Contract 34-05

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Title: **Bifunctional biomaterial design for spinal cord regeneration**

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1. Original aims of the project:

As stated in the proposal, the *broad, long-term objective* of this research is to develop a force-actuating, implantable biomaterial to spur regeneration and growth of the spinal cord. Two general tissue engineering strategies have been employed in attempt to restore spinal cord function following trauma. The first and most prevalent is the development of biomaterials that serve as bioactive scaffolds to stimulate neurites to regenerate and grow through the injury site. The second strategy is to employ mechanical force, or traction, to axons at very slow rates and physically force the nerves to grow. The second technique has been used *in vitro* to generate neural tissue in the hope of ultimately "splicing" together the spinal cord.

The *Specific Aims* of this proposal were as follows:

Specific Aim 1: To identify the force-actuating potential of DNA-crosslinked hydrogels. *Hypothesis: Increasing the stiffness of the hydrogel by introducing more crosslinks will cause the hydrogel to shrink and exert force.*

Specific Aim 2: To functionalize DNA-crosslinked hydrogels for axon attachment and growth.

Specific Aim 3: To determine the potential for the functionalized, force-actuating gels to control axon growth in vitro.

We were granted one year of funding, beginning December 15, 2004. Therefore, two of the three stated specific aims were accomplished.

2. Project Success:

Force-actuating potential of DNA-crosslinked hydrogels

A special chamber of 20x5x5 mm was designed to perform the force actuation experiment. A porous polymer block was rigidly glued to one end of the chamber and another porous block was placed at the other end. A calibrated force transducer was glued to the free block. Sufficient room was provided in the chamber for the hydrogel to swell as shown in figure 1. Two designs of the DNA crosslinked hydrogel are considered, D1 and D3, with base lengths of 20:20:40 and 10:10:20 for SA1, SA2 and L2 crosslinker respectively.

It was observed in a previous study that gelation at room temperature occurs at 50% crosslink for design D1 and at 30% crosslink for the design D3. This is used as a starting point and a 50% crosslinked D1 gel is cast inside the chamber. The gel gets interlaced in the porous polymer, thus attaching rigidly after gelation. 1X TE buffer is introduced in the chamber, until the gel is completely immersed, to swell the gel to equilibrium. After 2 hours of swelling, the TE buffer is drained out leaving just enough for keeping the gel hydrated. For the D3 gel, a 30% gel is cast in the chamber and a similar procedure is followed as for the D1 hydrogel for swelling to equilibrium.

DNA crosslinks are then slowly introduced by delivering them directly on to the gel, without disturbing the setup. Time-lapse digital images are continuously taken using a COHU CCD camera. The crosslinks are added in controlled increments increasing the concentration from 50% to 90% in steps of 10% every one hour for the D1 hydrogel and from 30% to 90% in steps of 10% every hour for the D3 hydrogel.

An optical fiber cable of length 25 mm, diameter 240 μm and Young's modulus of 7.1GPa is used as the cantilever. The force generated during shrinking is evaluated by measuring the displacement of the cantilever when the gel shrinks. The cantilever is calibrated by a force deflection curve obtained by using small weights. The results for the shrinking of the gels and the force produced are shown in Table 1. Also the stiffness of each of these gels is calculated using previously developed techniques [1, 2].

For the D1 hydrogel, the gel sample had an initial length of 4800μm. On addition of 1X TE buffer solution, the sample swelled to equilibrium at 5200μm. Upon addition of crosslinks a maximum contraction of 25.7% was observed at 90% crosslink density. The corresponding force generated was calculated to be 249μN. The contraction and force vs. crosslink density is shown in Figure 3(a). Similar results were obtained for the D3 hydrogel, which had an initial length of 2450μm and swelled by 50μm upon addition of TE buffer for 2 hrs. On adding 10% crosslinks every 1 hour, the gel contracted by a maximum of 160μm and the corresponding force was 24.6μN. The results for the behavior of the gel are shown in Figure 3(b).

The forces obtained above are much larger in comparison to the maximum force shown by Heidemann to induce extension of single neurons [3]. However we are applying the above force across the entire cross-section of the hydrogel and the force will be distributed among many neurites.

DNA-crosslinked hydrogels for neuronal attachment and growth

Introduction of a DNA crosslinking mechanism to polymer gel formation gives rise to many new possibilities, such as modifying DNA gels in a controlled temporal and spatial manner. Meanwhile, due to the similarity between bis- and DNA-crosslinked polyacrylamide hydrogels (Bis- and DNA-gel), neuronal cell culture on bis-gels could provide general guidelines for that on DNA gels. Therefore, we started our experiments by probing the sensitivity range of rat spinal cord cells with a popular bis-acrylamide hydrogel culture system. Bis polyacrylamide gels (5% (soft), 10%, and 20% (very stiff)) were prepared on 12 mm glass cover glasses, and the resulting gel thickness was approximately 100μm. Gels were functionalized with sulfo-SANPAH, a bifunctional linker, and then coated with poly-D-lysine (1 mg/ml) or collagen (0.2 mg/mL). E16 rat spinal cords were dissected, dissociated, and cells were plated on polyacrylamide gels in wells of a 24-well plate at a cell density of 50,000 cells/cm^2 in serum-containing medium, which is composed of minimum essential medium (MEM), 10% horse serum and 0.6% glucose supplemented by antibiotics (penicillin/streptavidin). Cells were cultured in a humidified CO_2 incubator at 37°C, and one day after cell plating, the medium was changed to Neurobasal medium with B-27 supplement, antibiotics, and β-mercaptoethanol (1μL per 500mL medium). On day 6, cells were fixed with 4% paraformaldehyde, followed by immunocytostaining with antibodies against MAP2 (a neuronal cell marker, 1:1000 in blocking solution), GFAP (a mature astroglial marker, 1:200), and Vimentin (an immature astroglial marker, 1:200). Labeled cells were visualized by immunofluorescence (Olympus IX50 microscope with a Cooke Sensicam charge-coupled device cooled camera, fluorescence, imaging system, and Image Pro software). Numbers of cells and primary dendrites were counted, and the length of the primary dendrites was measured. The results are shown in Figures 2 and 3. Immunocytostaining revealed typical multi-polar morphology of spinal cord neurons, and both neurons and non-neuronal cells, including mature and immature astroglia (Figs. 2&3), attached and spread on the gels, an observation different than a previous report. Our results indicate that although the number of MAP2+ neurons is not significantly different

across the stiffness range, neurite number varies on low, intermediate, and high stiffnesses (Fig. 3), which has important implications in the biomaterial design for spinal cord injuries. Additionally, pure neuron and pure astroglia cultures were studied and contrasted with mixed culture, and we found that the sensitivity of spinal cord neurons to mechanical stiffness is affected by the presence of cell-cell interactions (1), which can also be utilized in the design of scaffolds where the relative cell population distribution can be controlled.

Following this work, we then expanded our DNA gel design space by including crosslinker length as a design parameter. Mechanical characterization showed that a range of stiffness from a few hundred Pa to approximately 60 kPa has been made available from the current DNA gel designs. After immobilization of DNA gels onto cover glasses, we functionalized these DNA gels with extracellular matrix molecules, including collagen and poly-D-lysine, for cell attachment. We then plated rat spinal cord cells on these gels with different designs. Immunocytostaining revealed typical multi-polar morphology of spinal cord neurons (Fig. 4). We found that among the cell population that survived after dissection and on the gels, a relatively small percentage (20~30%) of them are neurons, and neurons and mature astroglia represent approximately 60% of the total cell population on DNA gels. Immunostaining using antibodies against Tau-1 revealed axonal growth on the DNA gels. Further assessment and quantification of neurite outgrowth is under way.

3. Project challenges:

There is a significant amount of information available on Bis crosslinked gels. We used this material to learn and perfect our fictionalization and evaluation methodologies. Properties of DNA gels are unknown for use as a biomaterial. We had to learn on our own as there are no data available. We had to do several trial runs to get our DNA gels to behave like a biomaterial. The results are discussed above.

The main challenge we have in front of us is the consistency and reproducibility in fabricating DNA crosslinked gels with the desired properties. We also discovered that unlike Bis crosslinked gels, DNA crosslinked gels are sensitive to incubator temperatures.

4. Implications for future research and/or clinical treatment.

We have been able to perform static *in vitro* cellular studies. At present, under the current NJSCR grant, we are investigating dynamic alteration in DNA gel properties and how these changes affect neuronal cell growth and attachment.

5. Plans to continue this research, including applications submitted to other sources for ongoing support.

DNA crosslinked gels remain challenging yet very exciting new biomaterials. Based on our NJSCR dynamic study results, we are making plans to submit NIH grants in very near future.

6. List and include a copy of all publications emerging from this research, including those in preparation.

One journal article is published and second one is ready to be submitted. In addition, five conference Proceedings articles have been published, these conference papers are valuable to disseminate the scientific information as quickly as possible and get feedback from the peer groups. There are nine other related conference publications and two invited talks.

Journal publications:

1. Jiang, X, Georges, PC, Li, B, Du, Y, Kutzing, MK, Previtera, ML, Langrana, NA and Firestein, BL, "Cell growth in response to mechanical stiffness is affected by neuron-astroglia interactions", The Open Neuroscience Journal, Vol. 1, pp 7-14, 2007.
2. Jiang, X, Yurke, B, Firestein, BL, and Langrana, NA, "Neurite outgrowth on a DNA crosslinked hydrogel with tunable stiffnesses", in preparation.

Conference Proceedings Publications:

1. Jiang, X, Du, Y, Firestein, BL, Shreiber, DI, Schloss, R, Yurke, B, Chippada, U, Li, L, and Langrana, NA, "Neural Cell Engineering Using a Polyacrylamide Hydrogel – A Preliminary Study", in the Proceedings of the 2006 Bioengineering Conference, Paper No. BIO2006-157302, ASME, Amelia Island, Florida, June 21-25, 2006.
2. Jiang, X, Li, B, Du, Y, Firestein, BL, Yurke, B, Chippada, U, Li, L, Shreiber, DI, Schloss, R, and Langrana, NA, "Design of DNA-Crosslinked Hydrogels For Neural Cell Study", Page 180, In the Proceedings of BMES Conference, Chicago, MD, October 2006.
3. Jiang, X, Firestein, B, Du, TY, Shreiber, D, Rosenson-Schloss, R, Yurke, B and Langrana, NA, "Longer axons of spinal cord neuron on stiffer DNA cross linked hydrogels", in the Proceedings of International Society for the Study of Lumbar Spine, June 2007.
4. Jiang, X, Du, Y, Chippada, U, Li, L, Firestein, BL, Yurke, B, Shreiber, DI, Schloss, R, and Langrana, NA, "Neurite Elongation And Branching On DNA Crosslinked Polyacrylamide Hydrogels", in the Proceedings of the 2007 Bioengineering Conference, Paper No. SBC2007-176153, ASME, Keystone, Colorado, June 20-24, 2007.
5. Jiang, X, Firestein, B, Du, TY, Shreiber, D, Rosenson-Schloss, R, Yurke, B and Langrana, NA, Cell growth in response to mechanical stiffness is affected by neuron-astroglia interactions", In the Proceedings of BMES Conference, Los Angeles, CA, September 2007.

Related Publications (on NIH grant):

6. Li, L, Sharma, N, Chippada, U, Jiang; X, Schloss, R, Yarmush, ML, and Langrana, NA, "Functional Modulation of ES Derived Hepatocyte Lineage Cells via Substrate Compliance Alteration", Annals of Biomedical Engineering, accepted for publication, January 2008.
7. Lin, DC, Langrana, NA, Shreiber, DI, and Yurke, B, "Cellular Engineering Substrate With Tunable Compliance And Force-Generating Capability", Page 967, In the Proceedings of BMES Conference, Baltimore, MD, October 2005.
8. Langrana, NA, Shreiber, DI, Jiang, X, Li, L, Schloss, R, Lin, DC, Firestein, B, and Yurke, B," DNA-crosslinked gel for spinal cord regeneration", in the Proceedings of International Society for the Study of Lumbar Spine, June 2006.

9. Chippada, U, Langrana, NA, Jiang, X, Shreiber, DI, Schloss, DI, Jiang, X, Li, L, and Yurke, B, "Obtaining the Local Properties of Soft Hydrogels using Non-Intrusive Methods", in the Proceedings of the 2006 Bioengineering Conference, Paper No. SBC2006-156951, ASME, Amelia Islands, Florida, June 21-25, 2006
10. Li, L, Jiang X, Chippada, U, Yurke, B, Schloss, R, and Langrana, NA, "Differentiation of Embryonic Stem Cells on Dynamic Polyacrylamide Gels", in the Proceedings of the 2006 Bioengineering Conference, Paper No. BIO2006-157398, ASME, Amelia Island, Florida, June 21-25, 2006.
11. Chippada, U, Langrana, NA, Jiang, X, Zubok, A, Li, L, Shreiber, D, Schloss, R, Yurke, B, "A non-intrusive method to obtain the local properties of Soft and Hard Hydrogels", in the Proceedings of BMES Conference, Chicago, October 2006.
12. Li, L, Chippada, U, Jiang X, Schloss, R, Yurke, B, Yarmush, M., and Langrana, NA, Effects Of Substrate Compliance On Hepatic Differentiation Of Embryonic Stem Cells", Page 308, In the Proceedings of BMES Conference, Chicago, MD, October 2006.
13. Chippada, U, Langrana, NA, Jiang, X, Shreiber, DI, Schloss, DI, Jiang, X, Li, L, and Yurke, B, "Force/Torque Field Generation To Obtain Local Properties Of Soft Hydrogels", in the Proceedings of the 2007 Bioengineering Conference, Paper No. SBC2007-176081, ASME, Keystone, Colorado, June 20-24, 2007.
14. Chippada, U, Langrana, NA, Jiang, X, Li, L, Yurke, B, Shreiber, DI, Schloss, R, "Force/Torque Applied on Microneedles Embedded in Soft Hydrogels", in the Proceedings of BMES Conference, Los Angeles, CA, September 2007.

Invited talks

15. "Strategies to modulate DNA-crosslinked hydrogel stiffness to understand cell behavior", invited seminar at RPI, Department of Biomedical Engineering, CCNY Presentation, March 7, 2007
16. "Biomechanical Strategies to understand and Promote Neuronal Cell Behavior", invite seminar at the Section on Tissue Biophysics and Biomimetics, National Institutes of Health, February 21, 2008.

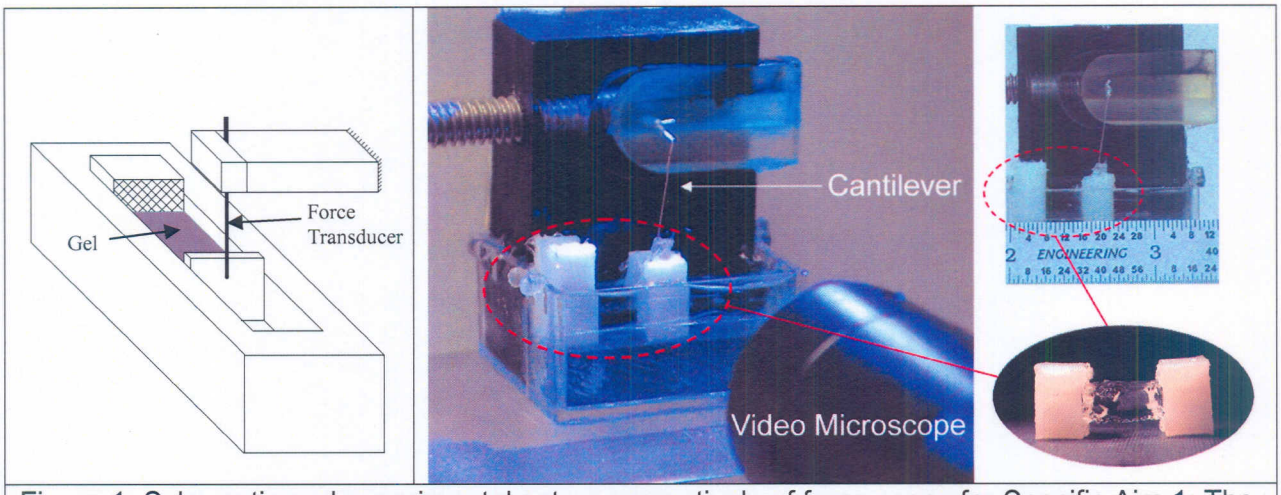

Figure 1: Schematic and experimental set up respectively of force assay for Specific Aim 1: The DNA-crosslinked gel is formed in a Teflon trough and then is exposed to buffer. One end of the gel is fixed to the assay chamber and the other is tethered to an ultra low force transducer. As DNA-crosslinking strands are introduced, the gel stiffens and shrinks as it can better withstand osmotic forces, and the crosslinks prevent expansion. The force associated with the shrinking gel is measured with the force transducer.

Table 1: Contraction and force generated by D1 and D3 gels at different crosslink densities

Gel	Crosslinker (%)	Stiffness (kPa)	Contraction (μm)	Force (μN)
D1	50	2.97	0	0
D1	60	5.09	263.97	41.83
D1	70	7.04	279.5	44.38
D1	80	8.96	698.73	116.8
D1	90	10.12	1381.86	249.89
D3	30	0.91	0	0
D3	45	4.01	15.71	2.41
D3	60	8.4	47.09	7.25
D3	70	12.13	62.86	9.70
D3	80	32.42	78.16	12.09
D3	90	55.12	94.34	14.63

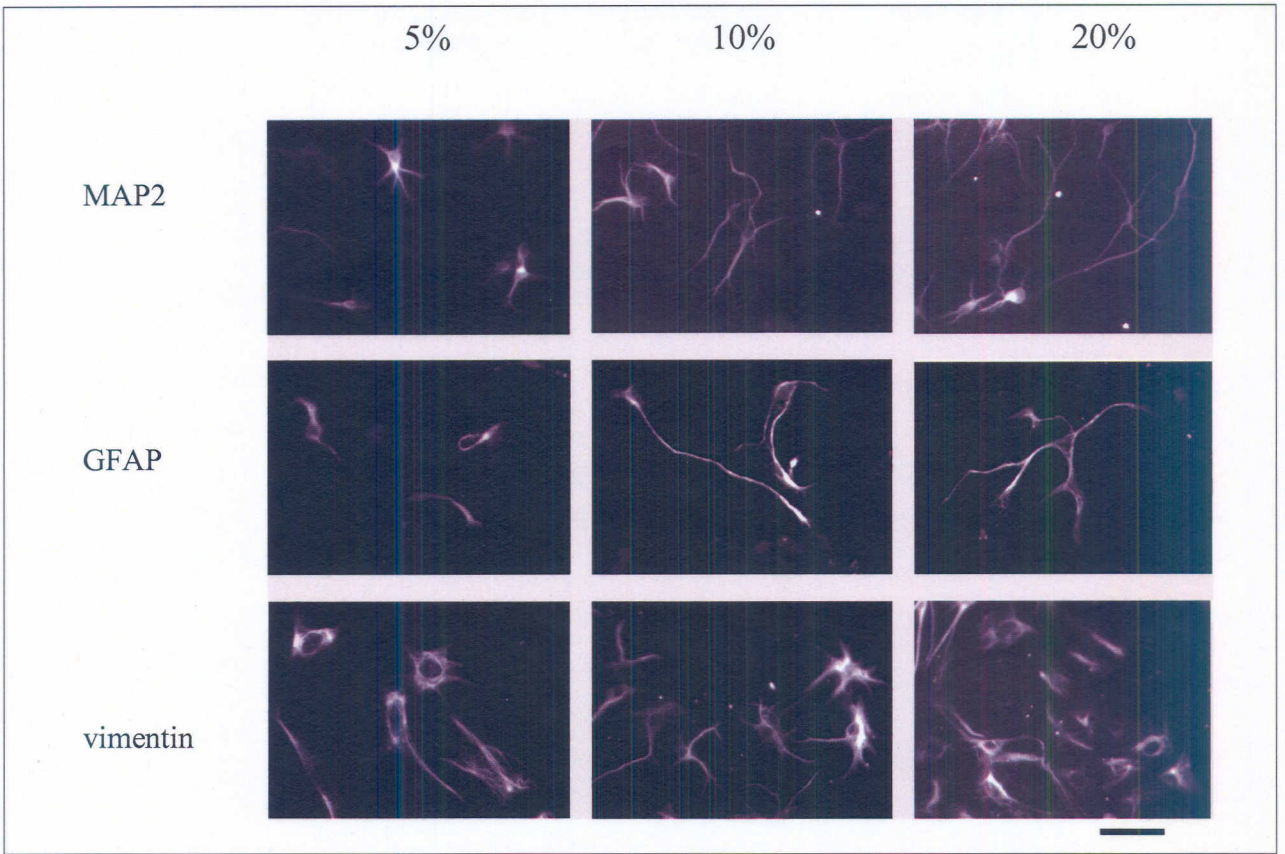

Figure 2: Mixed culture of rat spinal cord cells on bis-polyacrylamide gels. Upper panel: MAP-2 immunocytochemistry shows typical morphology of spinal cord neurons on all three gels (5%, 10%, and 20%). Middle: GFAP immunocytochemistry shows the growth of mature astrocytes on bis-crosslinked polyacrylamide gels. Lower panel: Vimentin immunocytochemistry shows the growth of immature astrocytes. Scale bar: 30 μm.

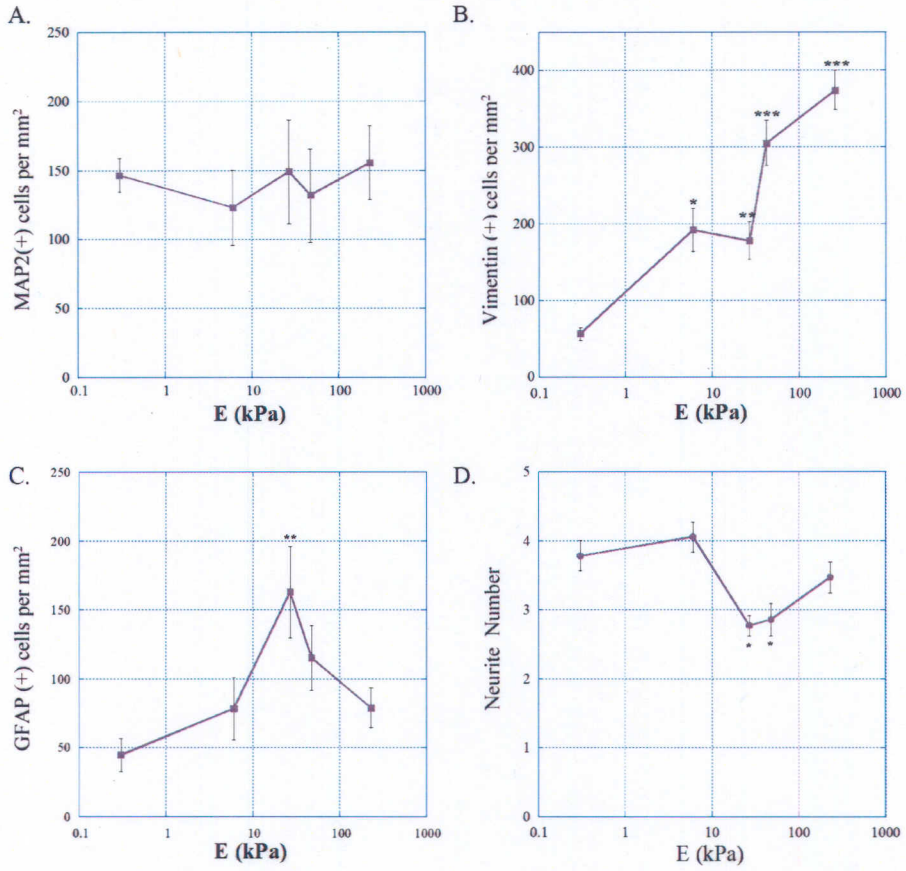

Figure 3: Effects of different gel stiffnesses on spinal cord cell growth. A: Quantification of MAP2+ neurons grown on bis-gels. The results indicate that neuronal cell number does not significantly change with gel stiffness. B: Quantification of vimentin+ cells identifies immature astrocytes growing on bis-gels. Immature astrocyte adhesion increases with gel stiffness. C: Quantification of GFAP+ cells to identify mature astrocytes growing on bis-gels. Mature astrocytes preferentially adhere to 27 kPa gels. D: Neurite number is lowest on 27 and 47 kPa gels compared to 300 Pa gels. *p□ 0.05, **p□ 0.01, ***p□ 0.001 by ANOVA followed by Tukey HSD test for multiple comparisons versus 300 Pa gel.

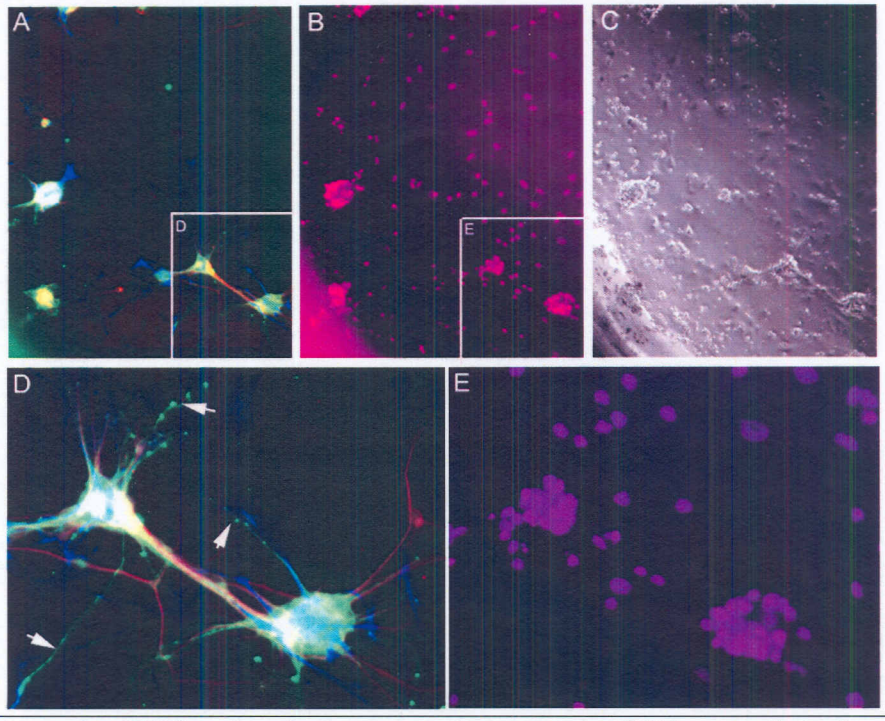

Figure 4: Embryonic (E16) rat spinal cord cells grew on DNA crosslinked poly-acrylamide hydrogels with stiffness of about 5kPa. Immunocytostaining images (A, 10× and D, 20×) have shown the axon, cell body and dendrite of neurons as well as astrocytes, and DAPI staining images cell nuclei (B, 10× and E, 20×) in the corresponding phase contrast images (C). Axons (Tau) in green indicated by arrow, dendrite and cell body (MAP2) in red, astrocytes (GFAP) in blue (A/D) and cell nuclei (nuclei acid with DAPI) in purple (B/E). Scale bar: 100□m..