Tailoring a chitosan-ECM hydrogel platform to mimic the tumor microenvironment of equine sarcoid for comparative oncology

Alessia Zanacchi[1], Floriana Fruscione[2], Elisabetta Razzuoli[2], Laura Pastorino[1], Donatella Di Lisa[1]

[1]*Department of Informatics, Bioengineering, Robotics and Systems Engineering (DIBRIS), University of Genoa, Genoa, Italy*

[2]*National Reference Center of Veterinary and Comparative Oncology (CEROVEC), Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Genova 16129, Italy*

Abstract

Equine sarcoid is the most common cutaneous neoplasm in horses, characterized by the lack of effective treatments [1]. Furthermore, it's associated with bovine papillomaviruses (BPVs), making it a valuable model for comparative oncology investigations of papillomavirus (PV)-associated neoplasms [2]. In line with the growing applications of three-dimensional (3D) systems, this study aimed to develop a biomaterial that more faithfully recapitulates the tumor microenvironment (TME) of equine sarcoid.

A protocol was optimized for the decellularization of tumor spheroids to obtain a decellularized extracellular matrix (dECM) [3, 4] derived from a primary cell line established from a BPV-1 induced equine sarcoid tumor. The resulting dECM was incorporated into previously developed and validated chitosan (CHITO) and chitosan-collagen (CHITO-COL) hydrogels [5] to enhance the biological and structural fidelity of the model.

The formulations were subjected to rheological, morphological via scanning electron microscopy (SEM), and mechanical analyses to assess the influence of dECM incorporation on material properties. Biological characterization, including confocal microscopy and real-time quantitative PCR (RT-qPCR), was performed to evaluate changes in cellular organization and gene expression. Data were analyzed by one-way ANOVA with Tukey's test (*$p \leq 0.05$).

Rheological analyses indicated that the inclusion of dECM did not significantly alter gelation kinetics, ensuring suitability for cell viability. Mechanical testing revealed decreased stiffness in CHITO-COL + dECM hydrogel, consistent with microstructural differences observed by SEM. Confocal microscopy highlighted better cellular morphology in CHITO + dECM hydrogel, and gene expression analyses suggested that dECM incorporation may contribute to the maintenance of mesenchymal features characteristic of the tumor phenotype.

Overall, these findings demonstrate that the developed dECM-enriched hydrogels represent a promising 3D model for mimicking the equine sarcoid TME, providing a relevant tool for future applications in comparative oncology.

Materials and Methods

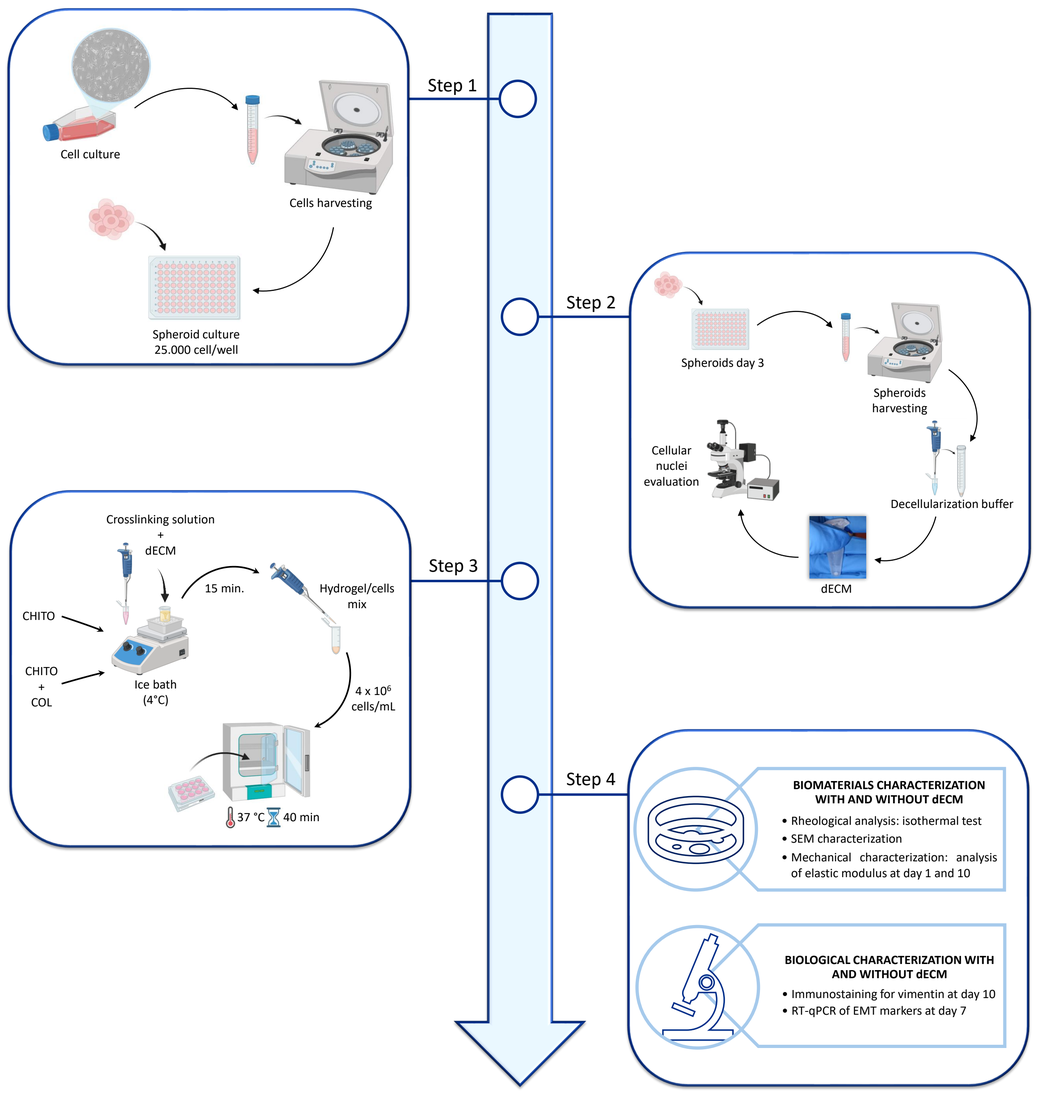


Fig. 1: Experimental workflow. Schematic representation of the study design, including spheroids formation, decellularization process, hydrogel preparation (CHITO and CHITO-COL with/without dECM) and subsequent physical and biological characterization.

Results

Optimization of the decellularization protocol

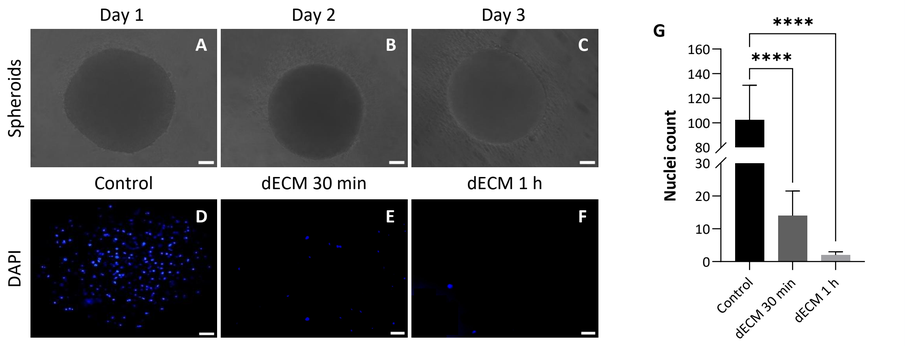
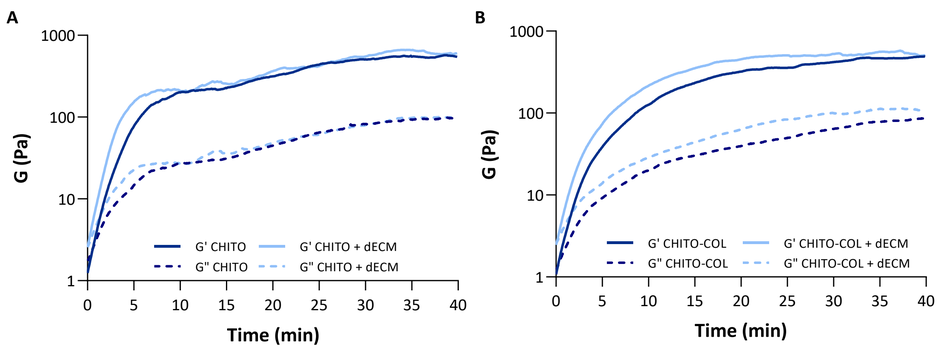


Fig. 2: Spheroid characterization and decellularization efficiency. (A–C) Optical microscopy images showing spheroid morphology at days 1, 2, and 3 (10x objective; scale bar: 200 µm). (D–F) Confocal microscopy images of (D) control spheroids, dECM after (E) 30 min and (F) 1 h of incubation with decellularization buffer. Nuclei are stained with DAPI (blue) to visualize DNA content (20x objective; scale bar: 100 µm). (G) Quantification of residual cell counts in control and decellularized spheroids. Images were processed using ImageJ/Fiji and data analyzed via GraphPad Prism with one-way ANOVA with Tukey's multiple comparison test (****$p < 0.0001$).

Rheological tests on hydrogels with and without dECM



	Initial gelation time (sec)	Final gelation time (min)
CHITO	0.62 ± 0.29	36.32 ± 3.94
CHITO + dECM	0.18 ± 0.13	38.46 ± 0.58
CHITO-COL	0.63 ± 0.19	38.63 ± 0.06
CHITO-COL + dECM	0.28 ± 0.03	38.56 ± 0.38

Table 1. The resulting initial and final gelation times are presented as mean ± standard deviation (SD). Data were analyzed via Matlab.

Fig. 3: **Rheological characterization of gelation kinetics**. Isothermal tests performed at a constant temperature of 37 °C for 40 min, with a constant shear strain of 10% and a frequency of 1 Hz. The plots illustrate the time evolution of the storage modulus (G') and loss modulus (G") for (A) CHITO hydrogels with and without dECM and (B) CHITO-COL hydrogels with and without dECM. These measurements analyze the impact of dECM on the sol-gel transition. Data were analyzed via GraphPad Prism.

SEM and mechanical characterization on hydrogels with and without dECM

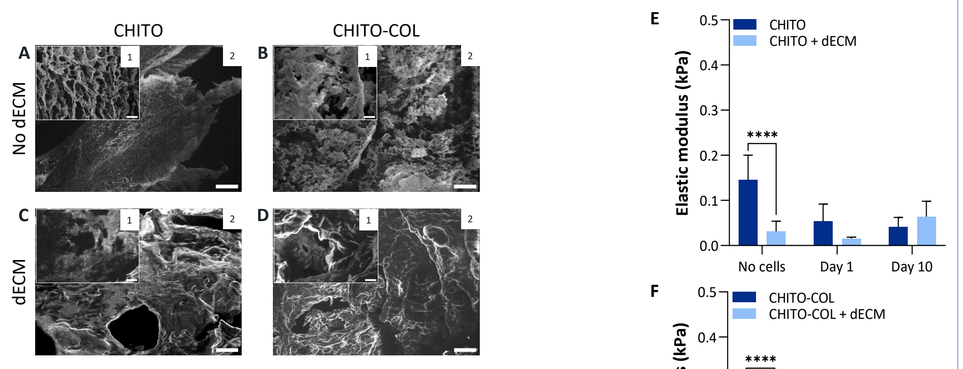


Fig. 4: Structural and mechanical characterization of hydrogels. (A–D) SEM images showing the inner microstructure (1, magnification 2000x) and surface morphology (2, magnification 250x) of (A, C) CHITO and (B, D) CHITO-COL hydrogels, with and without dECM (scale bar: 200 µm). Images were processed using ImageJ/Fiji.

(E–F) Mechanical characterization showing the elastic modulus (kPa) of acellular cell-laden hydrogels at days 1 and 10 for (E) CHITO and (F) CHITO-COL hydrogels with and without dECM. Statistical analysis was performed via GraphPad Prism using one-way ANOVA with Tukey's multiple comparison test (*** $p \leq 0.001$, ****$p \leq 0.0001$).

Morphological and gene expression analysis

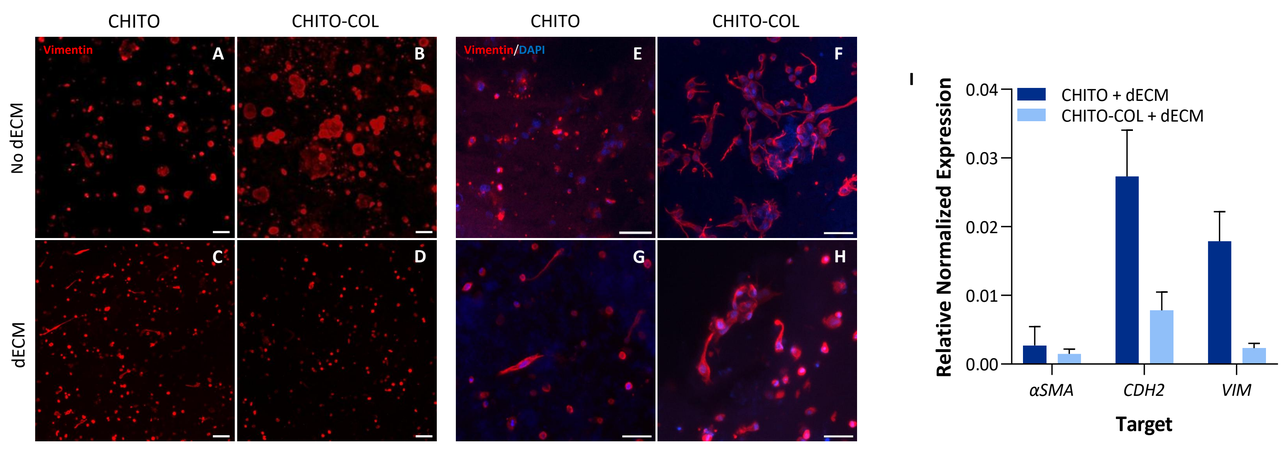


Fig. 5: Biological characterization of tumor cells cultured within 3D hydrogels. (A–H) Confocal microscopy analysis of tumor cells at day 10 (20x objective). (A–D) Images of CHITO and CHITO-COL hydrogels, with and without dECM, stained for Vimentin (red; scale bar: 50 µm). (E–H) Images of CHITO and CHITO-COL hydrogels, with and without dECM, stained with Vimentin (red) and DAPI (blue; scale bar: 100 µm). (I) RT-qPCR gene expression analysis of EMT markers (*α-SMA*, *CDH2*, and *Vimentin*) at day 7 for tumor cells cultured within dECM-enriched CHITO and CHITO-COL hydrogels. Statistical analysis was performed via GraphPad Prism using one-way ANOVA with Tukey's multiple comparison test (*$p \leq 0.05$).

Take-home message

The integration of spheroids-derived dECM into chitosan-based hydrogels successfully recreates a more biomimetic TME for equine sarcoid studies. Key findings include:

Effective decellularization: 1-hour protocol enables effective spheroid decellularization.

Rheological profile: Incorporation of dECM don't alter the gelation kinetics, which remain suitable for cell viability and growth.

Structural and mechanical tuning: Addition of dECM modifies the internal microstructure and mechanical stiffness of CHITO-COL matrices, likely due to an incomplete crosslinking.

Morphological and gene expression analysis: dECM enrichment promotes enhanced spatial organization in CHITO hydrogels, while gene expression suggests the maintenance of mesenchymal and EMT-related features.

References

[1] Ogłuszka, Magdalena, et al. "Equine sarcoids—causes, molecular changes, and clinicopathologic features: a review." Veterinary pathology 58.3 (2021): 472-482.

[2] Munday, J. S. "Bovine and human papillomaviruses: a comparative review." *Veterinary pathology* 51.6 (2014): 1063-1075.

[3] Zhang, Yanmin, et al. "Rapid formation of 3D: Decellularized extracellular matrix spheroids for enhancing bone formation." *Journal of Biomedical Materials Research Part A* 111.3 (2023): 378-388.

[4] Chiang, Cheng-En, et al. "Bioactive decellularized extracellular matrix derived from 3D stem cell spheroids under macromolecular crowding serves as a scaffold for tissue engineering." Advanced Healthcare Materials 10.11 (2021): 2100024.

[5] Zanacchi, Alessia, et al. "Engineered biopolymeric hydrogels for in vitro modeling of equine sarcoid tumors in comparative oncology." *International Journal of Biological Macromolecules* 348 (2026): 150840.

Ministerial Project funded by the Ministry of Health 22M07: "Development of a 3D model for the evaluation of the interaction between tumor and immune system", CUP J19I22001260001 and the Ministry of Health 05/24 IZS PLV: "Modulazione dei TLRs per indurre lo switch "from cold to hot" del microambiente tumorale: uso dei modelli 3D nell'ottica One Health", CUP J19I24001430001.

Contact info: alessia.zanacchi@edu.unige.it, laura.pastorino@unige.it, donatella.dilisa@edu.unige.it