**Subculturing adherent cells on GAG modified surfaces**

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**Abstract:** This experiment tested the biocompatibility of a chitosan scaffold versus a scaffold made with chitosan cross-linked with chondroitin sulfate. Flat scaffolds in tissue culture plates were made from chitosan solution and chitosan augmented with chondroitin sulfate (CSA). Human fibroblasts were seeded onto these scaffolds, as well as a control. The degree of cell proliferation was assessed one week after plating. The results were disappointing: not only did cells fail to proliferate on either scaffold but the fewest amounts of cells were present on the chitosan/CSA scaffolds.

*Keywords*: chitosan, chondroitin sulfate, cross-linking, cell culture, scaffolds

**1. Introduction**

 Chitosan is a polymer used often for tissue engineering scaffolds due to its high degree of customization; crystallinity and pore size, shape and structure can all be modified during the scaffold creation process. However, while chitosan exhibits excellent biocompatibility, its abilities to promote cell growth and proliferation are subpar when compared to other scaffold materials like collagen. But, due to the presence of positively charged amide groups in its monomer units, chitosan can be very easily modified by adding immobilized glycosaminoglycans (GAGs) to its surface. The immobilization of the GAGs on a scaffold (often done with EDC solution) is important because this more accurately reflects the positioning of GAGs *in vivo*. The GAG used in this experiment was chondroitin sulfate (CSA) [1]. It was predicted that while a chitosan scaffold would show little in the way of cell proliferation, a chitosan/CSA cross-linked scaffold would show high levels of proliferation over that of the chitosan and control groups.

**2. Materials and Methods**

*2.1 Materials*

* Chitosan (medium molecular weight) from Sigma Aldrich
* Chondroitin sulfate from Sigma Aldrich
* Human Peridontal Ligament Fibroblasts (HPDLFs)
* HEPES buffer from Sigma Aldrich
* Phosphate-buffered saline (PBS)
* 2% acetic acid solution
* Dulbecco’s Modified Eagle’s Medium (DMEM) from Invitrogen
* Fetal bovine serum (FBS)
* Pen-strep antibiotic
* Trypsin
* Sterile six-well culture plate

*2.2 Scaffold Fabrication*

 The scaffolds and control were contained in a six-well plate. The first two wells were designated as the control group and left unmodified. The second and third sets of two wells were coated with a 1% chitosan solution in 2% acetic acid. The plates were allowed to dry over the course of a week and the solutions hardened into a gel-like substance. Afterwards, a solution of 10% CSA solution was applied one set of the four chitosan wells. After allowing 18 hours for the CSA to crosslink with the chitosan, the scaffold and control wells were rinsed with PBS. Then, they were sterilized with 70% ethanol for one hour at 37 °C. After being rinsed with PBS again, the plates were ready for cell seeding.

*2.3 Cell Seeding*

 The target seeding density for each well was 30,000 cells. Cells were removed via trypsinization from a previously cultured plate with a cell density determined to be 411,500 cells per mL. Each well could hold 2 mL. This meant that the volume of cell solution to be added to each well was:

$$\frac{30,000 cells}{x mL}=\frac{823,000 cells}{2 mL}$$

Which yielded 0.0729 mL or 72.9 µL.

 The cell solution was added to each well and media was added to make up the difference of 2 mL. The media consisted of Dulbecco’s Modified Eagle’s Medium – which contains vitamins, glucose, and amino acids, fetal bovine serum – which contains growth factors required for cell proliferation, and pen-strep – a mixture of two antibiotics to prevent bacterial infection of the culture. The media was changed roughly every two days.

*2.4 Cell/Scaffold Characterization*

 Cell density was characterized via counting by hemocytometer 1 week after seeding. The wells were also visualized under a light microscope throughout the week to qualitatively measure cell proliferation. The cells were removed from the plates via trypsinization again, stained with Trypan blue, and counted. The purpose of the Trypan blue stain was distinguish between living and dead cells, as living cells would not uptake the dye. The areas counted on the hemocytometer consisted of four grids 1 mm2 each. The cells counted on all four grids could be translated to cell counts of each well with the following equation:

$$\frac{\# cells counted ({cells}/{mm^{2})}}{4}×\frac{1 cm^{2}}{100 mm^{2}}×10$$

The equation is multiplied by 10 because each well covers 10 cm2. The number of cells counted on the hemocytometer was averaged between each of the two wells for the control and two scaffolds. Comparisons were made between scaffolds for cell viability based on these results.

**3. Results and Discussion**

 Early light microscope visualization showed marked proliferation of cells in the control wells, but little to none in the wells with scaffolds. Cells that were present were likely to be found along the edges of the wells or in bubbles with no scaffold surface, suggesting that they were actively avoiding the material.

 Quantitative analysis supported these observations. Surprisingly, the chitosan cross-linked with CSA scaffolds showed the least amount of cell proliferation. Only the control wells showed an increase in cell number from the original 30,000 cell seeding density (Table 1).



This is unexpected because it was believed that the chitosan/CSA scaffolds would show an *improvement* in cell proliferation, at least over that of the plain chitosan scaffolds. The chitosan scaffolds were expected to produce very few viable cells. However, the data shows that the chitosan scaffolds provided a higher degree of cell viability over the cross-linked scaffold, though nowhere near that of the uncoated wells. In addition, the cells present on each of the scaffolds were likely from uncoated regions, like those visualized under the light microscope.

 One possible explanation of the chitosan/CSA scaffolds’ poor results is that the chondroitin sulfate was not adequately attached to the chitosan before seeding took place. It is also possible that there was retention of ethanol from sterilization in each of the scaffolds; this would have severely impaired the ability of cells to grow on them.

**4. Conclusion**

 Two scaffolds were created: one of chitosan and another of chitosan cross-linked with chondroitin sulfate. These scaffolds, and the control, were seeded with human fibroblasts at a density of 30,000 cells per well. One week later, the only wells showing growth were those of the control, while the scaffolds showed a marked decrease in cell density from the original seeding. The cross-linked scaffold, contrary to expectation, seemed to do the most poorly in promoting cell proliferation. Possible explanations include improper cross-linking or retention of ethanol in the scaffolds.

**References**

[1] Keong LC, Halin AS. *In Vitro* Models in Biocompatibility Assessment for Biomedical-Grade Chitosan Derivatives in Wound Management. Internations Journal of Molecular Science: Multidiscinplinary Digital Publishing Institute, 2009; 10:1300-1313.

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**Appendix**

“GAG modified surfaces by Matthew and Suh” protocol (see attachments)

“Subculturing adherent cells” protocol (see attachments)

I have neither given nor received any unauthorized aid in completing this work, nor have I presented someone else’s work as my own.

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