 1638 | Amide I band<br>Protein secondary-structure-related vibration | Collagen-rich ECM;<br>Possible contribution from other proteins (e.g. Elastin) |
| | 1516 | Amide II band | Collagen |
| | 1436 | $COO^-$ symmetric stretching | Collagen and proteoglycan/GAGs-associated components |
| | | $CH_2/CH_3$ deformation | Lipid |
| | 1226 | Amide III band | Collagen |
| | | Sulfate-associated carbohydrate vibration | Sulfated GAGs |
| | | Phosphate backbone | Genetic materials |

| Side of PC2 | Peak position ($cm^{-1}$) | Functional group vibrations | Corresponding biomolecules |
|---|---|---|---|
| Positive | 1632 | Amide I (C=O stretching) | Collagen |
| | 1410 | $COO^-$ symmetric stretching<br>Uronic acid-associated vibration | Proteoglycans/GAGs-associated components |
| | 1220 | Amide III band | Collagen |
| | | Sulfate-associated carbohydrate vibration | Sulfated GAGs |
| | 1132 | C–O, C–O–C, and sulfate-associated carbohydrate vibration | GAGs/carbohydrate-rich matrix components |
| Negative | 1394 | $CH_3$ bending | Collagen-rich ECM |
| | | $COO^-$ symmetric stretching | Proteoglycans |
| | 1314 | Amide III band | Collagen-associated protein structure |
| | 1148 | C–O, C–O–C, and sulfate-associated carbohydrate vibration | GAGs/carbohydrate-rich matrix components |

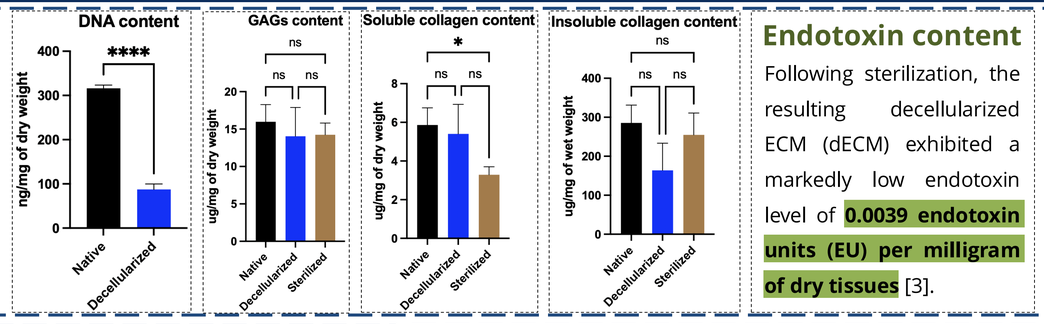
**Substantial preservation of the major ECM-associated molecular features, particularly the core collagenous and proteoglycan-rich matrix.**

### Proteomic analysis




A **substantial preservation of the core fibrocartilaginous meniscal ECM** is indicated following $scCO_2$ treatment, particularly **major fibrillar collagens, proteoglycans, and several key ECM glycoproteins**. Nevertheless, ***partial reduction or depletion of non-core and accessory ECM components*** was observed.

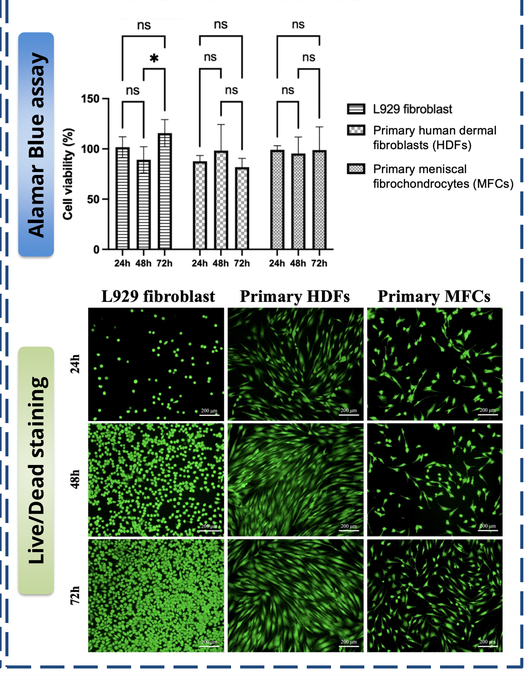
### QUANTITATIVE ASSESSMENTS



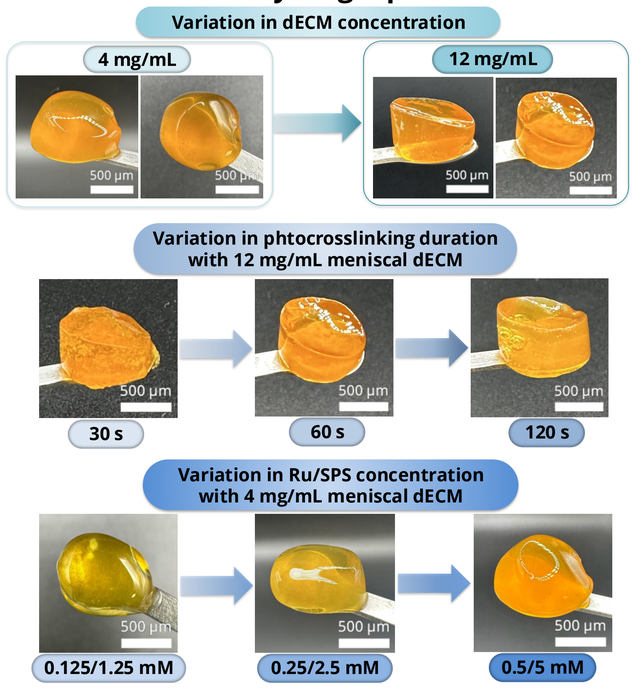

### Endotoxin content

Following sterilization, the resulting decellularized ECM (dECM) exhibited a markedly low endotoxin level of **0.0039 endotoxin units (EU) per milligram of dry tissues** [3].

### Cytocompatibility of the meniscal dECM




### ECM-based hydrogel production




## Conclusions

- $scCO_2$-assisted decellularization/sterilization process enabled the production of **a cytocompatible meniscal ECM** with **substantial DNA removal** and **low endotoxin burden**;
- The novel processing **preserved the principal biochemical and proteomic features of the native fibrocartilaginous matrix**;
- Despite some ultrastructural reorganization and selective loss of accessory ECM components, the processed dECM remained **suitable for hydrogel production**;
- dECM-based hydrogels were produced by tuning **dECM content, gelation time, and the Ru/SPS concentration**;
- This approach represents a promising and **greener** route for generating biomimetic dECM-based materials for meniscus regeneration.

## References

[1] Farey, John E., *et al.* "Outcomes of ACL Reconstruction Utilizing Supercritical CO2-Sterilized Allografts." Orthopaedic Journal of Sports Medicine 12.8 (2024): 23259671241254115.
[2] Fu, Cheng-Hsin, *et al.* "Kinetic and equilibrium analysis of peracetic acid formation at 1753 cm− 1 mid-infrared absorption peak using facile attenuated total reflection Fourier transform Infrared technique." Journal of the Chinese Chemical Society 70.6 (2023): 1427-1434.
[3] Jeong, Yun Hee, *et al.* "Establishing endotoxin limits to enhance the reliability of in vitro immunogenicity risk assessments." MAbs. Vol. 17. No. 1. Taylor & Francis, 2025.

## Acknowledgements

This work was supported by the IBEROS+ project (0072_IBEROS_MAIS_1_E), Instituto de Biofabricación en Red para el Envejecimiento Saludable, funded by Interreg VI-A España–Portugal (POCTEP) 2021–2027. F.A.P.R. acknowledges support from the FCT doctoral grant 2024.00742.BD. J.B.C. acknowledges contract ref. 2023.15056.TENURE.065, funded by national funds through FCT – Fundação para a Ciência e a Tecnologia, I.P., in partnership with PARALAB.