Longer axons of spinal cord neuron on Stiffer DNA crosslinked hydrogels

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INTRODUCTION

DNA crosslinked hydrogels

Our DNA-crosslinked hydrogel research project involves the development of novel biomaterial that can be used for a broad spectrum of biological and medical applications. In particular, it allows the design of materials that mimic biological tissues on the tissue, cell, and molecular levels, as well as the design of active biomimetic materials that can reversibly change shape.

Spinal Cord Injury and Axonal regeneration

Once described as 'a disease that cannot be treated' by ancient Egyptians, injuries to the spinal cords (SCI) have caused tremendous economic and emotional costs to the inflicted individuals and society. Researchers have been searching for cures and have identified axonal regeneration, and synaptogenesis and formation of the neuronal circuitry among the key steps in gaining functional recovery after SCI.

Various approaches have been attempted, to promote neuronal growth and protect neurons from further insults, which include designing bio-scaffold, delivering trophic factors, and other biological reagents and transplanting cells such as olfactory ensheathing cells and stem cells [2].

Our DNA crosslinked hydrogels have taken part in this effort. They have the potential as biomaterials for scaffold construction to integrate the major approaches including biological, cellular and guidance therapies. In this study, we have explored the potential of using these gels for axonal regeneration.

MATERIALS AND METHODS

Gel Preparation

DNA crosslinked poly-acrylamide hydrogels were prepared as described previously [1]. Briefly, Acrydite™ modified single stranded DNA, SA1 and SA2, were respectively co-polymerized with acrylamide monomer (10%) to form viscous fluids containing long polymer chains with SA1 or SA2 as side branches. After mixing these two, upon the addition of L2 crosslinker a hydrogel was created.

These hydrogels were immobilized on a a circular cover glass (18mm) by using optical glue (Optical Adhesive #72, Norland). The gels were finally kept in PBS at 4°C.

Gel Functionalization

In order to activate the gels for cell attachment, a bifunctional photoactivable crosslinker Sulfo SANPAH (Pierce, Rockford, IL) in HEPES buffer was applied allowing attachment of type I collagen (USB, Cleveland, OH) or poly-D-lysine to the gel surface.

Rat Spinal Cord Cell Culture

Rat spinal cord cells were obtained freshly from dissection of E16 rat embryos, which contain both glial cells and neurons. They were then plated on DNA gels at a density of 25K cells/cm^2 in serum-containing medium composed of MEM, 10% horse serum and 0.6% glucose supplemented by antibiotics. The plate was kept in a humidified CO_2 incubator at 37°C. One day after cell plating, the medium was changed to neural basal medium with B-27 supplement, antibiotics and β-mercaptoethanol to specifically promote neuronal growth.

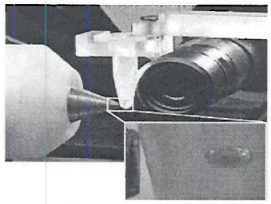

Figure 1. Experimental set up for mechanical measurement of DNA crosslinked hydrogels.

Immuno-cytochemistry and Characterization

On Day 5 cells were fixed using paraformaldehyde (4%), followed by incubating with primary antibodies against MAP2 (dendrite and cell body), Tau-1 (axons) and GFAP (mature astrocytes), and appropriate secondary antibodies. Labeled neurons were visualized by immuno-fluorescence. Cell number (as indicated by the appropriate immunostain in conjunction with DAPI stain) in each of the 10 pictures in each well will be counted at a magnification of 100X. Neurite length was measured by using the Scion Image software (Scion Corp.). Only those neurites with clear ending point were measured

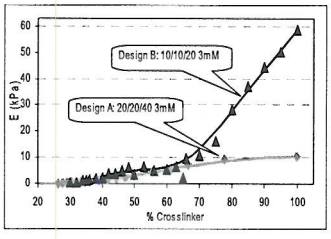

Figure 2. Changes of mechanical stiffness of DNA crosslinked polyacrylamide hydrogels with crosslinking density at room temperature.

Mechanical Characterization

Mechanical stiffness of DNA crosslinked hydrogels was probed by using a non-intrusive method developed in our lab (Figure 1).

By applying a known force with a magnet to a magnetic bead embedded in the gels, the moduli of the gels can be calculated by measuring the displacement of the bead, assuming homogenous, isotropic and elastic materials. The results of compressive moduli of DNA crosslinked polyacrylamide gels at two different designs are shown in Figure 2.

RESULTS

DNA Gel design

DNA crosslinked hydrogels used in this study were of design D1 (Table 1) and characterized to have a stiffness of 10 kPa, which is in the range of that of rat spinal cord [81], and D3 with a rigidity of 60kPa.

Table 1. DNA gel design (number of bases of single stranded DNA)

Design	SA1	SA2	L2	Initial DNA conc. (mM)	Stiffness (kPa) (100% crosslinking)
A	20	20	40	3	10
B	10	10	20	3	60

Spinal cord cell growth on DNA crosslinked gels

Cells were found to attach, spread and extend processes on all gel samples. Immunocytochemistry revealed that neurons exhibited typical morphology as shown in Figure . As the first step, we examined the specificity of Tau-1 staining of spinal cord neurons owing to the previous report that it stained both axons and dendrite of hippocampal neurons in in intro studies. We found that there was little overlap between MAP2 and Tau-1 stain indicating the specificity of Tau-1 stain on axons of rat spinal cord neurons, though Tau-1 was also found expressed in soma (**Figure 3**). Occasionally, relative long processes (over 1mm) were also observed in the neural cell culture on the DNA gel (**Figure 4**).

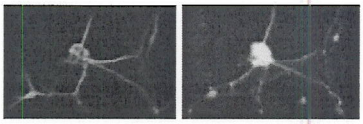

Figure 3. Immunostaining images of rat spinal cord neurons on DNA gels. (Left) Red for dendrites and cell body (MAP2) and (Right) Green for axons (Tau-1).

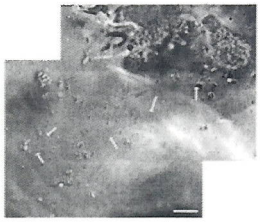

Figure 4. Rat spinal cord cells extended processes on DNA crosslinked polyacrylamide hydrogels, with the length of visible part about 1mm. Scale bar: 10p□m.

Cells were first subjected to DNA gels of two different designs (**Table 1**, both at 100% crosslinking) with average stiffness of 10 and 60 kPa (**Figure 2**). No significant difference was found in the number of neurons, astrocytes or total cells (**Figure 5**).

Though average neurite length did not display significant difference, the branching number was relatively higher on stiffer gels (**Figure 5**), which differs from previous reports [3]. However, the fact that the range of stiffness in this work (~10kPa to 60kPa) was different than that of the previous one (50Pa to 550Pa) [82] and that the specie difference (mice vs. rats) most likely contribute to the distinction.

Next, we investigated the cell response to DNA gels with distinctive crosslinking densities (50% and 100%) of the same design (B, **Table 1**). The resulting stiffnesses of these gels are 5 and 60 kPa, respectively. The results are summarized in **Figure 6**. Cells are more spread on gels of higher crosslink density based on average neuronal cell spreading area, which is in accordance with previous findings that cell spreading was reduced on softer gels [4] The soma is more elongated in shape as indicated by aspect ratio, which is not sensitive to stiffness (**Figure 6**). Neuronal branch number and neurite length were not significantly affected, while significantly longer axons were seen on fully crosslinked gels, suggesting the potential of promoting axonal elongation. The average neuronal branching number is about 2.7 (before normalization) due to large proportion of neurons exhibiting a bipolar morphology. Additionally, there was no significant difference in number of neurons, astrocyte or total cells on two different gels (data not shown). Many previous studies have shown that focal adhesions and integrin signaling are involved in cell mechanosensing, and elucidation of the underlying mechanism for neuronal cell behavior on DNA gels is under way.

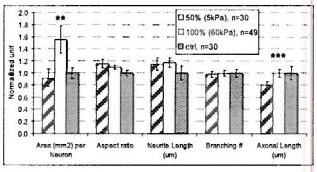

Figure 5. Phenotypical study of rat spinal cord cells on DNA gels with two designs (Soft: Design A; Stiff: Design B) and culture plate. All parameters are normalized with respect to the quantity on control culture plate. (*p<0.01)

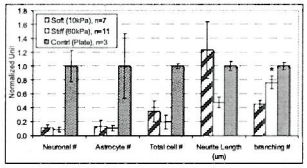

Figure 6. Rat spinal cord cell culture on DNA crosslinked hydrogels with different crosslinking density and culture plate. All parameters are normalized with respect to the quantity on control culture plate. (**p<0.05; ***p<0.02)

CONCLUSIONS

In summary, the observations confirmed that neuronal cells respond to differences in mechanical properties of the hydrogel substrate, and suggest that the sensitivity to stiffness might be affected by the nature the biomaterials. Longer axons have been found on the stiffer DNA gels about 60kPa. This work demonstrated the versatility in manipulating physical properties of DNA gels and will continue to be carried out to provide further information for the dynamic studies.

Acknowledgement

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