DEVELOPMENT OF SCHWANN CELL-ENCAPSULATED ALGINATE SCAFFOLDS FOR THE REPAIR OF PERIPHERAL NERVE INJURY

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INTRODUCTION

Nerve conduits for peripheral nerve repair have progressed from simple silicon tubes to complex engineered scaffolds. Recent advances in scaffold fabrication have enabled the incorporation of neurotrophins,[1] extracellular matrix components[2] and various cells[3] into scaffolds for enhanced biologic properties. Bioplotting is one of the emerging methods, where the scaffold material, in form of a solution, is dispensed from a needle, layer by layer forming a three-dimensional structure.[4] It enables the use of a wide range of materials, ranging from synthetic polymers (like polycaprolactone, polyglycolic acid, etc.) to naturally occurring polymers like alginate, chitosan, etc. Notably, the use of hydrogels gives the advantage of incorporating live cells during the dispensing procedure.[5] However, the use of hydrogel for the scaffold fabrication by bioplotting has shown to be a challenging task as their weak mechanical properties could cause collapse of the structure during the fabrication process.

To alleviate the collapse problem, a straightforward method is to use an additional temporary support structure to prevent collapse. But having temporary supports could complicate the dispensing procedure. Another promising method is to dispense hydrogel (e.g. alginate) into a medium of cross-linking solution to solidify hydrogel. In this method, the buoyant force on the alginate strands, once dispensed in the medium, helps prevent the strands from collapse. However excess buoyancy may cause them to detach from previously dispensed layers underneath. Thus the medium density must be properly selected to match that of the alginate solution. As a general rule, the medium density should be similar to that of the alginate solution to diminish the negative effects of the buoyant force.[6] On the other hand, another effective way is to enhance the attachment between the layers of strands. To do this, addition of polycationic materials such as polyethyleneimine (PEI) to the medium is rationale, as PEI has been shown to reinforce the crosslinking of alginate with calcium ions.[7] Besides, favoring the interaction with alginate (an anionic polymer) during the crosslinking process, PEI could also enhance the mechanical properties of cross-linked alginate. However the concern here is that the addition of PEI may not be favorable to cells that are to be encapsulated into the scaffolds during the fabrication process.

By addressing the above issue, this study is aimed at fabricating live-cell scaffolds with the addition of PEI during the fabrication process. The cells selected for encapsulation are derived from the rat Schwann cell-line (RSC96), as the scaffolds fabricated in this study would find potential applications in the repair of peripheral nerve injury. Particularly, we demonstrate the viability of the Schwann cells to short-term exposure to the solutions with PEI and show that dispensing alginate into minimal concentrations of PEI also enables well-defined scaffold structure.

MATERIALS AND METHODS

Sodium alginate, calcium chloride dihydrate, polyethyleneimine solution (average Mw of 750,000, 50 wt. % in H_2O) and Dulbecco's modified Eagle medium (DMEM) were purchased from Sigma Aldrich, Canada. Rat Schwann cell-line cells (RSC96) were obtained from the American Type Culture Collection (ATCC) and used after four passages. Sodium alginate was dissolved in DMEM at a concentration of 3% w/v, and calcium chloride,

was used to cross-link alginate at a concentration of 50 mM.

A 3D-bioplotter (EnvisionTec, Germany) shown in Fig. 1 was used to fabricate scaffolds with the designed patterns. Specifically, the alginate solution was dispensed through a precision conical needle (EFD, USA) with an inner diameter of 200 µm into dispensing medium consisting of calcium chloride, glycerol and varying concentrations of PEI. The pressure for dispensing was 0.1 bar and the needle was set to move at a speed of 8 mm/s. These parameters were set to ensure fabrication of the designed scaffold structures.

To facilitate the assessment of cell viability, scaffolds with a square contour pattern of 3 layers were fabricated to have a size of 6x6x0.4 mm (Fig. 2a). The effect of PEI on fabrication was demonstrated by constructing multi-layered scaffolds of size 10x10x3 mm. The direction of the alginate strands on each layer was at right angles to the preceding layer, until 31 layers were built (Fig. 2b,c).

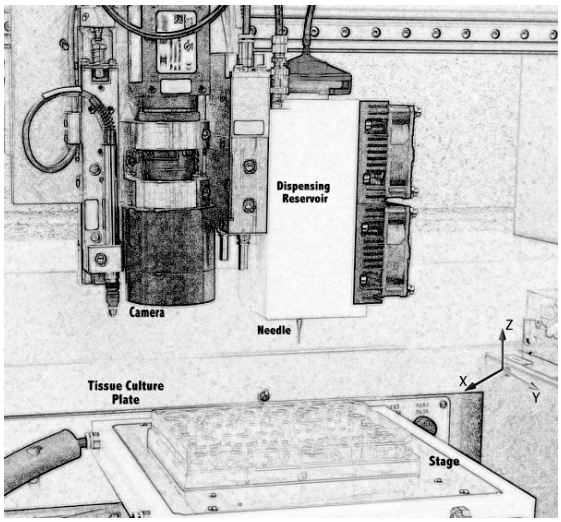

Figure 1: 3D-Bioplotter

A cell suspension of RSC96 cells was mixed with alginate to give a density of 7.8 x 10^5 cells/ml of alginate. This was dispensed into 12-well tissue culture plates coated with PEI, to enhance adhesion of alginate to the plate bottom. The dispensing media were prepared with varying PEI concentrations. Five minutes after the scaffold fabrication, the dispensing media was removed and all wells were washed thrice with DMEM. The cells were immediately stained using Calcein-AM. They were incubated at 37° C for half hour and imaged using a fluorescent microscope (Zeiss Axiovert 100). The plane of focus was adjusted to acquire images of the cells along the whole length of the scaffold pattern. These images were stacked using ImageJ (NIH, Bethesda, Maryland, USA) and using the function, grouped Z-project, the image stack was flattened. The number of cells was then quantified on all the planes.

Toxicological behavior of the PEI on RSC96 cells was examined using an MTT assay. Briefly, a suspension of RSC96 cells was plated on a 96-well tissue culture plate and incubated overnight. Various concentrations of PEI were added to the wells. MTT solution (5 mg/ml) was added to each well and the plate was again incubated for 5 hours. The cell media was aspirated and DMSO was added. The absorbance was read at 560 nm using a UV-visible spectrophotometric micro-plate reader. The control wells did not have any PEI added to the media.

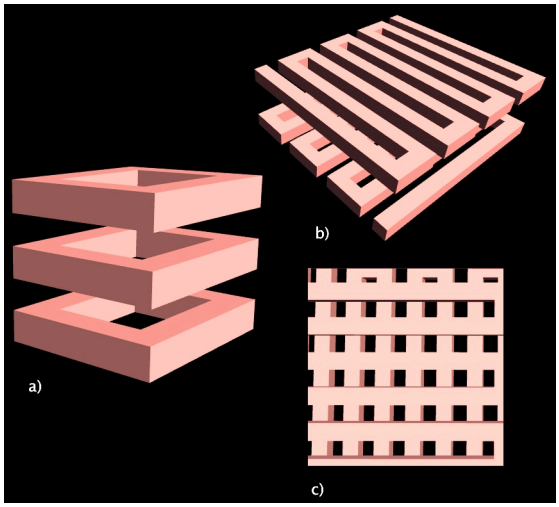

Figure 2: Patterns of scaffolds

The presented data were in the form of mean with standard error of the mean (SEM). Statistical significance was assessed by a one-way analysis of variance (ANOVA) followed by Tukey's HSD test. Statistically significant difference with level of $P<0.05$ was estimated

for the comparison between groups of data. Graphpad Prism 5.0a for Macintosh (GraphPad Software, San Diego, California, USA) was used for statistical analysis.

RESULTS

The optical density at 560 nm is proportional to the formazan produced by active reductase enzymes in live cells. The results in Fig. 3 show that on overnight incubation of cells in DMEM containing various concentrations of PEI, the absorbance is nearly zero, indicating the absence of formazan. This shows that prolonged exposure of cells to PEI results in their death.

After scaffold fabrication the viability of RSC96 cells was examined with the results shown in Fig. 4. It is seen there is no statistical significance between the control group and the groups with glycerol and varying concentrations of PEI. This suggest that the inclusion of PEI does not affect cell viability if the scaffold fabrication process is not prolonged and is less than five minutes, as examined in this study. The staining of RSC96 cells with Calcein-AM is shown in Fig. 5, suggesting the presence of live cells in the scaffold.

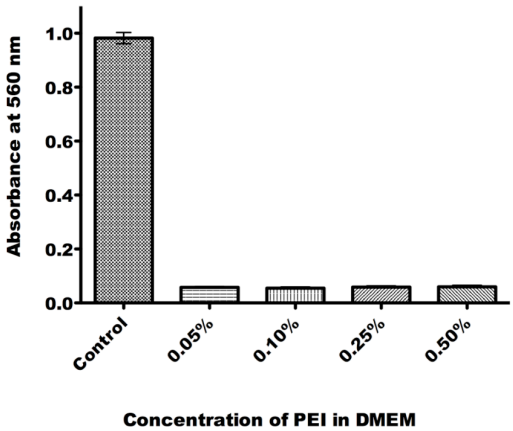

Figure 3: MTT Assay

Scaffold structure was examined with the results shown in Fig. 6. It is seen that with the presence of PEI, a three-dimensional structure of scaffold with 31 layers can be well formed. Light microscopic images of the scaffold (Fig. 6a) further confirm the presence of vertical pores. By simple tensile tests with hands, it was found the layers of alginate strands were attached firmly. This could be attributed to the interactions between PEI and alginate during the crosslinking process. In contrast, without PEI, the alginate strands seemed to be misaligned and did not form a pattern.

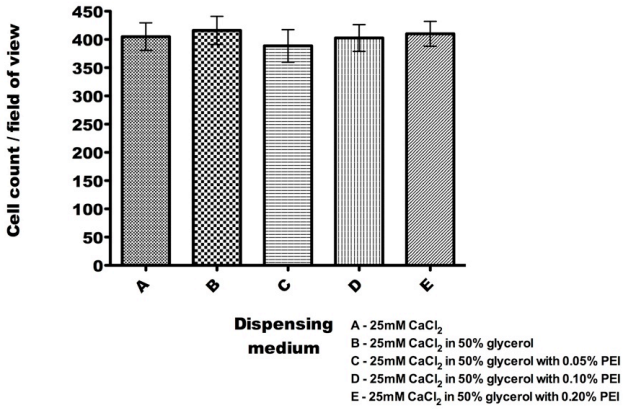

Figure 4: Cell Viability

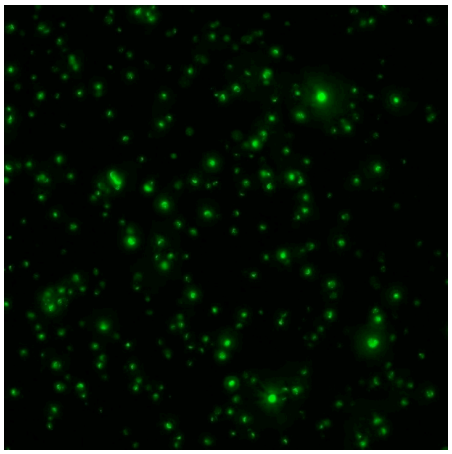

Figure 5: Calcein-AM staining of RSC96 cells in alginate

DISCUSSION

With almost zero absorbance of the wells containing PEI in the MTT assay, toxicity of PEI on RSC96 cells is confirmed. Even concentrations as small as 0.05% w/v were toxic, on an overnight incubation. Calcein-AM is a fluorescent dye that selectively labels viable cells.[8] On transport into the cell membrane, the acetomethoxy component of calcein-AM is removed by intracellular esterases (seen only in

live cells) and it fluoresces. Staining the alginate patterns containing cells, after few minutes of dispensing showed similar number of cells in each field of view, irrespective of the inclusion of PEI.

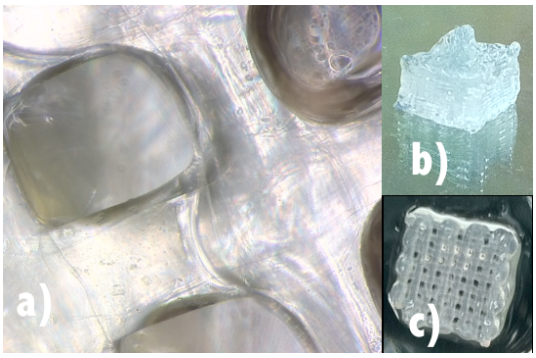

Figure 6: Fabricated alginate scaffold in presence of PEI. a) Microscopic image of scaffold. b, c) 31-layered scaffold.

Thus short-term exposure to PEI does not seem to affect cell viability. PEI has also been shown to increase the mechanical properties of alginate scaffolds.[9] During the process of crosslinking, strong ionic interactions occur between alginate and the cationic PEI.[7] The presence of these interactions help with the easy fabrication of multi-layered scaffolds of alginate using bioplotting.

CONCLUSION

We present the development of a method to fabricate alginate scaffolds for peripheral nerve repair using low concentrations of PEI in the dispensing solution. Though toxic on prolonged incubation, short exposure during the bioplotting technique does not seem to be a concern. The addition of PEI in the dispensing medium helps prevent the collapse of the stacked layers of alginate. The reason is attributed to the interaction of PEI with the alginate during the crosslinking procedure. Further studies will consist of assessing the mechanical properties and degradation rates of scaffolds fabricated with PEI incorporated and *in vivo* implantation studies in rat sciatic nerve models.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the financial support from the Natural Sciences and Engineering Research Council of Canada (NSERC) and the Saskatchewan Health Research Foundation (SHRF).

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