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Evaluation of corneal keratocytes response to time-dependent mechanical properties in a hydrogel system

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Introduction

The **cornea** constitutes the outermost part of the eye. It is composed of 5 layers: epithelium, Bowman's layer, stroma, Descemet's membrane and endothelium (**Figure 1**). Cornea is a **viscoelastic** tissue, therefore it exhibits the mechanical properties of both a **solid** and **viscous fluid**.

Problem:

It has long been established that **environmental mechanical cues** can affect cellular behaviour.[1] Being able to understand how these cues affect cells and use this knowledge to **tailor biomaterials** that suit our needs is a crucial part of tissue engineering.

In the case of the cornea, the effect of the solid like properties (such as substrate stiffness) on the **stromal cells** (CSCs or keratocytes), have already been investigated**.** However there is still a lack of knowledge regarding how the **viscous** or **time-dependent mechanical properties** can influence these cells.

Aim:

To fabricate **hydrogels** with **different viscoelastic properties** to evaluate how cornea stromal cells respond to changes in these features.

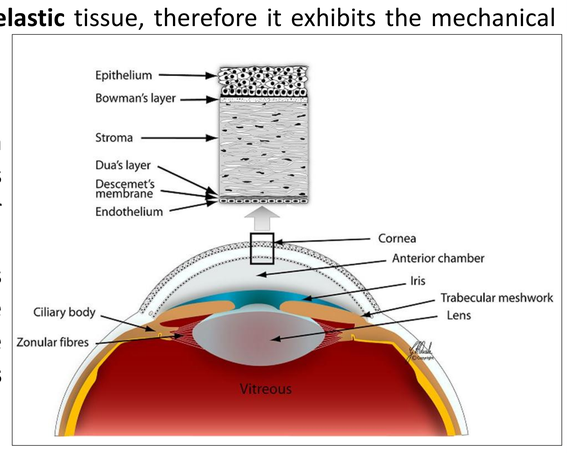


Figure 1. Cornea schematics, illustrating the macro architecture of the eye and the micro architecture of the cornea, subdivided into 5 layers (3 cellular and 2 connective). This image was adapted from *Navaratnam et al. 2015* .[2]

Materials and Methods

Hydrogels with tuneable time-dependent mechanical properties were made using **alginate of different molecular weight (MW)** which was obtained through **controlled autoclaving**. These alginates were then blended with **type I collagen and PEG** Succinimidyl Glutarate (**Figure 2**). The mixture was then pre-crosslinked at 37 °C for 30 minutes in a PDMS mould, before being fully crosslinked with $CaCl_2$ for 1h. In cell gels, **primary corneal keratocytes** were added to the mixture before the pre-crosslinking step.

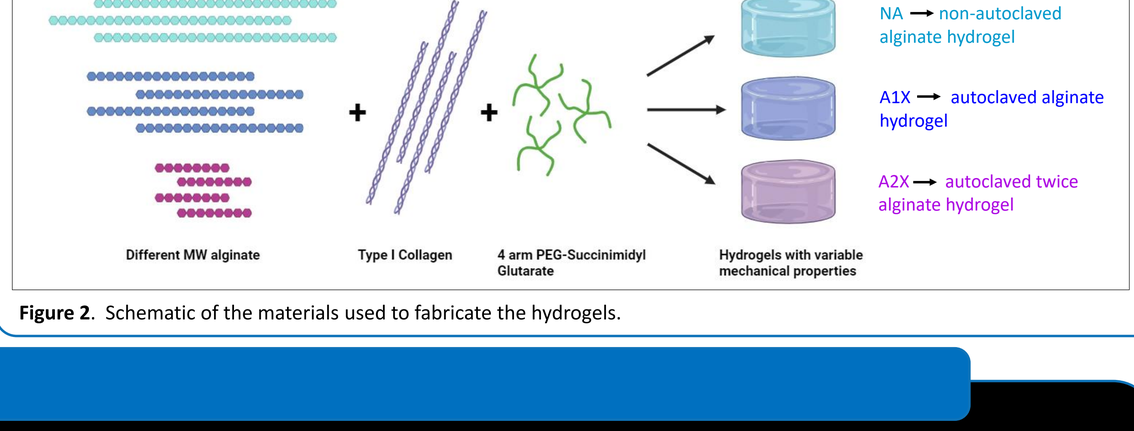


Figure 2. Schematic of the materials used to fabricate the hydrogels.

Results

Mechanical properties of the hydrogels

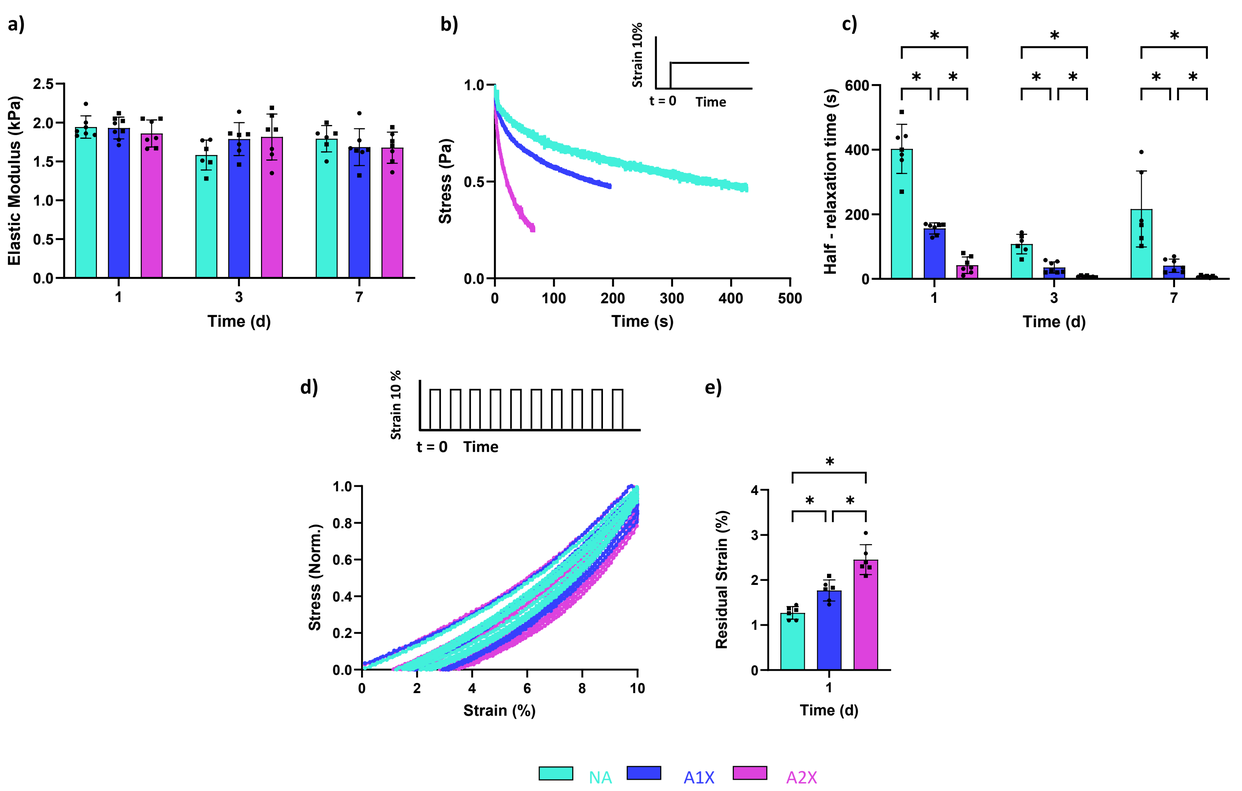


Figure 3. Evaluation of the mechanical properties of hydrogels under compression (a) Gels show constant elastic modulus. **(b)** Representative stress relaxation profiles for hydrogels. (**c)** Stress Relaxation time is largely different among gels and decreases with each autoclaving cycle. **(d)** Representative cyclic strain profiles of the hydrogels. **(e)** Permanent deformation of hydrogels after 10 cycles of compression, show increased deformation in more viscoelastic samples.* Indicates statistically significant difference between groups ($p < 0.05$).

Biochemical analysis of cell embedded hydrogels

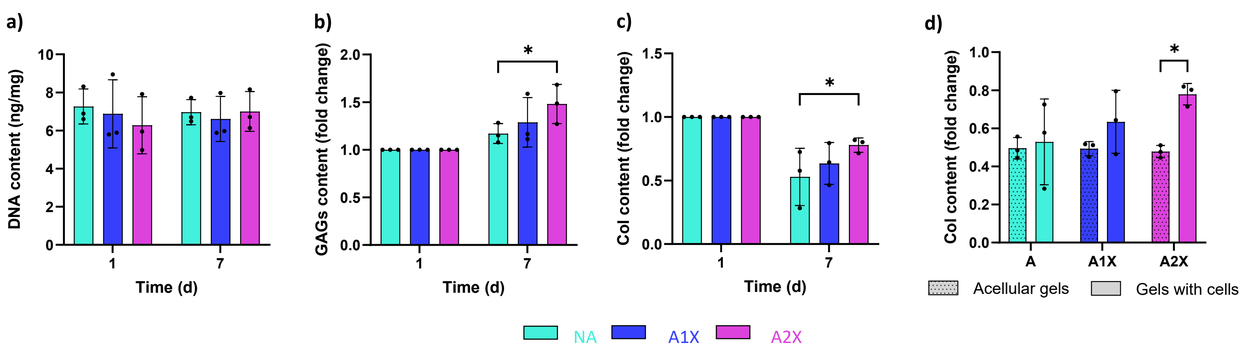


Figure 4. Analysis of DNA, GAGs and collagen of cornea stromal cells embedded in hydrogels of varying viscoelasticity(a) DNA content shows no significant differences occur among the groups between day 1 and 7. **(b)** Glycosaminoglycans content analysis shows higher production of GAGs in faster relaxing hydrogels. **(c, d)** Hydroxyproline assay shows that even though there's an overall decrease in collagen content, faster relaxing gels loose significantly less, probably due to higher deposition by cells as seen in (d) where collagen loss between acellular and cell gels is compared. * Indicates statistically significant difference between groups ($p < 0.05$).

Live/dead analysis

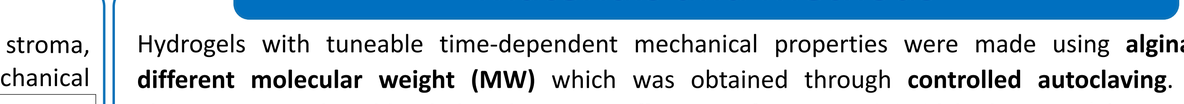


Figure 5. Live/dead imaging shows high viability for all groups, also indicating that changes in viscoelastic properties doesn't affect cell vitality.

Immunofluorescence of typical keratocytes markers

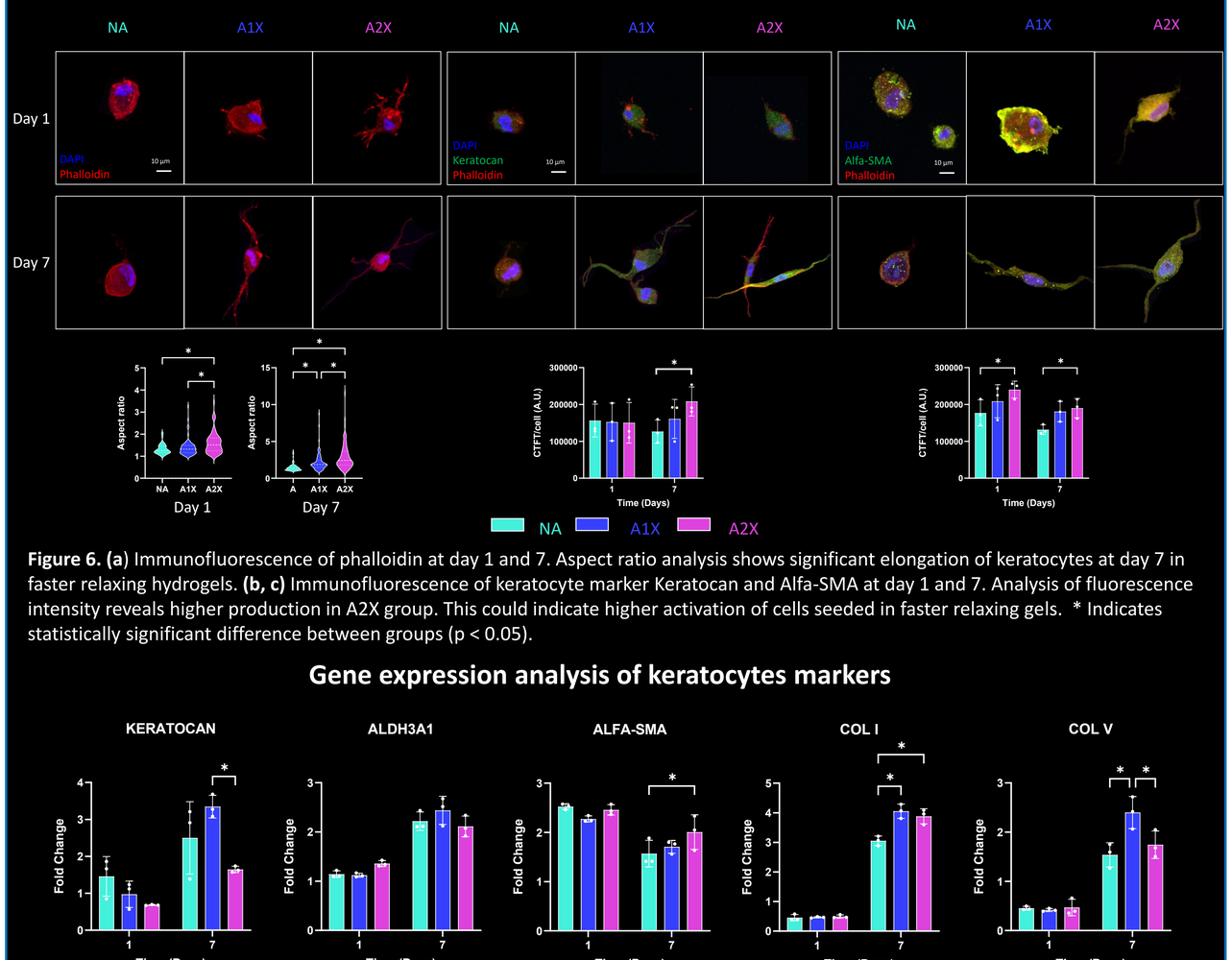


Figure 6. (a) Immunofluorescence of phalloidin at day 1 and 7. Aspect ratio analysis shows significant elongation of keratocytes at day 7 in faster relaxing hydrogels. **(b, c)** Immunofluorescence of keratocyte marker Keratocan and Alfa-SMA at day 1 and 7. Analysis of fluorescence intensity reveals higher production in A2X group. This could indicate higher activation of cells seeded in faster relaxing gels. * Indicates statistically significant difference between groups ($p < 0.05$).

Gene expression analysis of keratocytes markers

Figure 7. qRT-PCR of CSCs seeded in hydrogels. Graphs show overall higher expression of key ECM markers (Keratocan , Col I and Col V) and alfa smooth muscle actin suggesting a general higher activation of cells in faster relaxing gels * Indicates statistically significant difference between groups ($p < 0.05$).

Conclusions

- **Autoclaving ⟶ reduce** alginate **molecular weight ⟶** hydrogels with **tuneable time-dependent mechanical properties**[3]
- Tuning **concentration of $CaCl_2$ ⟶** gels with the **same elastic modulus**, but **different stress relaxation**.
- **Elastic modulus constant** over 7 days **⟶ stress relaxation decreases** slightly, probably due **⟶ ionic exchanges**
- Biochemical analysis **⟶ DNA** remains **constant**, but **higher** production of **GAGs** and **lower collagen degradation** in A2X group
- Embedded stromal cells **⟶** very **good viability**
- Day 7 cultures **aspect ratio analysis** of phalloidin stained keratocytes **⟶ significant elongation** in gels with **faster stress relaxation**
- Immunostaining of keratocytes markers **Keratocan** and **Alfa-SMA ⟶ significantly higher production in A2X** group
- PCR analysis **confirms** immunostaining and biochemical evaluation **⟶ higher expression** of EMC markers and activation marker in faster relaxing gels

In conclusion we have shown a simple method **to tune the viscoelastic properties** of hydrogels while keeping other parameters constant. We have also shown that these gels have **very good cytocompatibility**, and that **viscoelastic** properties can **affect keratocytes phenotype.**

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2. **Navaratnam et al. 2015**, *J. Funct. Biomater. 6*
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