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Directed cell growth on protein-functionalized hydrogel surfaces

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Abstract

Biochemical surface modification has been used to direct cell attachment and growth on a biocompatible gel surface. Acrylamide-based hydrogels were photo-polymerized in the presence of an acryloyl-streptavidin monomer to create planar, functionalized surfaces capable of binding biotin-labelled proteins. Soft protein lithography (microcontact printing) of proteins was used to transfer the biotinylated extracellular matrix proteins, fibronectin and laminin, and the laminin peptide biotin-IKVAV, onto modified surfaces. As a biological assay, we plated LRM55 astrogloma and primary rat hippocampal neurons on patterned hydrogels. We found both cell types to selectively adhere to areas patterned with biotin-conjugated proteins. Fluorescence and bright-field modes of microscopy were used to assess cell attachment and cell morphology on modified surfaces. LRM55 cells were found to attach to protein-stamped regions of the hydrogel only. Neurons exhibited significant neurite extension after 72 hr *in vitro*, and remained viable on protein stamped areas for more than 4 weeks. Patterned neurons developed functionally active synapses, as measured by uptake of the dye FM 1-43FX. Results from this study suggest that hydrogel surfaces can be patterned with multiple proteins to direct cell growth and attachment.

Keywords

Hydrogel; microcontact printing; acrylamide; LRM55; neuron; FM1-43FX

Introduction

In recent years, considerable progress has been made in the fabrication of electronic devices at the micro- and nano-scales. In particular, microfabricated neural prosthetic devices have been used to stimulate localized regions and record from areas of the central nervous system. However, the functioning of these devices has been limited, due to their inability to effectively and chronically interface with host nervous tissue. The long-term establishment of electrical contacts between implanted prosthetic devices and host neurons is restricted by reactive cellular encapsulation. It therefore remains to be discovered how the design of the prosthetic devices needs to facilitate complete interaction with the host tissue.

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It is envisaged that the next generation of implantable devices will comprise electrical and/or biological components capable of enhancing biocompatibility. The ability to tailor the chemical and structural properties of these surfaces, so as to control various aspects of cell attachment, survival, proliferation and differentiation will be crucial (for review, see Tang and Hu, 2005). It has been shown that both biochemical and topographical cues can be used to direct cell growth. Microcontact printing (μ CP) is a technique that offers a way to control the patterning of molecules onto a surface. The use of μ CP has been used to print a range of biomolecules and cells into specific arrangements (Singhvi et al., 1994). The μ CP technique uses the relief pattern on the surface of an elastomeric stamp (typically composed of polydimethylsiloxane, or PDMS) to deposit a molecule in a specific pattern onto a surface. The stamp is “inked” with the desired solution, dried, and brought into contact with the surface. The molecule of interest is only patterned where the PDMS stamp is in close contact with the surface (for review, see Kane et al., 1999).

Previous work on the patterning of molecules has involved the use of either monolayers of amino and methyl-terminated alkylsiloxanes on glass (Branch et al., 1998; Cornish et al., 2002) or else self-assembled monolayers of alkanthiols on gold (Mrksich et al., 1997). However, these procedures are often limited in their ability to pattern multiple biomolecules, and in the long-term stability of the deposited molecules. Hydrogels are a novel class of polymers which are being more extensively used in the bioengineering field (Kashyap et al., 2005). They are hydrophilic, crosslinked polymers that show minimal bioreactivity *in vivo*. Hydrogels can also retain large quantities of water without dissolution of the polymer network, as a result of their cross-linked nature and hydrophilicity. Due to this high water content hydrogels exhibit enhanced biocompatibility, by facilitating the exchange of gases and nutrients in a biological environment. Photo-polymerized hydrogels have been investigated in a variety of biomedical applications, including drug delivery (Soppimath et al., 2002; Li et al., 2006), wound management and healing (Ishihara et al., 2001), and in the fabrication of the coatings for biosensors (Charles et al., 2004; Yoshimura et al., 2004).

In the present study, the biotin-streptavidin system has been adapted to create biomimetic hydrogel surfaces functionalized with streptavidin. Modified surfaces were then treated with the biotinylated extracellular matrix proteins laminin and fibronectin, to enable cell attachment to the otherwise non-permissive hydrogel surfaces. The results presented here describe a novel method for the specific patterning of multiple proteins in order to direct cell growth and attachment on a biomimetic hydrogel surface.

2. Materials and Methods

2.1 Photolithography and elastomeric stamp casting

Silicon wafers were patterned using the following technique of Kleinfeld et al., (1988), with minor modifications. Briefly, the surfaces of 10 cm silicon wafers were pre-treated with hexamethyldisilazane (HMDS). The wafer was held in a vacuum chuck, 1 mL of HMDS was applied to the surface, and the silicon substrate spun to dryness in a wafer spinner (Headway Research, TX) operating at 2000 rpm for approximately 20 sec. Positive photoresist, Microposit 1818 (Shipley, PA), was immediately applied to the surface, and the wafer was spun at 4000 rpm for 30 sec. The resist coating was cured for 1 min at 90°C. A high-resolution pattern of either 10 μ m-wide bars or 15 μ m-diameter discs (nodes) at the intersections of orthogonal 2 μ m-wide lines was transferred to each wafer by forming a contact print of the substrate with a mask containing the lithographic pattern. The substrate and mask were positioned in a contact aligner (Karl Suss, VT), and the resist was exposed with collimated UV light, for 15 sec. Following exposure, photoresist was developed in an automatic wafer developing system (Steag-HamaTech, Germany). Elastomeric polydimethylsiloxane (PDMS; Dow Corning, Midland, MI) stamps were then cast from patterned silicon masters. PDMS was

mixed in a 10:1 ratio (elastomer:curing agent), degassed for 1 hr, and then poured over the silicon master pattern. The mixture was left to cure for 2 hr at 60°C, before the PDMS was peeled away from the master pattern.

2.2 Protein biotinylation

Human fibronectin (Sigma, St Louis, MO) was biotinylated by mixing 1 mg protein in 100 mM Na₂CO₃, pH 8.5, with 0.25 mg EZ-Link™ Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) and incubating with gentle rocking for 2 hr at room temperature. Mouse laminin (1 mg; Sigma) was dialyzed against 100 mM Na₂CO₃ containing 0.2% azide for 1 hr before being biotinylated as detailed for fibronectin (above). Non-reacted biotin-linker was removed by dialysis of each sample against PBS containing 0.2% azide for 6 hr at 4°C, with two buffer changes. The biotin-conjugates laminin epitope, biotin-IKVAV (Ile-Lys-Val-Ala-Val), was purchased from New England Peptide (Gardner, MA).

2.3 Hydrogels

Borosilicate glass coverslips (18 mm diameter; Fisher Scientific, Albany, NY) were used as a backing template during hydrogel polymerization. Prior to use, coverslips were treated 3-(trimethoxysilyl)-propyl acrylate ("Bind-Silane"; Sigma) for 1 hr, and then rinsed extensively in dH₂O (distilled water) and dried at room temperature. A polyacrylamide gel matrix consisting of 50% acrylamide (40% solution; acrylamide:bisacrylamide, 19:1), 50% polyethylene glycol diacrylate (PEGDA, M_w = 3400; Nektar, Huntsville, AL), 0.1% Irgacure 2959 (Ciba Speciality Chemicals, Tarrytown, NY) in *n*-vinyl pyrrolidone (Sigma), and 0.1 M Tris-Cl, pH 8.5, was prepared. A total of 150 pmol of streptavidin-conjugated acrylamide (Molecular Probes, Carlsbad, CA) was added to 8 µL of acrylamide solution. The mixture (10 µL) was applied to a quartz slide pre-treated with PlusOne Repel-Silane ES (Amersham Biosciences, Piscataway, NJ). A Bind-Silane treated glass coverslip was placed on top of the solution. The acrylamide solution was photo-polymerized by exposure to UV light (9 mW/cm²) for 5 min on a Spectrolinker XL-1000 at 365 nm (Spectronics Corporation, Westbury, NY). Following polymerization, the hydrogel-bound coverslip was detached from the slide using a scalpel and washed in dH₂O for 2 min to remove any un-polymerized gel solution. Hydrogels were then placed in Tris-Cl (pH 8.5), buffer containing 0.2 % azide and stored at 4°C until use.

2.4 Protein patterning

Biotinylated fibronectin, laminin, and the biotinylated peptide, biotin-IKVAV, were inked separately onto PDMS stamps for 30 min at room temperature. The stamping solution consisted of 100 µg biotinylated protein or 1000 pmol peptide in 300 µL Tris-Cl, pH 8.5 buffer. After 30 min, the stamp was removed from the inking solution and carefully air-dried under a stream of filtered air. Hydrogels were removed from storage, and excess buffer was removed from the surface by transferring to 37°C for 10 min. A PDMS stamp was then lowered onto the hydrogel, and left for 30 min, to allow protein transfer. After incubation each stamp was carefully lifted from the gel. Hydrogels were then briefly immersed in Tris-Cl buffer to remove unbound protein. For detection of patterned proteins, hydrogels were incubated in either anti-fibronectin primary antibody (0.7 mg/mL, rabbit polyclonal, 1:500, human immunoaffinity; Sigma) or anti-laminin (0.6 mg/mL, rat polyclonal, mouse immunoaffinity, 1:500; Sigma) for 60 min at 37°C. Coverslips were rinsed three times in HEPES-buffered Hanks' saline (HBHS) and incubated in secondary antibodies (AlexaFluor 488 anti-rabbit and Texas Red anti-rat and, both at 1:1000 dilution) for 45 min at room temperature.

2.5 Cell culture

All cell attachment assays were carried out in 12-well tissue culture plates (Falcon, Cockeysville, MD). Patterned hydrogels were sterilized in 0.1% gentamycin solution for 2 hr, and then rinsed thoroughly in sterile PBS. Cell cultures of rat LRM55 astrogloma cells were used to initially characterize adhesion to protein-modified hydrogels (Martin and Shain, 1979). Cultures were maintained in 20 mm tissue-culture dishes under standard tissue culture conditions (37°C, in humidified 5% CO₂/95% air). Culture medium for LRM55 cells consisted of Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum, penicillin (10,000 units/mL), streptomycin (10 mg/mL) and amphotericin B (25 µg/mL; Sigma). Before use, semi-confluent monolayers of LRM55 astrogloma cells were trypsinized for 2 min in TrypLE (Invitrogen), pelleted by centrifugation at 750 rpm for 5 min, and resuspended in DMEM. Cells were plated at 1.0×10^4 cells/gel. After 2 hr, gels were rinsed twice in 37°C HBHS solution, to remove unattached cells, and were then transferred to a new culture dish containing complete DMEM.

Primary rat hippocampal neurons (E18) were obtained and cultured as previously described (Brewer et al., 1993). Briefly, hippocampi were removed and enzymatically dissociated in 0.25% trypsin for 20 min at 37°C. In order to obtain a single cell suspension, cells were triturated through a fire-polished, fine-bore glass pipette. For cultures involving primary neurons, cells were initially plated onto hydrogels in neuron plating medium at 37°C (MEM supplemented with 10% horse serum and 1 mM pyruvic acid) at a density of 2.5×10^4 cells/gel. After 4 hr, the medium was carefully aspirated, and replaced with pre-warmed Brewer's culture medium (Neurobasal medium containing 2% B27 supplement (Stem Cell Technologies, Vancouver, BC) and 2.0 mM GlutaMAX (Invitrogen). Neuron cultures were maintained under standard conditions, with one half of the medium replaced every 4 days with fresh Brewer's medium.

2.6 Cell fixation and imaging

Adherent cells were fixed *in situ* using standard fixation procedures. Briefly, hydrogel coverslips were rinsed in Ca²⁺ Mg²⁺-free HBHS pre-warmed to 37°C for 2 min. Cells were fixed in 4% paraformaldehyde containing 0.5% Triton for 1 min at 4°C, and in 4% paraformaldehyde for 15 min at 37°C. Cells were next rinsed in HBHS for 5 min and blocked in 6% BSA for 30 min at room temperature. Coverslips were rinsed once in HBHS and incubated in either anti-fibronectin primary antibody (0.7 mg/mL, rabbit polyclonal, 1:500, human immunoaffinity, Sigma) or anti-laminin (0.6 mg/mL, rat polyclonal, mouse immunoaffinity, 1:500, Sigma) for 1 hr at 37°C. Coverslips were rinsed three times in HBHS and incubated in secondary antibodies (AlexaFluor 488 anti-rabbit and Texas Red anti-rat; 1/1000 dilution) for 30 min at 37°C. Cell nuclei were stained with Hoechst 33342 (5.0 µL of 5 mg/mL stock in dH₂O). Coverslips were mounted onto slides with a mounting medium consisting of HBHS and glycerol with *n*-propyl gallate. Fluorescently stained cells were imaged using an Olympus wide-field microscope (Olympus, Center Valley, PA) equipped with a mercury vapor lamp.

2.7 FM1-43 Uptake Studies

Neurons on patterned hydrogels were analyzed for the presence of functional synapses at 14 days *in vitro*, using the fluorescent styryl pyridinium dye, *N*-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl) pyridinium dibromide (FM1-43FX, fixable version; Invitrogen). Prior to labelling, neurons were rinsed in an external solution containing 2 mM CaCl₂ and 4 mM MgCl₂, in PBS pre-warmed to 37°C. To label active synapses, neurons were incubated in a hyperkalemic solution consisting of 15 mM KCl, 30 mM NaCl, 30 mM D-glucose, 25 mM HEPES, 2 mM CaCl₂, 4 mM MgCl₂, and 10 µM FM1-43FX for 60 sec. Excess dye was removed with five rinses for 1 min each in external solution at 37°C, two of which contained

10 mM Advasep-7 (Sigma). To confirm that dye-uptake was depolarization-dependent, cells were stimulated using a second round of depolarization. Immediately after rinsing, neurons were fixed in 4% paraformaldehyde for 15 min at 37°C.

Results

3.1 Patterning of Extracellular Matrix Proteins

PDMS stamps (1 cm³) complementary to silicon masters were successfully fabricated, containing relief patterns of either 10 µm-wide lines with a 90 µm-gap-spacing or a grid pattern consisting of orthogonal 2 µm-wide lines intersecting at 15 µm-diameter nodes, with a repeat spacing of 50 µm. Hydrogel surfaces were microcontact printed with the biotinylated extracellular matrix proteins laminin, fibronectin, and the laminin peptide IKVAV, using soft protein lithography. Epifluorescence imaging of patterned hydrogels with antibodies to either laminin (this antibody also recognized the laminin epitope, IKVAV) or fibronectin revealed that molecules localized to the patterned regions only (Figs. 1A, B). Pattern fidelity was high; while patterning complied closely with the dimensions of the PDMS stamps used for protein deposition. For the line-only pattern, stamped widths were found to be 10.0 ± 0.5 µm, while for the grid pattern, nodal areas were 15.0 ± 0.6 µm in diameter, and orthogonal lines were 2.0 ± 0.3 µm. The dimensions of each PDMS stamp result in a stamping area of ~1 cm².

While areas between the stamped patterns were typically devoid of proteins, we occasionally (<2% of stamped patterns) observed non-specific protein deposition in interline regions. We suggest that this deposition is due to a) slight irregularities in the hydrogel surface (resulting in areas that have contact with the stamp between the relief structures), b) excessive pressure applied to the hydrogel during stamping, or c) insufficient drying of the stamp (resulting in areas of protein-containing buffer flowing across the hydrogel surface) (Fig. 1C).

Due to the hydrated nature of the gel no significant loss of biotin binding capacity has been noticed for times up to 1 hr (unpublished data). To ensure maximum transfer of biotinylated proteins to the hydrogel, stamps were inked and stamped for 30 min each. In these studies, shorter inking- and stamping-times typically result in sub-optimal transfer of large molecular weight proteins, such as fibronectin and laminin. The typical weight of each stamp was ~300 mg. This weight was sufficient to ensure conformal contact with the hydrogel surface during the stamping procedure. The volume of inking solution has been defined as the minimum volume required to cover the stamping side of each elastomeric stamp. Typically, a 1 mg/mL solution of protein was used for all cell culture studies. Less dilute solutions of protein typically resulted in the attachment of fewer cells to the hydrogel substrate. On gels printed with stamps inked in PBS alone, cells were not seen to adhere to the surface of the hydrogel. This demonstrates that the interaction of cells with the hydrogel surface is dependent on cell-protein interactions, and not with physical deformations of the hydrogel substrate.

No significant diffusion of stamped biotinylated proteins was observed over time periods of up to 4 weeks *in vitro*. In addition, binding of molecules was not observed when hydrogels were stamped with non-biotinylated proteins (data not shown). On hydrogels sequentially patterned with laminin and fibronectin, the fluorescence signal for fibronectin when it was stamped perpendicular to the laminin lines, appeared continuous and undiminished at the intersections {Hynd et al., 2006}. This suggests that hydrogels retain sufficient binding capacity, following immobilization of a first protein, to permit additional protein immobilization. By variation of the streptavidin-acrylamide content of the hydrogel, protein density within patterns can be optimized.

3.2 Cultures of LRM55 Astroglioma Cells on Non-patterned Hydrogel Surfaces

On un-patterned hydrogel surfaces, LRM55 cells remained spherical, indicative of non-attachment. While lamellipodia were seen extending from cells following plating, no cell adhesion to unpatterned hydrogels was observed after 4 hr (Fig. 2A). In order to confirm that cells were still capable of surface adhesion, we removed cells after 4 hr from the hydrogel surface by gentle agitation, and re-plated them on poly-L-lysine coated glass coverslips. Such cells (>90 %) then attached successfully to the glass coverslip surfaces (Fig. 2B).

3.3 Cultures of LRM55 Astroglioma Cells on Patterned Hydrogel Surfaces

Rat astroglioma LRM55 cells were plated onto protein-functionalized hydrogel surfaces. On hydrogels patterned with both laminin and fibronectin, cells were seen to attach and extend on regions stamped with either protein or at the junctions of the two stamped proteins (Fig. 3A). After 24 hr on patterned hydrogel surfaces, LRM55 cells appeared to be well spread along the patterned regions. Cell morphologies compared well with those of control LRM55 cells cultured on poly-L-lysine-coated glass coverslips. After 3 days *in vitro*, fewer than 1 % of cells showed signs of compromised membranes, as assessed by Live/Dead staining (Molecular Probes). Patterned cells also incorporated BrdU, indicative that they were actively synthesizing DNA prior to cell division on hydrogel surfaces (Fig. 3B). Cells (typically >85 %) were found to maintain compliance to the patterned regions of the hydrogel for over 4 weeks *in vitro* (Fig. 3C).

3.4 Cultures of Primary neurons on Patterned Hydrogel Surfaces

Primary hippocampal neurons were plated onto hydrogels stamped with the laminin peptide, biotin-IKVAV. After 4 hr in culture, neurons were predominantly localized to the peptide-stamped regions with few neurons exhibiting neurite processes. At 24 hr, growth-cone lamellipodia were extensive and extended (Fig. 4A). Cell somata were predominantly sited nodes of the grid pattern. The distribution of neurite trajectory showed no preference for any of the four possible directions. While neuron cell bodies were very occasionally seen to adhere to the 2 μ m-wide lines, cell bodies had by 72 hr, migrated along the lines to adhere to the nodes (Fig. 4B). By 10 days in culture, neurons exhibited extensive process outgrowth, typically in compliance with the patterned areas of the hydrogel (Fig. 4C). After 4 weeks in culture, neurons extended over the stamped region of the hydrogel. While pattern fidelity was predominantly maintained at 4 weeks, the occasional neuron process was observed to deviate from the patterned areas (Fig. 4D, arrow).

3.5 FM1-43 functional studies

To ensure that patterning of neurons on hydrogels resulted in the formation of functional networks, we tested neurons for the presence of active synapses using the FM1-43FX dye (Fig. 4E, F). FM1-43 is a non-toxic, cationic, fluorescent, styryl pyridinium dye that has been used to observe synaptic vesicle recycling *in vitro* (Betz et al., 1992; Schote and Seelig, 1998). While the FM1-43 dye is essentially non-fluorescent in water, its quantum yield increases >350 times after the dye incorporates into the plasma membrane of cells (Henkel et al., 1996; Audrey et al., 2004). Hydrogel-patterned neurons were found to have discrete FM1-43FX labeled puncta, indicative of functional synapses. To assess whether FM1-43 labeled puncta were confined to patterned areas of the hydrogel, we performed immunocytochemistry on paraformaldehyde-fixed cells. Staining for neuron-specific β III tubulin revealed that puncta were localized only to neuron processes on hydrogel areas patterned with biotin-IKVAV.

Discussion

The present work describes a novel methodology for the efficient immobilization of multiple proteins and peptides onto a biocompatible gel surface for the controlled growth of several types of neural cells. The use of acrylamide gives rise to a highly hydrophilic surface with low protein absorption, and hence low cell-binding capacity. Co-polymerization of acrylamide with poly(ethylene) glycol diacrylate (PEGDA; 10–50% v/v) can further increase the resistance of the hydrogel surface to non-specific protein absorption (Drumheller and Hubbel, 1995). PEGDA also presents an ideal material for which to tailor the physical properties of the hydrogel to suit specific applications; for example, the pore size can be easily altered, either by varying the molecular mass of the PEGDA (ranging from 575 Da to >3400 Da), or else by varying the ratio of cross-linker to monomer (Cruise et al., 1998).

While the acrylamide monomer has been reported to induce a variety of neurotoxic effects in animal species following absorption via the respiratory, dermal, and oral routes (LoPachin, 2005), polymerization of this acrylamide monomer (both acrylamide and PEGDA) results in a non-toxic and biologically inert material (e.g. stable in a physiological environment, non-degradable). Once polymerized, PAA/PEGDA hydrogels are also highly resistant to degradation. We have kept co-polymer gels in solution, at both 4°C and 37°C, for periods exceeding 3 months, without any significant signs of polymer degradation or loss of biotin-binding capacity (unpublished data). In this study, PAA/PEGDA hydrogels were found to be cyto-compatible under standard tissue-culture conditions.

The interaction of cells with a surface is governed by a variety of factors, including the surface's topography, chemical qualities, and mechanical properties. Firm adhesion between the surface and the contacting cell must be developed, to ensure long-term stability. In the current report, we demonstrate that 'patterns' of the extracellular matrix molecules, laminin and fibronectin, and the laminin epitope, IKVAV, can be formed on hydrogel surfaces polymerized in the presence of streptavidin-acrylamide. The biotin/streptavidin linkage is one of the strongest ($K_d = 10^{-15}$ M) non-covalent interactions known. Immunocytochemical observation of patterned biomolecules confirmed that the patterns were stable for at least 4 weeks *in vitro*.

In addition, patterned hydrogel surfaces supported the long-term survival of neural cells. Both LRM55 rat astrogloma cells and primary hippocampal neurons were found to adhere to hydrogel areas stamped with biotinylated molecules while virtually no cells were seen to attach to non-patterned areas of the hydrogels. Primary rat hippocampal neurons formed functional networks on hydrogels stamped with the laminin epitope, IKVAV. Active synapses were present only on neurites on patterned areas. This observation is consistent with previous reports of stamped IKVAV on functionalized glass surfaces (Heller et al., 2005). We found that neurons on patterned hydrogels attached predominantly to the 15 μ m nodes of the stamped grid pattern, with few cells attaching to the 2 μ m-wide lines of the grid. Neurons self-organized into networks by 10 days and survived on the hydrogel surface for at least 4 weeks. These neurons displayed normal neuronal morphology and were immunoreactive with anti-serum against the neuron-specific protein β III-tubulin. The use of a grid pattern has previously been used to localize the cell bodies of individual neurons above the stimulating and recording electrodes on microelectrode arrays (Jun et al., 2006).

The attachment of cells to hydrogels stamped with biomolecules supports the observation that stamped proteins retain higher-order structural integrity (Hynd et al., 2006). For example, laminins are $\alpha\beta\gamma$ heterotrimeric proteins in which the globular domains of the β and γ chains are important in laminin polymerization, while the functional domains at the ends of the α chain bind to cell-surface receptors. In contrast to other techniques for the immobilization of molecules on biocompatible surfaces (e.g., electron beam patterning (Hong et al., 2004),

robotic protein spotting (Flaim et al., 2005), soft protein lithography has the following advantages: the method permits multiple proteins to easily patterned, without the use of specialized laboratory equipment; the method is relatively inexpensive; and, lastly, the method enables patterning of proteins to feature sizes required for the control of cell growth and attachment (~2–100 μm). PAA/PEGDA hydrogels also exhibit minimal interactions with surface immobilized proteins, thus ensuring retention of protein structure and function following μCP .

In summary, this work describes a technique for the patterning of a biomimetic hydrogel surface with multiple proteins and peptides. Patterned biomolecules were found to retain sufficient biological properties to permit cell adhesion. These findings suggest that the methodology can be successfully used for the rational design of functional neural networks.

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References

- Audrey C, Brumback AB, Janet L, Lieber C, Joseph K, Angleson C, Betz WJ. Using FM1-43 to study neuropeptide granule dynamics and exocytosis. *Meth Enzymology* 2004;33:287–294.
- Betz WJ, Bewick GS. Optical analysis of synaptic vesicle recycling at the frog neuromuscular junction. *Science* 1992;255(5041):200–3. [PubMed: 1553547]
- Branch DW, Corey JM, Weyhenmeyer JA, Brewer GJ, Wheeler BC. Microstamp patterns of biomolecules for high-resolution neuronal networks. *Med Biol Eng Comput* 1998;36(1):135–41. [PubMed: 9614762]
- Brewer GJ, Torricelli JR, Evege EK, Price PJ. Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. *J Neurosci Res* 1993;35(5):567–76. [PubMed: 8377226]
- Caulfield MJ, Qiao GG, Solomon DH. Some aspects of the properties and degradation of polyacrylamides. *Chem Rev* 2002;102(9):3067–84. [PubMed: 12222982]
- Charles PT, Goldman ER, Rangasammy JG, Schauer CL, Chen MS, Taitt CR. Fabrication and characterization of 3D hydrogel microarrays to measure antigenicity and antibody functionality for biosensor applications. *Biosens Bioelectron* 2004;20(4):753–64. [PubMed: 15522590]
- Cornish T, Branch DW, Wheeler BC, Campanelli JT. Microcontact printing: a versatile technique for the study of synaptogenic molecules. *Mol Cell Neurosci* 2002;20(1):140–53. [PubMed: 12056845]
- Cruise GM, Scharp DS, Hubbell JA. Characterization of permeability and network structure of interfacially photopolymerized poly(ethylene glycol) diacrylate hydrogels. *Biomaterials* 1998;19(14):1287–1294. [PubMed: 9720892]
- Drumheller PD, Hubbell JA. Densely crosslinked polymer networks of poly(ethylene glycol) in trimethylolpropane triacrylate for cell-adhesion-resistant surfaces. *J Biomed Mat Res* 1995;29(2):207–215.
- Flaim CJ, Chien S, Bhatia SN. An extracellular matrix microarray for probing cellular differentiation. *Nature* 2005;2(2):119–125.
- Heller DA, Garga V, Kelleher KJ, Lee TC, Mahbubani S, Sigworth LA, Lee TR, Rea MA. Patterned networks of mouse hippocampal neurons on peptide-coated gold surfaces. *Biomaterials* 2005;26(8):883–9. [PubMed: 15353199]
- Henkel AW, Simpson LL, Ridge RM, Betz WJ. Synaptic vesicle movements monitored by fluorescence recovery after photobleaching in nerve terminals stained with FM1-43. *J Neurosci* 1996;16(12):3960–7. [PubMed: 8656290]

- Hong Y, Krsko P, Libera M. Protein Surface Patterning Using Nanoscale PEG Hydrogels. *Langmuir* 2004;20:11123–11126. [PubMed: 15568866]
- Hynd MR, Frampton JP, Burnham M-R, Martin DL, Dowell-Mesfin NM, Turner JN, Shain W. Functionalized hydrogel surfaces for the patterning of multiple biomolecules. *Biomed Mat Res Part A*. 2006;10.1002/jbm.a.31002
- Ishihara M, Ono K, Sato M, Nakanishi K, Saito Y, Yura H, Matsui T, Hattori H, Fujita M, Kikuchi M, Kurita A. Acceleration of wound contraction and healing with a photocrosslinkable chitosan hydrogel. *Wound Repair Regen* 2001;9(6):513–21. [PubMed: 11896994]
- Jun SB, Hynd MR, Dowell-Mesfin N, Smith KL, Turner JN, Shain W, Kim SJ. Low-density neuronal networks cultured using patterned poly-l-lysine on microelectrode arrays. *J Neurosci Methods*. 2006;10.1016/j.jneumeth.2006.09.009
- Kane RS, Takayama S, Ostuni E, Ingber DE, Whitesides GM. Patterning proteins and cells using soft lithography. *Biomaterials* 1999;20(23–24):2363–76. [PubMed: 10614942]
- Kashyap N, Kumar N, Kumar MN. Hydrogels for pharmaceutical and biomedical applications. *Crit Rev Ther Drug Carrier Syst* 2005;22(2):107–49. [PubMed: 15862110]
- Li J, Li X, Ni X, Wang X, Li H, Leong KW. Self-assembled supramolecular hydrogels formed by biodegradable PEO-PHB-PEO triblock copolymers and alpha-cyclodextrin for controlled drug delivery. *Biomaterials* 2006;27(22):4132–40. [PubMed: 16584769]
- LoPachin RM. Acrylamide neurotoxicity: neurological, morphological and molecular endpoints in animal models. *Adv Exp Med Biol* 2005;561:21–37. [PubMed: 16438286]
- Martin DL, Shain W. High affinity transport of taurine and beta-alanine and low affinity transport of gamma-aminobutyric acid by a single transport system in cultured glioma cells. *J Biol Chem* 1979;254(15):7076–84. [PubMed: 457670]
- Mrksich M, Dike LE, Tien J, Ingber DE, Whitesides GM. Using microcontact printing to pattern the attachment of mammalian cells to self-assembled monolayers of alkanethiolates on transparent films of gold and silver. *Exp Cell Res* 1997;235(2):305–13. [PubMed: 9299154]
- Schote U, Seelig J. Interaction of the neuronal marker dye FM1-43 with lipid membranes. Thermodynamics and lipid ordering. *Biochim Biophys Acta* 1998;1415(1):135–46. [PubMed: 9858712]
- Singhvi R, Kumar A, Lopez GP, Stephanopoulos GN, Wang DI, Whitesides GM, Ingber DE. Engineering cell shape and function. *Science* 1994;264(5159):696–8. [PubMed: 8171320]
- Soppimath KS, Aminabhavi TM, Dave AM, Kumbhar SG, Rudzinski WE. Stimulus-responsive “smart” hydrogels as novel drug delivery systems. *Drug Dev Ind Pharm* 2002;28(8):957–74. [PubMed: 12378965]
- Tang L, Hu W. Molecular determinants of biocompatibility. *Expert Rev Med Devices* 2005;2(4):493–500. [PubMed: 16293087]
- Yoshimura I, Miyahara Y, Kasagi N, Yamane H, Ojida A, Hamachi I. Molecular recognition in a supramolecular hydrogel to afford a semi-wet sensor chip. *J Am Chem Soc* 2004;126(39):12204–5. [PubMed: 15453719]

Fig. 1.

Patterned hydrogel surfaces. (A) A streptavidin-acrylamide hydrogel surface was microcontact printed with the biotinylated extracellular matrix proteins laminin and fibronectin. PDMS stamps were used to transfer proteins in a pattern corresponding to 10 μm -wide lines with 90 μm -gap spacing. Proteins were detected using primary antibodies to laminin and fibronectin, and secondary antibodies for laminin (Alexa 488 secondary antibody, green) and fibronectin (Texas Red secondary antibody, red). Scale bar = 40 μm . (B) The laminin peptide biotin-IKVAV was stamped onto a hydrogel using a PDMS stamp grid pattern consisting of orthogonal 2 μm -wide lines intersecting at 15 μm diameter nodes, with a repeat spacing of 50 μm . The IKVAV epitope was visualized using an anti-laminin primary antibody and Alexa 488-conjugated secondary antibody (green). Imaging revealed that the peptide localized to stamped regions only. Scale bar = 50 μm . (C) Examples of non-specific protein deposition that can occur following stamping of hydrogel surfaces. Panel 1 (left). Protein diffusion across hydrogel surface during μCP . The result is areas of protein-containing buffer flowing across the hydrogel surface. Panel 2 (right). Areas of hydrogel are devoid of stamped biomolecules due to the presence of a defect (bubble) in the PDMS stamp. Bubbles in the stamp are due to incomplete de-gassing of the elastomer mixture prior to curing. Defect-containing stamps produce areas not in close contact with the hydrogel surface. Scale bar = 50 μm .

Fig. 2.

Cell attachment to hydrogel surfaces (A) LRM55 astroglioma cells plated onto PAA/PEGDA hydrogel surfaces. Non-patterned hydrogel surfaces do not allow cell attachment. However, cells still have the ability to attach to cell-permissive (protein-stamped) hydrogel surfaces. Cells were stained using Alexa 568-conjugated phalloidin (cytoskeleton, red) and Hoechst 33342 (nuclei, blue). Scale bar = 50 μm . (B) Unattached cells from (A) were plated onto poly-L-lysine coated glass coverslips. Cells were able to successfully attach to glass coverslips. Scale bar = 50 μm .

Fig. 3.

Cell attachment to protein-functionalized hydrogel surfaces. (A) At 24 hr, rat astroglioma LRM55 cells were seen to attach and extend on regions stamped with either the laminin (left to right) or the fibronectin (top to bottom) proteins. Cells were stained using Alexa 568-conjugated phalloidin (cytoskeleton, red) and Hoescht 33342 (nuclei, blue). Scale bar = 25 μm . (B) Patterned LRM55 cells were shown to incorporate BrdU, denoting that cells were actively synthesizing DNA prior to cell division. BrdU-positive cells are indicated by arrows. Scale bar = 25 μm . (C) At 4 weeks *in vitro* cells were found to maintain compliance with the stamped patterning of the hydrogel. Cells were observed to extend along both the laminin and the fibronectin stampings, or they spread where the two proteins intersected (arrow), indicating that μCP proteins retain long-term functionality. Scale bar = 50 μm .

Fig. 4.

Neurons on hydrogel peptide-stamped hydrogel surfaces. (A–C) Neurons at 24 hr, 48 hr and 10 days in culture. At 24 hr, growth-cone lamellipodia were extensive and extended. After 48 hr in culture, cell somata were predominantly located at the nodes of the grid (15 μm -diameter discs at the intersections of 2 μm -wide orthogonal lines). By 10 days *in vitro* neurons had formed into neuronal networks. Cells were stained for neuron-specific βIII -tubulin (red) and nuclei were stained with Hoescht 33342 (blue). Scale bar = 50 μm . (D) Hydrogel patterned neuronal network at 4 weeks *in vitro*. Arrows indicate where neuronal processes have deviated from the patterned areas. Pattern fidelity was maintained over a 1cm^2 area of the hydrogel. Neurons were stained with βIII -tubulin (red). Scale bar = 100 μm . (E) Neurons at 10 days *in*

vitro were analyzed for the presence of active synapses, using the FM1-43FX dye. Neurons were depolarized in the presence of FM1-43FX, rinsed extensively to remove excess dye, and stimulated for 2 min in a high K^+ solution. Following stimulation and washing, cells were fixed with 4% paraformaldehyde. Active synapses as revealed by the FM1-43FX dye are labelled in green (arrows). Neurons were imaged using an Olympus widefield upright microscope with ImagePro software (MediaCybernetics, Silver Spring, MD). Scale bar = 50 μm . (F)

Depolarization-dependent FM1-43FX labelling. Neurons from Figure 4E were subject to a second round of depolarization prior to fixation. FM-143FX synapses (green) were co-labelled with antibodies to post-synaptic density protein 95 (PSD95; red). Putative synapses (yellow; arrows) were identified by the co-localization of both FM 1-43FX and PSD95. Scale bar = 10 μm .