Pore Forming Alginate Gels with Tunable Porosity Affect Tendon Derived Cell Behavior

Nikhil Mahtani[2], David J. Mooney[1,2], Benjamin R. Freedman[1,2]
[1]Wyss Institute for Biologically Inspired Engineering at Harvard University, Boston, MA USA
[2]John A Paulson School of Engineering and Applied Sciences, Harvard University, Cambridge, MA, USA
Disclosures: Nikhil Mahtani (N), David J. Mooney (N), Benjamin R. Freedman (N)

INTRODUCTION: Increases in life expectancy have led to an increase in aging tendon injuries, negatively impacting millions of people worldwide [1]. While several new surgical, conservative, and advanced biological therapies have been proposed to improve the prognosis of tendon injuries, these treatments have not yet been able to effectively reduce high tendon re-rupture, re-tear, and re-injury rates. Promising advancements in biomaterial-based scaffolds have shown potential for tendon regeneration. In particular, porosity has been identified as a crucial characteristic of many of these scaffolds, as it influences cell behavior, proliferation, differentiation, and fate. Nonetheless, there is still a need in the field of tendon tissue engineering for scaffolds that can control the rate of pore formation and for studies on the optimal pore size for tendon cells. Therefore, the objective of this study was to engineer hydrogel scaffolds with tunable pore size and use them to study the effect of pore size on tendon-derived cell proliferation and release. We hypothesized that pore size would significantly affect tendon-derived cell behavior and that there would be an optimal pore size range for cell proliferation and release.

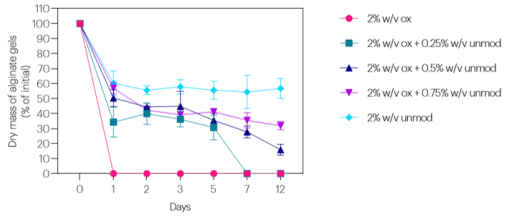

Figure 1 : Degradation rate of alginate can be modulated by varying amount of added unmodified alginate. In vitro degradation kinetics of gels constituted of 2% w/v oxidized (ox) alginate, 2% w/v unmodified (unmod) alginate, or mixtures of unmodified and oxidized alginate (n=4; mean ± SD shown)

METHODS: *Alginate Oxidation:* Medical grade, high molecular weight, high guluronic acid content (MVG) was oxidized and reduced to 7.5% [2]. Sodium periodate is added to a 1% w/v solution of alginate and left to stir and react overnight, protected from light. A tangential flow filter (TFF) was used to purify out the salt by-product of the reaction. Ammonium borane was then added to this solution and left to stir overnight. After another TFF step, the oxidized alginate is sterile filtered, frozen, lyophilized, and stored at -20ºC until use. *Alginate Porogen Fabrication:* Unmodified MVG alginate (0-0.75% w/v) in Dulbecco's Modified Eagle Medium (DMEM) is mixed with 2% w/v of 7.5% oxidized alginate and reduced alginate. Porogens are fabricated through a glass nebulizer (Meinhard) which is supplied with nitrogen at 10-30psi. Porogens are crosslinked for 5 in a calcium crosslinking bath of 100×10^{-3} M $CaCl_2$ and 100×10^{-3} M HEPES (pH 7.2) before being size separated with cell strainers (Pluriselect), washed and pelleted. *Pore Forming Alginate Hydrogel Fabrication:* A 1:1 mixture of 2% w/v of unoxidized MVG alginate and porogens was crosslinked by mixing with a sterile calcium sulfate slurry (1.2 M $CaSO_4 \cdot 2H_2O$ in H_2O) at 4% v/v relative to the bulk alginate. The solution was mixed back and forth rapidly before being injected between silanized glass slides separated by 1mm spacers. Gels are allowed to crosslink for 45 minutes before a 6mm biopsy punch is used to obtain samples (≈100 µL) for in vitro testing. *Bulk Gel Degradation Assay:* Alginate gels (100 uL) were incubated in 1 mL of DMEM at 37ºC. At set time points, gels were collected, rinsed, frozen, lyophilized, and then weighted to obtain dry mass. *Pore-Forming Gel Degradation Assay:* Void-forming gels made with FITC-labeled porogens are incubated in media. Media and gels were collected at each time point after the gels were digested with Ethylenediamine tetraacetic acid (EDTA). The amount of FITC present in the gel and in the media was measured using a BioTek plate reader with an excitation at 490nm and an emission of 520nm. *Proliferation Assay:* Cell proliferation was assessed after incubation with Edu for 24h at 7 days after encapsulation and was then imaged to evaluate the total number of proliferating cells (Confocal, Zeiss). *Cell Release Assay* The cells that were released from the gels and adhered to the bottom of the well plate were fixed and stained with a Hoechst DNA stain. *Statistical Analysis:* One-way analysis of variance (ANOVA) and Welch's t-test were used to compare multiple groups with post hoc t-tests with Bonferroni correction. Prism 8 (GraphPad) was used to perform all the statistical analyses.

RESULTS: Pore-forming gels were created by generating hydrolytically degradable alginate porogens and encapsulating them into an alginate gel. By modulating the amount of non-oxidized alginate added to these porogens, it was discovered that the rate of degradation of these porogens could be tuned (**Fig. 1**). The rate of degradation of porogens was found to decrease as the amount of unoxidized alginate added was increased. For tendon tissue engineering, porogens created with 2% oxidized alginate and 0.5% unmodified alginate were deemed to be most suitable since they demonstrated substantial degradation only after 7 days (the duration of the inflammatory phase of tendon repair). Cell strainers were used to gate porogens created by the nebulizer into three distinct size categories. 40, 100, 200, and 300-micron cell strainers allowed for the collection of porogens that were less than 40 microns, between 100 to 200 microns, and greater than 300-micron with very small variation and overlap between these sizes (not shown). These porogens were successfully fabricated and encapsulated into gels to create alginate hydrogels with these distinct pore sizes that form over time (**Fig. 2**). With this system, tendon-derived cells were incorporated into these gels to study the effect of pore size on tendon cell proliferation and release. These studies revealed that these tendon cells proliferated significantly more in the gels with pores that were greater than 300 microns. Moreover, cell release was also significantly higher in the greater than 300 micron condition compared to all conditions except the 100–200 micron condition (**Fig. 3**).

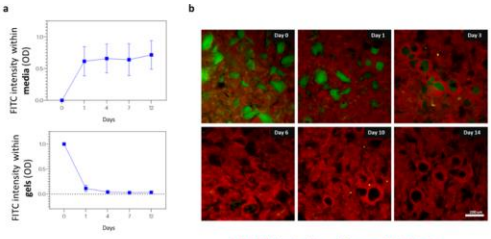

Figure 2 : Porogens exhibit controllable degradation kinetics when encapsulated within bulk alginate gels. (a) Quantitative analysis of FITC released in media over time from unlabeled bulk gels containing FITC-labeled porogens and FITC maintained in these same gels. (b) Qualitative confocal images of rhodamine-labeled bulk gel (red) and FITC-labelled porogens (green) were taken at different time points. n=3 gels/group

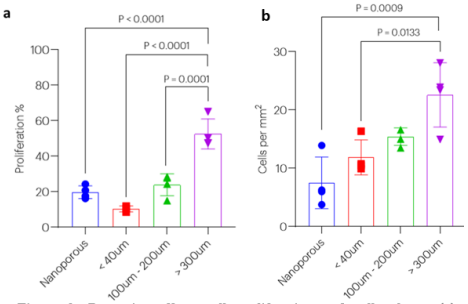

Figure 3: Pore size affects cell proliferation and cell release. (**a**) Quantification of proliferation rate by comparing the percentage of proliferating cells over total cells for different pore sizes. (**b**) Quantification of cell deployment counting attached cells using Image J for different pore sizes. Data are shown as mean ± standard deviation. Significance has been determined via one-way ANOVA. n=3 gels/group.

DISCUSSION: This study investigated the effect of pore formation on tendon cell proliferation and delivery. These results reveal that tendon cells are indeed sensitive to pore size and also pave the way for future studies on these macroporous gels seeded with tendon cells. Past work has shown that pore size may play an important role in cell differentiation, fate, viability, and behavior [3]. Accordingly, future studies will use this system to culture cells for longer periods and perform more extensive RNA-based assays to obtain information about cell health and behavior. Lastly, in vivo studies could be carried out where these gels are injected into animal models of tendon injuries to observe the functional beneficial effects of using these porous alginate hydrogels for tendon tissue engineering.

SIGNIFICANCE/CLINICAL RELEVANCE: This study elucidated the effects of pore size on tendon-derived cells in pore-forming alginate gels for tendon tissue regeneration. This knowledge can be used to optimize and improve hydrogel-based cell delivery systems for tendon tissue repair.

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