Advancing prevascularised skin model with functionalised chitosan-based bioinks

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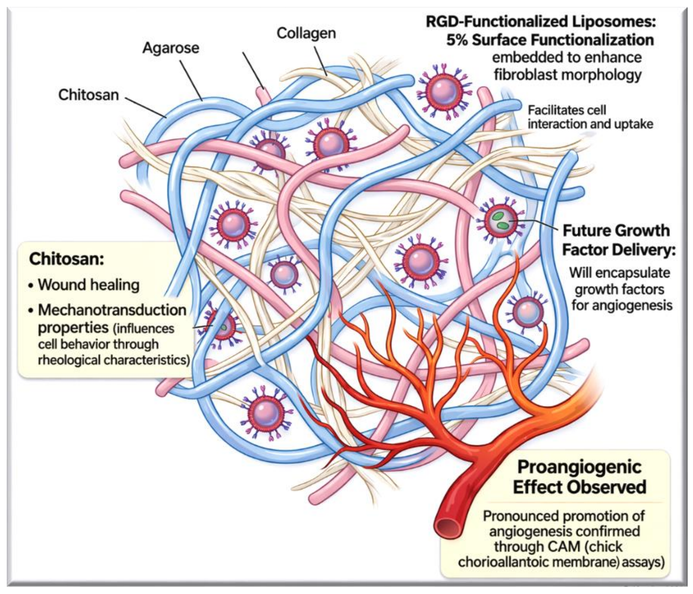
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1 Introduction

Prevascularized skin models are promising platforms for in vitro studies and future regenerative applications, but their development remains challenging. Chitosan/agarose (CS/AG) hydrogels are attractive bioinks due to their printability, biocompatibility, and gelation near physiological temperature without chemical crosslinkers. However, their lack of intrinsic adhesive cues limits cell attachment and spreading. To address this, CS/AG bioinks were functionalized with collagen and RGD-based components, including RGD-functionalized liposomes.



2 Methodology

- **Material preparation:** Chitosan was prepared by the CO_2 saturation method according to patent EP2920240A2 and combined with agarose based on patent application EP.2346004. To improve bioactivity, type I collagen (Symatese Lab, 4 mg/mL) was mixed with chitosan/agarose systems at 1:3, 1:1, and 3:1 volumetric ratios after neutralization with the supplied buffer.
- **Cell viability study:** Primary fibroblasts were embedded in hydrogel constructs at a density of 1 × 10^6 cells per construct and cultured under standard conditions. Cell viability was assessed after 2 and 7 days using Calcein AM/Propidium iodide staining, allowing visualization of live cells in green and dead cells in red.
- **Rheological characterization:** Complex viscosity (η*) of agarose solutions (0.25–1.0% w/v) and selected chitosan/agarose systems was measured during controlled cooling to evaluate sol–gel transition. Oscillatory measurements in the LVE region (0.1–100 rad/s) were used to determine G' and G", while time sweep tests assessed gel stability over time.
- **CAM assay:** Angiogenic response was evaluated in the *ex ovo* CAM model at 0, 48, and 72 h. Vascular parameters were quantified using AngioTool.
- **Liposomes and confocal microscopy**: DSPC/DSPE-RGD liposomes (~100 nm) were incorporated into hydrogels at a final concentration of 200 µg/mL, while free RGD peptide served as a control. DiI-labeled liposomes were visualized by confocal microscopy after phalloidin staining of F-actin and Hoechst staining of nuclei to assess cell morphology and liposome distribution.

3 Chitosan / Agarose composition

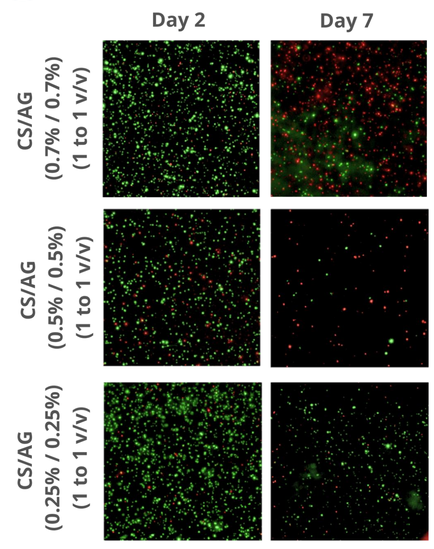


Figure 1. Live/dead staining of fibroblasts cultured in chitosan–agarose (CS/AG) constructs for 2 and 7 days.

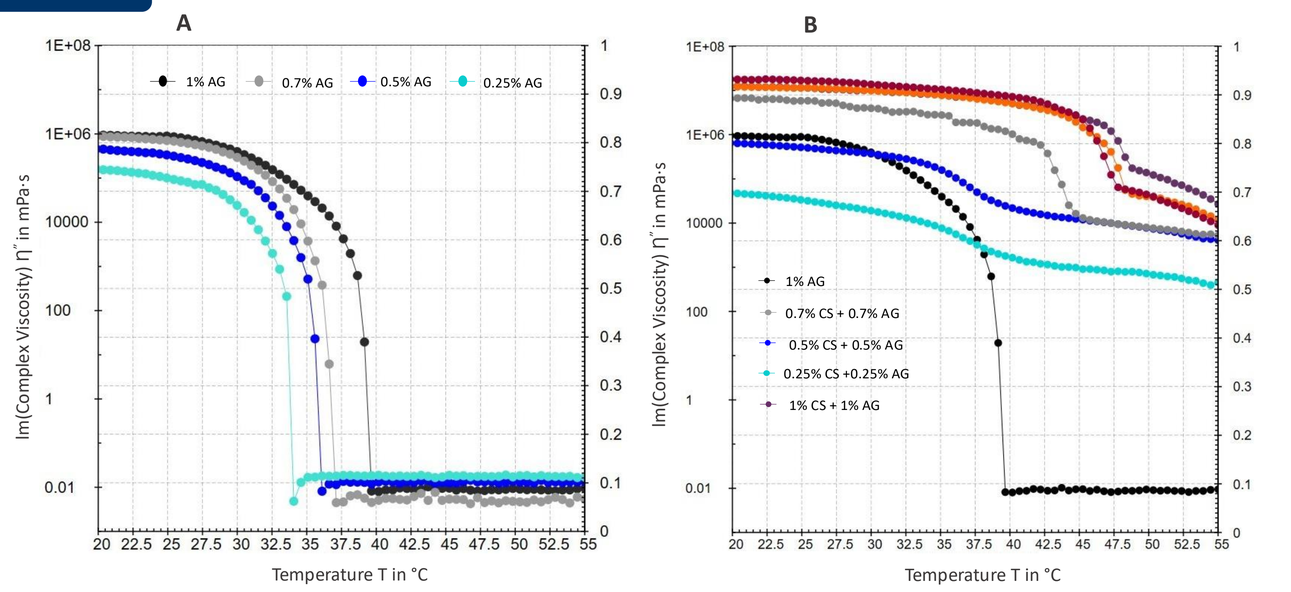


Figure 2. Temperature-dependent complex viscosity (η) profiles of (a) agarose solutions (0.25–1.0% w/v), (b) agarose–chitosan systems at different polymer ratios, measured during cooling, showing concentration-dependent sol–gel transition behavior.

4 Chitosan/Agarose with Collagen

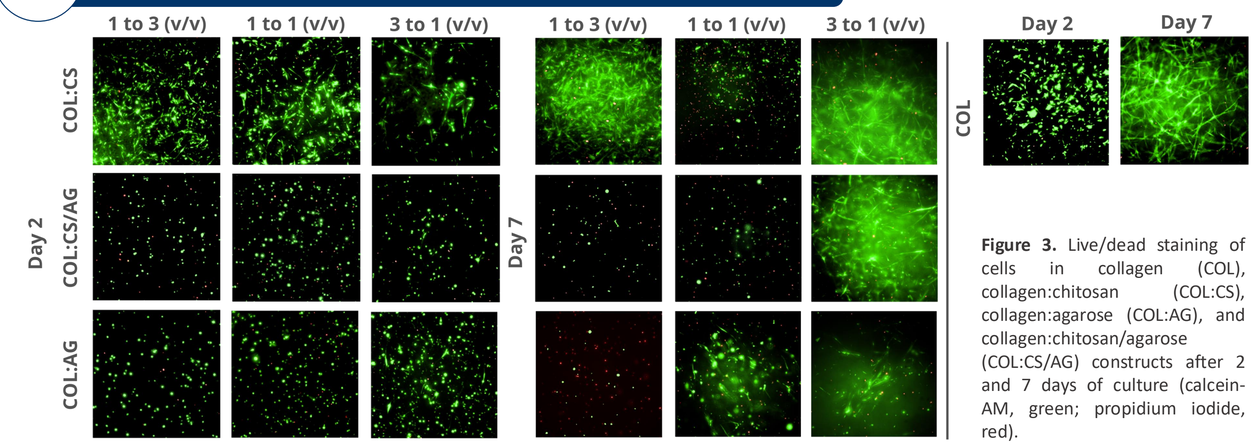
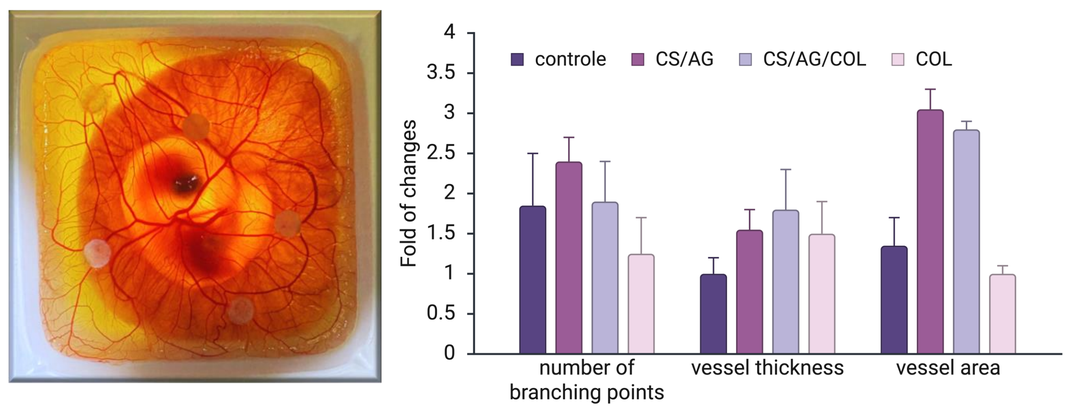


Figure 3. Live/dead staining of cells in collagen (COL), collagen:chitosan (COL:CS), collagen:agarose (COL:AG), and collagen:chitosan/agarose (COL:CS/AG) constructs after 2 and 7 days of culture (calcein-AM, green; propidium iodide, red).

Figure 4. Fold change in selected CAM vascular parameters at 72 h relative to day 0 for control (saline), CS/AG, CS/AG/COL, and COL.



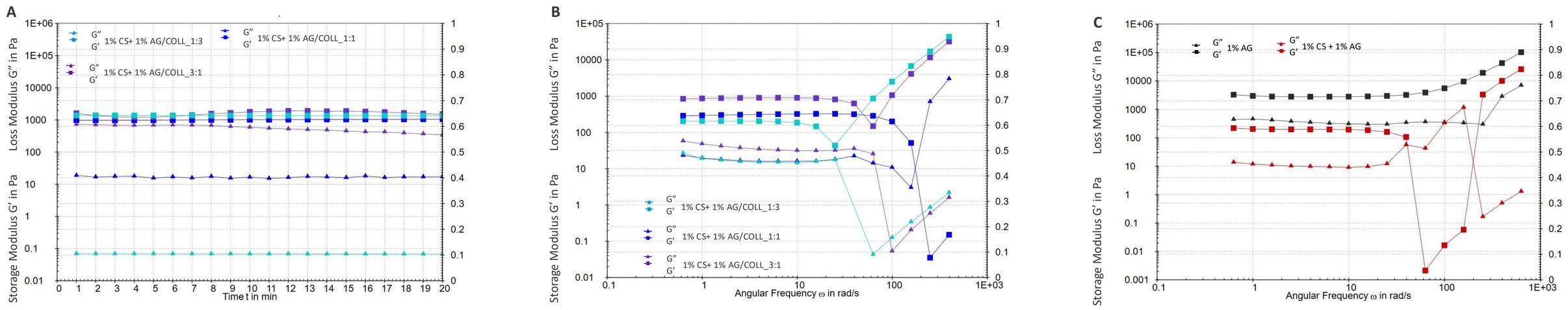


Figure 5. Rheological characterization of the studied hydrogels: **(A)** time sweep of G' and G" for agarose–chitosan–collagen systems; **(B)** frequency sweep ofagarose based systems; **(C)** frequency sweep of agarose–chitosan–collagen systems.

5 Chitosan/Agarose/Collagen with RGD and liposomes

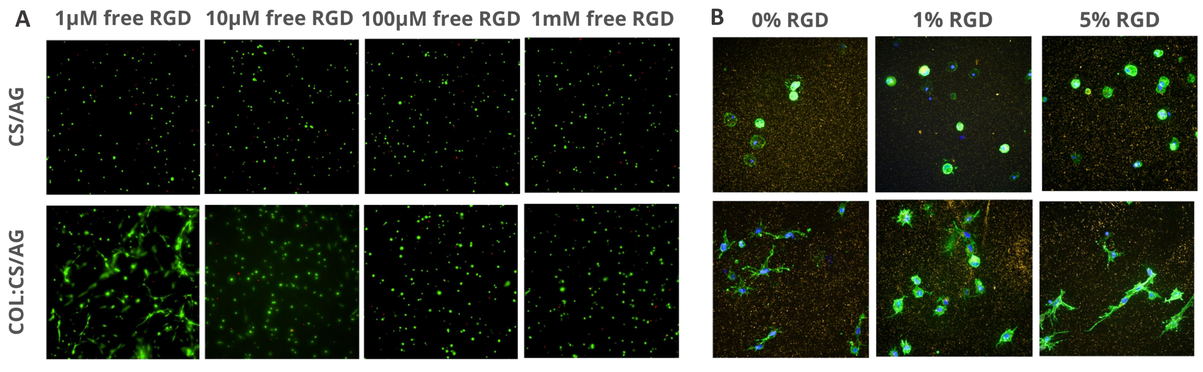


Figure 5. (A) Cell viability in chitosan/agarose (CS/AG) and collagen:chitosan/agarose (COL:CS/AG) hydrogels with free RGD (1 µM, 10 µM, 100 µM, and 1 mM), stained with calcein-AM (green) and propidium iodide (red) **(B)** Confocal images of cells in hydrogels containing DiI-labeled liposomes with 0%, 1%, and 5% RGD; F-actin is shown in green, nuclei in blue, and liposomes (DiL) in orange.

6 Conclusions

- The **0.7% CS/0.7% AG** formulation was selected as the best base matrix due to its favourable rheological profile, with sufficient viscosity, controlled gelation, and stable network formation.
- **Collagen-containing** systems showed stable gel-like viscoelasticity (G' > G"), higher mechanical strength, and good structural stability over time, supporting their further biological evaluation.
- **Collagen functionalization** improved fibroblast adhesion and morphology, with the **1:1 CS/AG/COL** system identified as the most promising biological variant.
- The **CS/AG/COL** composition showed a balanced proangiogenic response, combining increased vessel thickness with enhanced branching and vessel area.
- **Free RGD** was ineffective in restoring proper fibroblast elongation and spindle-like morphology.
- **RGD-functionalized liposomes** improved cellular response in collagen-containing hydrogels, indicating that ligand presentation on a carrier is more effective than soluble RGD alone.
- The best results were obtained for **5% RGD liposomes**, supporting further development of this strategy in prevascularized skin models.

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