Conjunctiva mimetic hydrogel for the delivery of extracellular vesicles to the ocular surface

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Subvencionado por la Junta de Castilla y León a través del proyecto "CLU-2023-1-04 - Unidad de Excelencia del Instituto de Oftalmobiología Aplicada (IOBA)" de la Universidad de Valladolid y cofinanciado por el Fondo Europeo de Desarrollo Regional (FEDER)

INTRODUCTION

Hydrogels are effective platforms in regenerative medicine and nanomedicine. They are frequently used as scaffolds that can mimic extracellular environments and support tissue repair.

The development of biomaterials that replicate conjunctival extracellular matrix (**ECM**) could open new strategies for treating **ocular surface diseases**, especially those characterized by fibrosis and scarring.

We used a hydrogel based on conjunctival ECM components (**ConjECMh**) previously characterized by our group and evaluated its potential **for delivering extracellular vesicles (EVs)** via subconjunctival injection.

Our final goal is to achieve a novel regenerative therapy for ocular surface pathologies based on hydrogels and EVs.

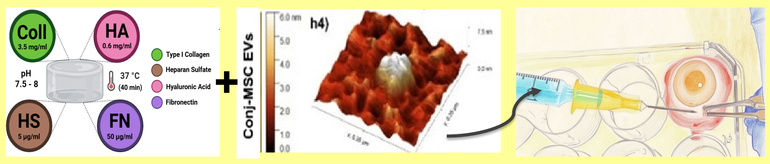


Figure 1. *Graphical abstract depicting the incorporation of conjunctival EVs to Conj-ECMh for subconjunctival injection.*

METHODS

- **Hydrogel preparation:** Conj-ECMh was prepared with collagen type I, hyaluronan, fibronectin, and heparan sulphate (Figure 1).
- **Conj-MSCs and EVs isolation:** Human conjunctival mesenchymal stromal cells (**Conj-MSC**) were isolated from cadaveric donor tissue and characterized following ISCT criteria.[1] EVs were isolated from Conj-MSC secretome by ultrafiltration and precipitation.[2]
- **Incorporation of EVs into Conj-ECMh:** Conj-ECMh were loaded with Conj-MSC-derived EVs (200 µg/ml) either blank or labeled with IRDye® 800 CW to enable microscopic identification.
- **Subconjunctival injection:** A total of 25-30 µl of Conj-ECMh either blank or loaded with 200 µg/ml EVs were subconjunctivally injected into *ex vivo* rabbit eyes obtained from a local slaughterhouse. After 2 hours, eyeballs were subjected to histopathological evaluation.
- **Histological analysis:** To facilitate hydrogel visualization in ocular tissues, two dyes (Coomassie blue or Sirius red) had been added into the Conj-ECMh prior to gelation.
- **All experiments were n = 3, in triplicate.**

RESULTS

1. Conj-ECMh preparation and staining

Conj-ECMh were successfully stained with either Coomasie blue or Sirius red dyes, which allowed macroscopic identification of the hydrogel (**Fig. 2**).

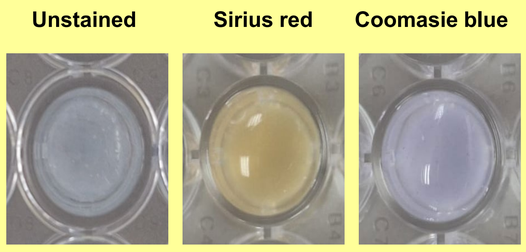
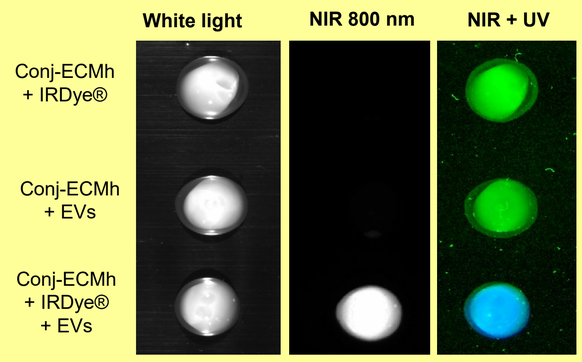


Figure 2. *Gelled Conj-ECMh with no dye (unstained), Sirius red or Coomassie blue. Dyes were used to facilitate Conj-ECMh identification.*

2. Incorporation of Conj-MSC-derived EVs to Conj-ECMh

IRDye® labelling confirmed EV retention within the Conj-ECMh hydrogel matrix (**Figure 3**).

Figure 3. *Conj-ECMh loaded with EVs and stained with IRDye® 800 CV to visualize the Evs (left: white light; middle: NIR-800 signal; right: NIR-800 signal overlaid with UV-B illumination for contrast).*



3. *Ex vivo* subconjunctival injection of Conj-ECMh

The subconjunctival injection of Conj-ECMh was successfully performed in *ex vivo* rabbit eyes using a 25G needle, allowing precise delivery of the Conj-ECMh into the subconjunctival space (**Figure 4A**).

Stained Conj-ECMh effectively gelled *in situ* after subconjunctival injection. Sirius red demonstrated better hydrogel retention at the injection site than Coomasie blue up to 24 hours (**Figure 4B**). For that reason, Sirius red was used in subsequent experiments.

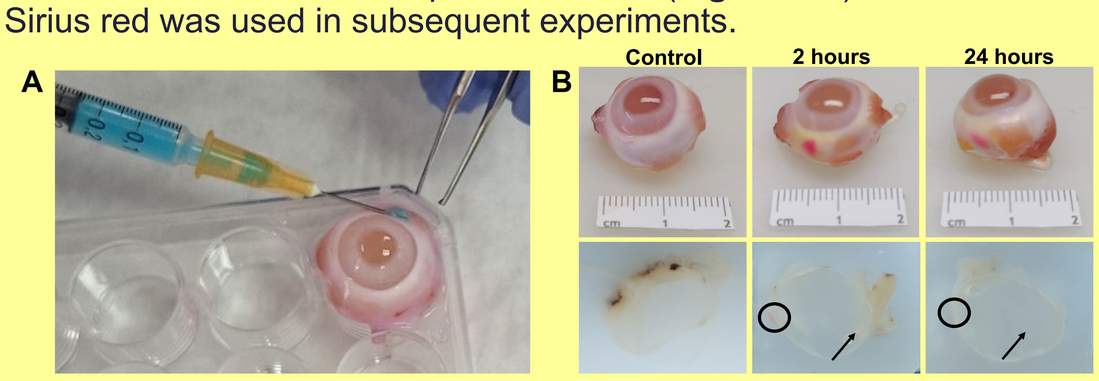


Figure 4. A) *Subconjunctival injection of Conj-ECMh with Coomassie blue in ex vivo rabbit eyes.* ***B)*** *Macroscopic identification of the subconjunctival injection site (arrows) of Conj-ECMh stained with Sirius Red (2.5%) in a control eye (immediately after collection) and eyes collected 2 and 24 hours after Conj-ECMh injection.*

4. Histopathological analysis

Stained hydrogels, either blank or loaded with EVs were visualized on tissue sections, demonstrating Conj-ECMh retention at the injection site up to 24 hours (**Figure 5**).

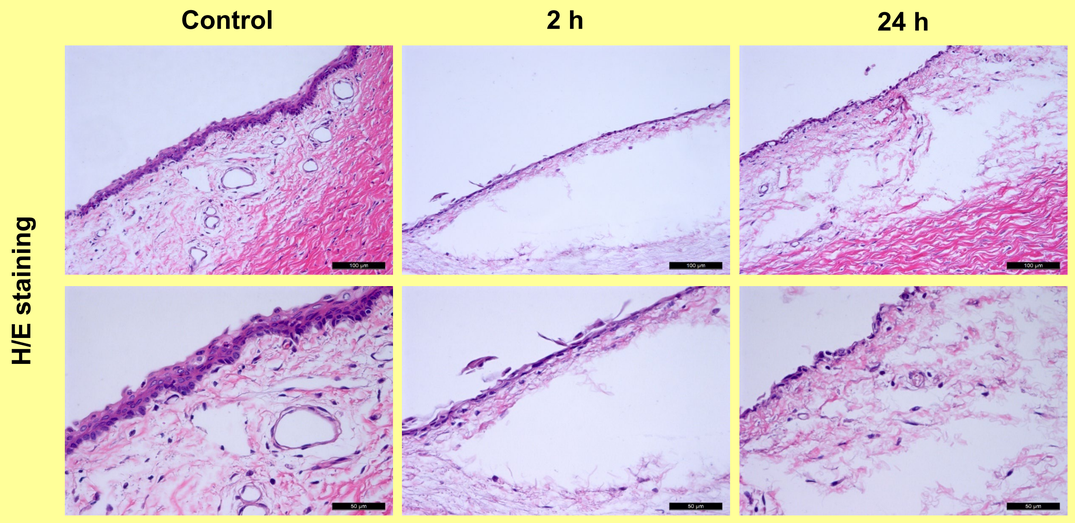


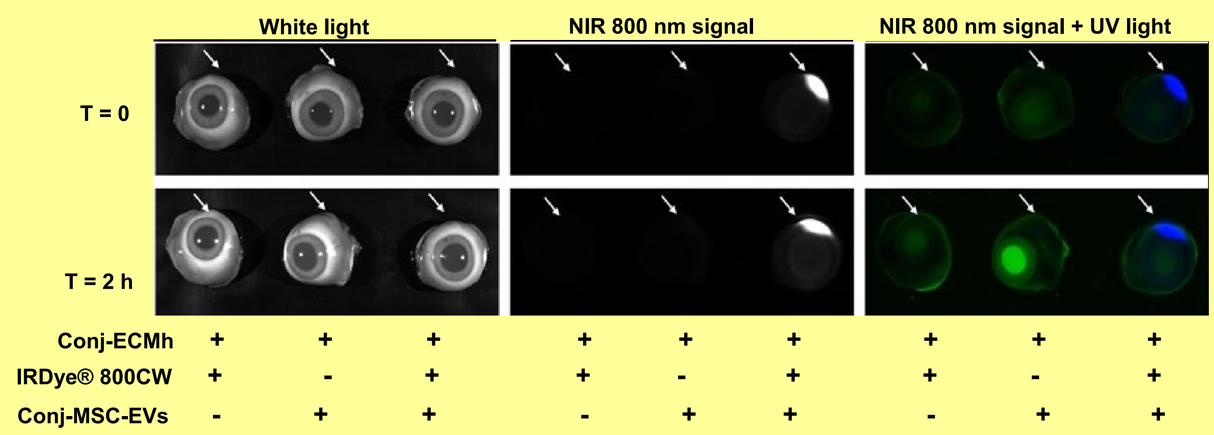
Figure 5. *Histopathological evaluation of rabbit ocular surface sections stained with hematoxylin and eosin. A small ampule at the injection site was observed. After 24 hours cells repopulate the area. Scale bar = 100 µm (top row) and 50 µm (bottom row).*

5. EVs identification in *ex vivo* rabbit eyes after subconjunctival injection of loaded Conj-ECMh

Conj-ECMh loaded with EVs stained with IRDye® 800 effectively gelled *in situ* after subconjunctival injection.

Fluorescence was used to monitor the localization of labelled EVs. Strong NIR fluorescence signal was detected at the injection site, confirming the presence of the labeled Conj-ECMh-EVs within the subconjunctival space (**Figure 6**).

Figure 6. *Detection of labelled EVs incorporated into Conj-ECMh in ex vivo rabbit eyes. Images are arranged by rows = time post-injection (0 and 2 hours) and columns = imaging mode (left: white light; middle: NIR-800 signal; right: NIR-800 signal overlaid with UV-B illumination for contrast). Arrows indicate the location of the Conj-ECMh.*



CONCLUSIONS

Our hydrogel based on conjunctival ECM components, Conj-ECMh, shows promising potential as a delivery platform for EVs in ocular surface therapies, offering efficient *in situ* gelation and targeted localization.

References:

1. García-Posadas L, Romero-Castillo I, Brennan K, Mc Gee MM, Blanco-Fernández A, Diebold Y. Isolation and Characterization of Human Conjunctival Mesenchymal Stromal Cells and Their Extracellular Vesicles. Invest Ophthalmol Vis Sci. 2023;64(12):38;
2. Romero-Castillo I, López-García A, Diebold Y, García-Posadas L. Enrichment protocols for human conjunctival extracellular vesicles and their characterization. Sci Rep. 2024;14(1):28270.

Funding:

This work was supported by projects RTI2018-094071-B-C21 and PID2023-148252OB-C21 funded by MICIU/AEI /10.13039/501100011033 and FEDER, UE.

Commercial Relationship Disclosure: None.