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Pre-crosslinking of alginate for the development of storable and printable cell-laden bioinks for on-demand corneal bioprinting

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Introduction

3D Bioprinting

The principle of 3D bioprinting integrates biomaterials, live cells and controlled motor systems for creating complex structures. The aim of 3D bioprinting is to recapitulate native corneal tissue. 3D bio-printing has tremendous potential for future regenerative medicine applications, such as bioprinting corneal equivalents on-demand.

Bioink

A bioink is a mixture of cells, biomaterials and bioactive molecules that create the printed construct. Bioinks must be sufficiently viscous to be dispensed as a free-standing filament and have sufficient strength and stiffness to maintain structural integrity after printing. Biofabrication window for rational design of bioinks requires compromise between printability and biocompatibility.

Alginate

Alginate is obtained by treatment of the cell walls of brown algae (**Figure 1**). Alginate is a linear anionic polysaccharide composed of β-D-mannuronic acid (M block) and α-L-glucuronic acid (G block). M and G blocks can be consecutive or interchanging and affect the physiochemical properties of the produced gels. The versatility of alginate hydrogels, as well as their ECM-like features, renders them efficient for the use as bioinks.

Here we investigate whether combining the function of alginate as a bioink with its cell preservation potential generates a storable and printable bioink for on-demand corneal bioprinting. Similar approaches have been taken with alginate encapsulated cells prior to bioprinting.[1]

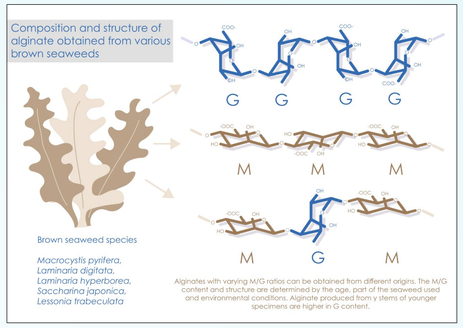


Figure 1 - Composition and structure of different alginates obtained from brown seaweeds.

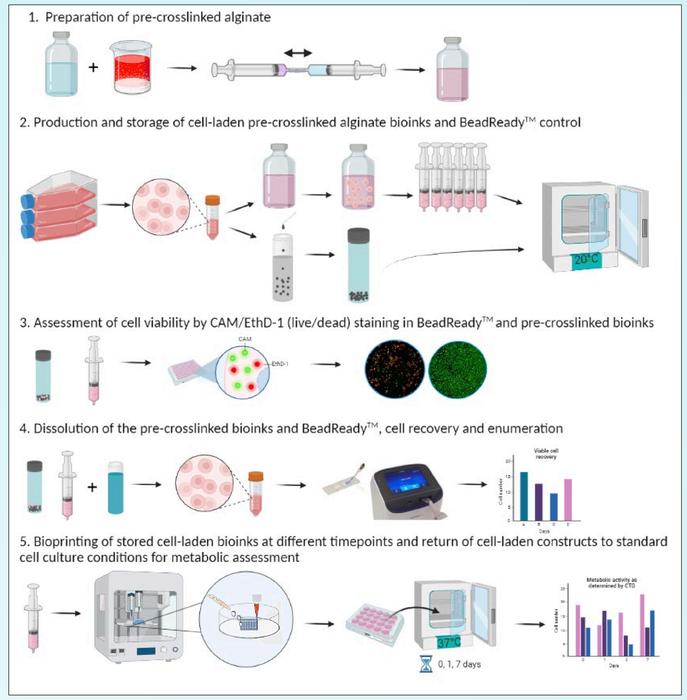
Methodology

Preparation of cell-laden pre-crosslinked alginate gel

1. Using the dual syringe mixing method, 2.4% medium viscosity high-M (MVM) alginate and 0.001M calcium sulphate ($CaSO_4$) solutions were mixed 50 times prior to 3-hour curing at room temperature (RT), producing the pre-crosslinked alginate bioink.
2. Primary human corneal stromal fibroblasts (hCSFs) were used to assess the feasibility of the modified gel to maintain cell viability during RT storage. Seeding density of hCSFs into $CaSO_4$ pre-crosslinked alginate gels was 1 million cells/mL of gel. hCSFs were encapsulated at the same density in BeadReady (a commercial product for hypothermic storage) to act as a positive control and medium only for the negative control. Cell-laden pre-crosslinked alginate gels were stored in bioprinting cartridges in a non-humidified incubator for 0, 1 and 3 days. Cells were encapsulated in BeadReady™ as per standard protocol provided by Atelerix and were stored in the same manner as the bioinks.[2]

Assessment of cell viability after storage

3. Cell viability was assessed in the gel on days 0, 1 and 3 with calcein AM and ethidium homodimer-1 (live/dead) staining in both BeadReady™ and pre-crosslinked bioink.
4. Viable cell recovery was assessed after gel dissolution before enumerating using an automated cell counter with trypan blue exclusion.
5. Following storage, pre-crosslinked cell-laden bioinks were 3D bioprinted using a pneumatic extrusion-based 3D bioprinter and returned to standard culture conditions. Cell viability in the 3D bioprinted constructs was assessed with CellTiterGlo.



Results

- Cell viability was maintained for up to 3 days within pre-crosslinked bioinks (**Figure 2a**) with some appearance of dead cells at day 3. Conversely when cells were stored in medium alone, there was a rapid increase in dead cells and very few live cells by day 3 (**Figure 2b**). Viability in pre-crosslinked bioinks was comparable to the positive control (BeadReady, **Figure 2c**) although storage in BeadReady resulted in no incidence of cell death.

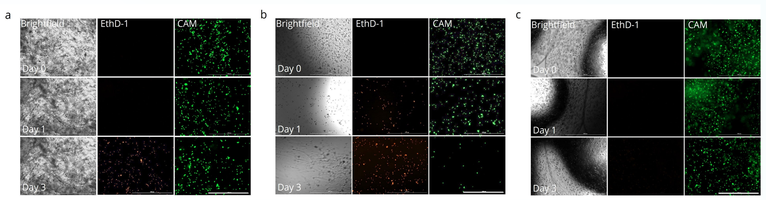


Figure 2 – Evaluation of hCSF viability by live/dead staining.

Encapsulated cells were assessed for viability by staining with CAM and Ethd-1. Pre-crosslinked bioink (a), non-encapsulated medium only negative control is presented in (b), Positive control (BeadReady) is presented in (c) Scalebars represent 1000µm.

- After gel dissolution viable cell recovery was assessed in the 3 storage conditions **Figure 3**. Viable cell number in the pre-crosslinked bioinks was significantly higher than the medium control over the 3-day time course ($p = < 0.05$) with no significant difference to the positive control at days 0 and 1 (90%, 80% cell recovery). At day 3, 60% of the cells were recovered in the pre-crosslinked condition.

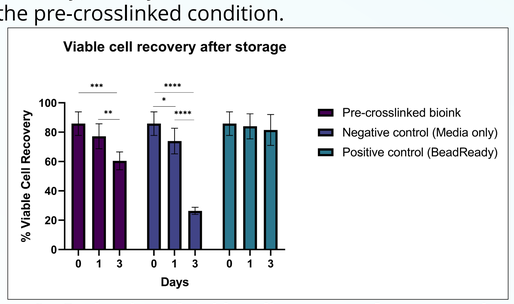


Figure 3 – Evaluation of viable cell recovery after storage by Trypan Blue enumeration.

Values are expressed as mean ± SD from three separate experiments using different cell donors.

- Following storage, cell-laden bioinks were successfully bioprinted using an extrusion system and cells exhibited high viability with no difference compared to the non-stored control in the same storage condition. Metabolic assessment after storage and 3D bioprinting has revealed hCSFs remain viable in standard culture conditions in pre-crosslinked bioinks with marginal cell death after 7 days (**Figure 4**).

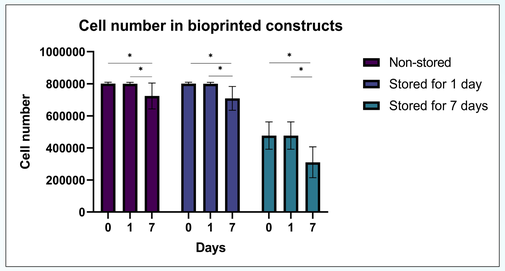


Figure 4 - Evaluation of hCSF viability in stored and 3D bioprinted constructs in culture. Values are expressed as mean ± SD from three separate experiments using different cell donors.

Conclusions

- Pre-crosslinked alginate bioink is suitable for storage of hCSFs for 3 days at room temperature with minimal cell death observed compared to the medium only control, where only 30% of encapsulated cells remained viable after 3 days of storage.
- No significant difference in cell recovery was observed in the pre-crosslinked bioink compared to the positive control on the day of encapsulation and after 1 day of storage, with a 20% decrease in cell viability observed after 3 days of storage.
- hCSFs remain viable in culture after storage for 0, 1 and 7 days and bioprinting with marginal cell loss.
- This simple, cost-effective, adaptable method demonstrates the feasibility of integrating storage and bioprinting in the same bioink, which can have wide-ranging applications for on-demand corneal bioprinting in the future.

References

1. Kostenko, A., Connon, C.J., Swioklo, S., 2023. Storable Cell-Laden Alginate Based Bioinks for 3D Biofabrication. Bioengineering 10, 23. https://doi.org/10.3390/bioengineering10010023
2. https://www.atelerix.co.uk/products/suspended-cells-beadready/