INJECTABLE CRYOGELS FOR NEURAL TISSUE ENGINEERING APPLICATIONS

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ABSTRACT

We show novel millimeter-sized injectable neural scaffolds based on cryogels exhibiting interconnected macropores, for neural tissue engineering applications. The polymer-scaffolds are designed to be compressible, allow cell adherence and it injection through a syringe needle while preserving neural cells viability and neurite integrity. The system is a potential answer to a major dilemma encountered in neural tissue engineering for the central nervous system: cellular scaffolds must be pre-organized and potentially large, but at the same time they should be delivered in a minimally invasive fashion to avoid further tissue damage.

KEYWORDS: Neurons, cryogels, tissue engineering, alginate

INTRODUCTION

In the field of tissue engineering, experimental strategies employing cell transplantation have notably held great promise for repairing the injured central nervous system. Cell transplant alone is not sufficient to regenerate a functional neuronal network in a lesion cavity: cell survival after graft is very low and transplanted cells appear to build chaotic constructs only around the lesion area [1]. Scaffolds made from various polymers and hydrogels have been proposed in order to support and protect the transplanted cells, guide graft growth and facilitate its integration with the host tissue. However, the transplantation of such scaffolds remains challenging. One proposed solution to this problem consists in minimally invasive delivery of in-situ gelling formulations or scaffold suspensions [2] through narrow-bore needles. However, without the guidance of a large-scale organized scaffold, the cells typically build disorganized structures rather than repairing the native tissue architecture as desired. The scaffolds developed here are both: organized at a large size scale, and nevertheless injectable thanks to their compressibility. To the best of our knowledge, the injectable neuronal cryogels reported here are the first to combine macroscopic scaffold size and injectability through a narrow-bore conduit for neuronal tissue engineering applications.

EXPERIMENTAL

Cryogel synthesis

Cryogels were synthesized based on established carbodiimide chemistry [3]. Briefly, alginate is dissolved in deionized (DI) water to the desired concentration, and crosslinking initiated by adding adipic acid dihydrazide (AAD) and an excess of the carbodiimide EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide). The reaction mixture is placed at -20°C in a mold. This results in ice crystal formation prior to completion of gel crosslinking. After 24h, the cryogels are thawed, washed and autoclaved in PBS.

Cryogel coating

Autoclave-sterilized cryogels were coated prior to cell culture. A poly-L-ornithine (PLO, 1mg/mL diluted in sterile deionized water) droplet was then deposited on top of each cryogel and left for 1 hour at 37°C. The PLO droplet was then removed, and the cryogel samples rinsed with DI water. The cryogels were again partially dehydrated, and laminin (1µg/mL in sterile DI water) was added (1x cryogel sample volume) and left for four hours at 37°C.

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Cell culture and cell behavior analysis

Mouse primary cortical neurons were prepared from embryonic day 17 Of1 fetal mouse brains. Cells were plated in neurobasal medium supplemented with 2% B27 and 2 mM Glutamax. Neurons were seeded onto cryogel and control substrates at a density of 4000 cells/mm^3. Half-media was changed twice weekly. At experimental endpoints, the cellular scaffolds or control substrates were fixed with 4% paraformaldehyde. Actin cytoskeleton and cell nuclei were stained. Fluorescence images of at least 200 cells on four randomly chosen observation fields were captured and analyzed for each experimental condition and for each experiment (n=3). Cell density was quantified as the number of cell nuclei in a given volume. Based on confocal images, neurite length was measured using the plugin NeuronJ of ImageJ. For cell survival analysis, samples were rinsed with HEPES-buffered salt solution (HBS), and incubated in a trypan blue solution (0.4% in HBS). The cells of five independent observation fields were observed and counted on the whole sample thickness using a bright field microscope with x20 objective. Cell viability (in %) resulted from the ratio between the number of non-stained cells and the total cell number.

RESULTS AND DISCUSSION

The polymer-scaffolds are designed to be highly compressible. The macroporous scaffolds can be partially dehydrated, reducing the size of the scaffold and allowing to perform injection through a syringe needle, as schematized in Figure1a-b. After injection, scaffolds retrieve their initial shape and volume, as shown on the pictures of Figure 1b. Figure 1c-d presents SEM images showing the microscopic architecture of the alginate cryogel scaffolds. The pores dimension are comprised between 30µm and 500µm and mainly depend on the kinetics of the cryogelation process. After a coating step with adhesive molecules, the scaffolds were partially dehydrated and seeded with primary cortical mouse neurons, as schematized on Figure 2a. At the initial time of 3D plating, the dissociated neurons had a spherical shape. Over time in culture, neurites sprouted and developed leading to interconnected neural networks. Figure 2b shows confocal images of the cellular scaffold obtained after 7 days in culture. The analysis demonstrates a complex network of neurites with an approximately homogeneous cell density throughout the 1mm thick scaffold. Cell densities of cells developing neurites was above 40.10^6 cells/mL. It approaches the ones found in the mouse cortex (92*10^6 cells/mL[4]), and are higher than the live densities that can be obtained with homogeneous gels by one order of magnitude. Neurite development was comparable for 3D scaffold and 2D glass control surfaces. Figure 2c indicates that mean neurites length increased with time also showing the compatibility of the developed scaffolds with long term neuronal culture (21 days in vitro). The main interest of the developed scaffolds is that they are injectable. We thus investigated cell survival to the injection of the 3D cellular construct through a 16Gauge needle performed after 7 days of culture. Results are presented in Figure 3. 2D controls consisted in 2D alginate layers of the same composition as the 3D scaffolds. Figure 3a shows a confocal image of neuronal networks morphology after the injection step. Figure 3b indicates that the injection step does not affect the viability of primary neurons and figure 3c further shows that even the developed long neurites did not suffered from the compression and expansion step performed for the injection. The 3D structure, with its high available surface area and high pore space for nutrient diffusion is responsible for sustaining high viability at these high cell densities. The gels are soft at the macroscopic scale, enabling syringe injection and potentially good cellular scaffold integration in the target tissues. At the same time, the scaffold material has a high local Young modulus, allowing to protect the neurons during injection.

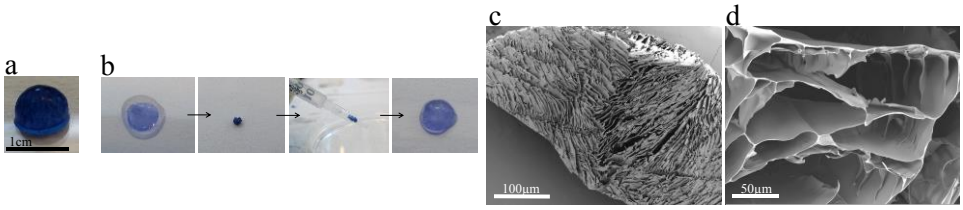

Figure 1: Cryogels based on alginate developed for neuronal cell culture. a : picture of a spherical cryogel, colored in blue. b : 3D scaffold before dehydration, after partial dehydration, inserted in a syringe and after it injection through the syringe. c-d : SEM images of the alginate cryogel.

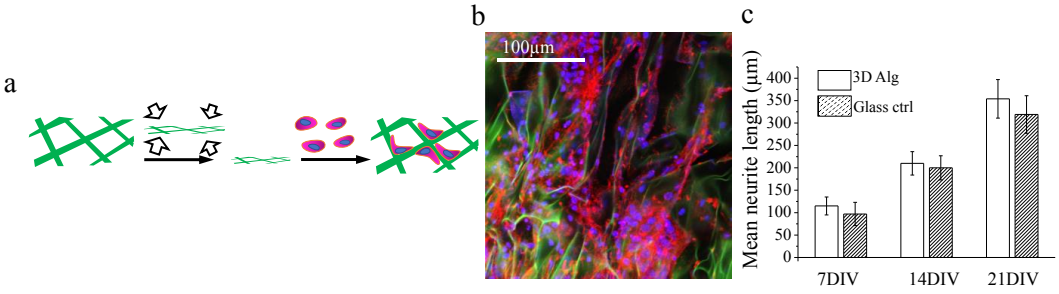

Figure 2: a : seeding step. b : confocal image of the scaffold after 7 days in vitro (z- projection of a 50□m thick stack),green : cryogel, red : actin cytoskeleton, blue : cell nuclei. c : neurite length obtained on 3D scaffolds and on 2D glass control substrates, after 7, 14 or 21 days of culture.

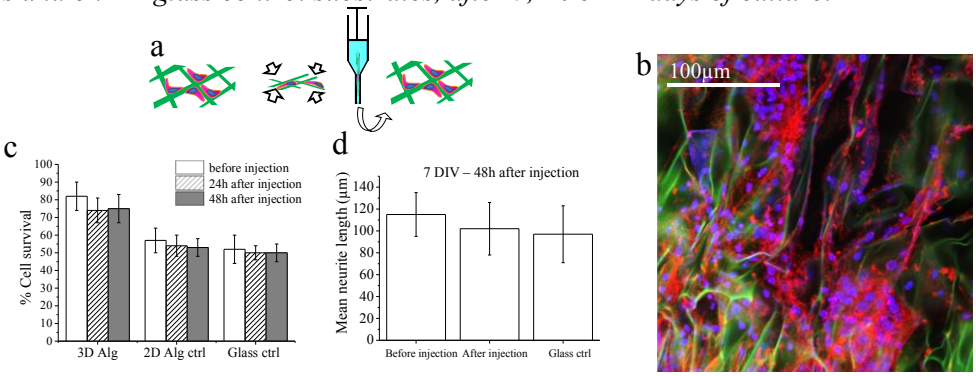

Figure 3: a : injection step. b : confocal image of the neuronal network after 7 days in vitro and 48hours after injection (z- projection of a 50µm thick stack), green : cryogel, red : actin cytoskeleton, blue : cell nuclei. c : cell survival before and after injection. d : neurite length analysis before and after injection.

CONCLUSION

Developed 3D injectable scaffolds show high cell survival rates, normal neurite development and are compatible with long term neuronal cultures. Cell density, neurite development and scaffold organization are preserved throughout the injection process. The scaffolds will thus allow to transfer in vivo not only the cells, but also their precise spatial organization.

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