Chapter VI. Preliminary cell culture studies on hydrogels assembled through aggregation of leucine zipper domains

Abstract

The engineering flexibility to precisely and systematically control structure and properties makes genetically engineered leucine zipper protein hydrogels excellent biomaterials. They have potential utility for solving clinical problems or addressing fundamental biological questions. Viability assay for mammalian 3T3 fibroblast cells cultured in the presence of artificial proteins containing leucine zipper domains showed no evidence of toxicity. An RGD cell-binding domain was successfully incorporated into the midblock backbone. Anchorage-dependent epithelial cells spread well on hydrogel films bearing an RGD cell-binding domain. In contrast, cells remained round on films without the cell-binding domain; significant apoptosis was induced. Encapsulated 3T3 fibroblast cells remained viable inside the hydrogel for at least 12 hours, suggesting that these materials have proper permeability for transferring oxygen, nutrients, and metabolic waste. Micropatterning of a hydrogel containing the RGD domain on a PEG-modified glass surface limited cell adhesion to the hydrogel region, demonstrating the potential of using these hydrogels to micropattern cells. Preliminary cell culture results demonstrate that appropriately engineered leucine-zipper hydrogels are promising biomaterials for cell culture and tissue engineering.

1. Introduction

Hydrogels have been successfully used in biomedical fields due to their high water content and the consequent biocompatibility. Successful examples include soft contact lenses[1], wound dressings[2,3], superabsorbents[4-6], and drug-delivery systems[7,8]. The most recent and exciting applications of hydrogels are cell-based therapeutics[9,10] and soft tissue engineering[11]. The biomaterial used to grow the first living, tissue-engineered skin product was a collagen hydrogel[12]. Efforts to engineer other soft tissues have not been as successful. Nevertheless, the field remains extremely active. Regarding potential utility for engineering different tissues, chondrocytes[13-21], hepatocytes[22-24], smooth muscle cells[25-28], epithelial cells[29], valvular interstitial cells[30], marrow stromal cells[31,32], bone marrow-derived mesenchymal stem cells[33], and embryonic stem cells[34] have been cultured either on surfaces of or inside different hydrogels. In addition to tissue engineering, encapsulating living cells that secrete therapeutic products, such as insulin[35-40] and IL-4/IL-13[41], into biocompatible hydrogels for immunoisolation is another type of intriguing application.

Hydrogels used in biomedical fields must meet more stringent requirements than those used for traditional industrial applications. Design variables include both physical and biological properties[42]. Physical properties include appropriate mechanical strength and integrity, and appropriate mass transport properties to allow diffusion of nutrients and metabolites. Biological properties include nontoxicity and ability to carry and control biological determinants, such as cell binding domains and enzyme recognition sites.

The slow progress in tissue engineering, in large measure, is limited by inappropriate properties of the biomaterials currently available. Hydrogels currently used

in research and clinical trials are formed from either natural biopolymers (such as collagen, hyaluronate, fibrin, alginate, agarose, and chitosan) or chemically synthesized polymers (such as poly(acrylic acid) and its derivatives, poly(ethylene oxide) and its copolymers, poly(vinyl alcohol), and polyphosphazene)[11,43]. Natural biopolymers often have biological functionality and are nontoxic, but sources are limited, properties are variable, opportunities for systematic control of structure and properties are limited, and concerns about viral contamination restrict the use of animal products in biomedical applications. On the other hand, chemically synthesized polymers address these shortcomings at the expense of biological functionality. Toxic residual monomers can compromise their safety.

In addition to the design variables mentioned above, their amenability to microfabrications is also important, and might be critical for eventual success of the tissue engineering field. Many tissues and organs have complex architectures in which cells are precisely organized. These structural features are critical for their normal functions. To mimic these architectures in tissue engineering, cells and their environmental cues need to be delivered precisely on micrometer or nanometer scales. Hydrogels are particularly suitable for microfabrications. Mironov et al.[44] demonstrated the concept of constructing organs by printing single cell and cell aggregates in a thermo-reversible poly(NiPAM) gel. Liu et al.[22] photopatterned hepatocytes in poly(ethylene glycol) diacrylate gels with feature sizes between 50 and 200 μm.

Genetic engineered artificial protein hydrogels assembled through aggregation of leucine zipper domains provide an opportunity to combine the advantages of hydrogels from both natural polymers and chemically synthesized polymers. The biosynthetic

method used to create these artificial proteins allows biological determinants readily incorporated and structure and properties systematically controlled. These hydrogels are reversible in response to pH and temperature[45], implying their compliance to microfabrications. Fast erosion behavior of originally designed $AC_{10}A$ gels in open solutions, which was a major hurdle for $AC_{10}A$ gels to be assessed for applications in cell culture and tissue engineering, has been addressed in Chapter IV and Chapter V. Therefore, in this chapter we assess the toxicity of artificial proteins containing leucine zipper domains and demonstrate the engineering flexibility to incorporate biological determinants. The viability and morphology of mammalian cells cultured on hydrogel films or encapsulated within hydrogels are investigated; cell responses to the incorporated cell-binding domain are examined. The potential of using these materials to micropattern cells is demonstrated.

2. Materials and methods

2.1. Incorporation of the RGD cell-binding domain of vitronectin into a leucine zipper hydrogel

DNA oligonucleotides encoding the vitronectin cell-binding domain (VN)[46] (Figure 6.1 (a)) were synthesized at the DNA synthesis facility in the Beckman Institute at the California Institute of Technology. VN was digested with *Nhe*I and *Spe*I, and ligated into the *Spe*I site of the plasmid $pQE9AC_{10}$cys(L11C) to yield $pQE9AC_{10}$(VN)cys(L11C). DNA segments encoding C_{10} and A(L11C) were excised from $pQE9C_{10}$trp[47] and pQE9Acys(L11C), respectively, by digestion with *Nhe*I and *Spe*I (New England Biolabs, Beverly, MA). The C_{10} and A(L11C) segments were sequentially

ligated into the plasmid pQE9AC$_{10}$(VN)cys(L11C) at the *Spe*I site to yield pQE9AC$_{10}$(VN)C$_{10}$Acys(L11C). The sequences were verified at the DNA sequencing core facility of the Beckman Institute at the California Institute of Technology.

2.2. Protein synthesis and purification

pQE9AC$_{10}$Atrp, pQE9AC$_{10}$Acys(L11C), and pQE9AC$_{10}$(VN)C$_{10}$Acys(L11C) were each transformed into *Escherichia coli* strain SG13009, which carries the repressor plasmid pREP4 (Qiagen, Chatsworth, CA). Proteins AC$_{10}$Atrp, AC$_{10}$Acys(L11C), and AC$_{10}$(VN)C$_{10}$Acys(L11C) (the amino acid sequence is shown in Figure 6.1 (b)) were expressed as previously described, and purified by affinity chromatography on a nickel nitrilotriacetic acid resin (Qiagen, Chatsworth, CA) following the denaturing protocol provided by Qiagen.

2.3. Cell maintenance

3T3 fibroblast cells and MCF10A epithelial cells were obtained from the Asthagiri Laboratory at the California Institute of Technology. 3T3 fibroblast cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Corporation, Carlsbad, California) with high glucose, supplemented with 10% (v/v) fetal bovine serum (Invitrogen) and 1% (v/v) penicillin/streptomycin (Invitrogen). MCF10A cells were cultured in DMEM /Ham's F-12 containing HEPES and L-glutamine (In-vitrogen), supplemented with 5% (v/v) horse serum (Invitrogen), 20 ng/mL epithelial growth factor (EGF) (Peprotech), 0.5 μg/mL hydrocortisone (Sigma), 0.1 μg/mL cholera toxin (Sigma), 10 μg/mL insulin (Sigma), and 1% penicillin/streptomycin. All cells were maintained in a

humidified incubator at 37 °C and 5% CO_2. Cells were grown to preconfluence and trypsinized in 0.05% trypsin/0.53 mM Ethylenediaminetetraacetic Acid Sodium Salt (EDTA) solution in PBS, followed by re-suspension in fresh medium and seeding at a density of ca. 3,500 cells/cm^2.

2.4. WST-1 viability assay

The viability of 3T3 cells cultured in the presence of artificial protein AC_{10}Atrp was assessed using WST-1 assay (Roche, Mannheim, Germany). Cells were seeded in 96-well cell culture plates at a density of 45,000 cells/cm^2, and covered with 100 μL cell culture medium containing the AC_{10}Atrp protein at concentrations up to 4.3 mg/mL. The stock AC_{10}Atrp solution was sterilized by filtration through 0.2 μm filters at pH 12.5 and brought back to neutral pH. Tests under all conditions were run in triplicate. Cells were cultured for 24 hrs in a humidified incubator at 37 °C and 5% CO_2. Before the WST-1 assay, the medium in each sample was replaced with fresh medium that did not contain the AC_{10}Atrp protein. WST-1 reagent (10 μL) was added into each well, followed by incubation at 37 °C for 3 hrs. The absorbance of each sample at 450 nm was measured on a SpectraMax Plus^{384} absorbance microplate reader (Molecular Devices Corporation, Sunnyvale, CA).

2.5. Cell culture on hydrogel films

To prepare hydrogel films, solutions of AC_{10}Acys(L11C) and AC_{10}(VN)C_{10}Acys(L11C) were prepared in PBS buffer (containing 1% penicillin/streptomycin) at a concentration of 4%. The pH of each solution was brought to

12 and the solution was filtered through a 0.2 μm filter. Each solution (20 μL) was dried in a sterile circular reservoir (8 mm in diameter) in a laminar flow hood. The dried protein was re-hydrated in PBS buffer and mounted on an aminated coverslip (sterilized with 70% ethanol) that fits into the chamber of the 8-chamber Lab-Tek* II Chamber Slide System (VWR). The film and coverslip were moved into the Lab-Tek* II Chamber Slide System and covered with serum-free DMEM/Ham's F-12 medium supplemented with 1% (v/v) penicillin/streptomycin and 0.1% bovine serum albumin (Sigma).

To examine the morphology of cells cultured on different hydrogel films, MCF10A cells were seeded onto hydrogel films in 150 μL serum-free DMEM/Ham's F-12 medium at a density of 43,000 cells/cm^2. Cells were cultured for 6 hrs and labeled with BCECF (2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein)) AM (acetoxymethyl) ester derivative (Molecular Probes). The BCECF AM stock solution was made in Dimethyl Sulfoxide (DMSO) at a concentration of 100 μM, and 0.8 μL of the stock solution was added into each sample. The samples were incubated in the cell culture incubator for 40 minutes and washed with PBS twice, followed by imaging using a Zeiss Axiovert 200 fluorescence microscope equipped with a FITC filter and a AxioCam MRm CCD camera.

2.6. Apo-ONE Caspase-3/7 assay for apoptosis

To investigate cell survival on different hydrogel films, MCF10A cells were seeded onto hydrogel films in 150 μL serum-free DMEM/Ham's F-12 medium at a density of 43,000 cells/cm^2. Poly(2-hydroxyethyl methacrylate) (polyHEMA) treated surfaces were prepared as positive control for the apoptosis assay[48]. A polyHEMA

(Sigma) solution was made in 95% ethanol at a concentration of 5.7 mg/mL. The solution (35 μL) was added into the chamber of the Lab-Tek* II Chamber Slide System and dried in a laminar flow hood overnight, followed by UV sterilization for half an hour and rinsing with PBS twice. Fibronectin treated surfaces were prepared as negative control. A fibronectin solution (10 mg/mL) was adsorbed onto the bottom surface of the chamber of the Lab-Tek* II Chamber Slide System at 4 °C overnight and rinsed with PBS twice. Cells were cultured for 42 hrs on hydrogel films and control surfaces, followed by the apoptosis assay with the Apo-ONE™ Homogeneous Caspase-3/7 Assay kit (Promega, Madison, WI)[a]. The assay reagent (150 μL) was added into each sample, followed by incubation at room temperature for 20 minutes on an Eppendorf Thermomixer (500 rmp). The supernatant of each sample (175 μL) was transferred to a black 96-well plate. The fluorescence of each sample at an excitation wavelength of 485 nm and an emission wavelength of 530 nm was monitored for 10 minutes on a Gemini XPS fluorescence microplate reader (Molecular Devices Corporation, Sunnyvale, CA). The kinetics of the caspase enzymes in each sample was determined on the basis of the fluorescence signals using SoftMax Pro® software provided by Molecular Devices.

An alternative positive control was prepared by inducing apoptosis using anisomycin[49]. Cells were cultured on AC_{10}Acys(L11C) and $AC_{10}(VN)C_{10}$Acys(L11C)

[a] Aspartic acid-specific caspase-3/7 play key role in apoptosis in mammalian cells. The assay kit provides buffer that rapidly lyses cultured mammalian cells and a profluorescent, aspartic acid-containing substrate that quantifies enzymatic activity of caspase-3/7.

hydrogel films, respectively, for 39 hrs. An anisomycin (Sigma) solution was made in DMSO at a concentration of 0.4 μg/μL, and 3μL of the solution was added into each cell culture. The samples were incubated for 3 hrs, followed by the Apo-ONE assay.

2.7. Cell encapsulation and fluorescence live/dead assay

A casting chamber was made with two Poly(dimethylsiloxane) (PDMS) slabs, each of which has a 8 mm × 8 mm square window in the middle, two pieces of dialysis membrane, and four clamps. The PDMS slabs and the dialysis membrane were exposed to UV illumination for sterilization (half an hour for each side). The clamps were autoclaved. One PDMS slab was stacked on the other with their windows aligned. The two pieces of dialysis membrane were placed between the two PDMS slabs. The three edges of the resulting assembly were clamped. The other edge was left open for sample loading.

An $AC_{10}(VN)C_{10}$Acys(L11C) solution (3.5% w/v) was made in PBS buffer, and its pH was adjusted to 5.5. Under these conditions, the solution remained fluid to allow homogenous cell seeding. 3T3 fibroblast cells were seeded into this solution at a concentration of 75,000 cells/mL. The mixture of the material and the cells (20 μL) was immediately loaded into the assembled casting chamber; the fourth edge was clamped. The sample was dialyzed against PBS buffer (pH 7.5) for half an hour. The chamber was dissembled and the gelled material containing cells was transferred to a cell culture dish. The encapsulated cells were cultured in 12 mL DMEM/serum medium for 12 hrs. For fluorescence live/dead assay, the medium was replaced with 4 mL fresh medium, followed by addition of BCECF AM (100 μM, 20 μL) and Ethidium homodimer (EthD-

1) (100 μM, 20 μL). Samples were incubated at 37 °C for 1 hr and washed with PBS twice, followed by imaging using a Zeiss Axiovert 200 fluorescence microscope equipped with FITC and Rhodamine filters and an AxioCam MRm CCD camera. Live cells were identified by bright green fluorescence through the FITC filter; dead cells were identified by bright red fluorescence through the Rhodamine filter.

2.8. Cell culture on micropatterned hydrogels

An $AC_{10}(VN)C_{10}$Acys(L11C) hydrogel was micropatterned on a PEG-modified surface on the basis of its reversible gelation in response to pH. A glass coverslip was cleaned and aminated as described in chapter V, followed by PEGylation using PEG-β-nitrophenylcarbonate (molecular weight 5000). The treated surface was brought into contact with the surface of a PDMS device that had grooves (50 μm wide and 20 μm deep) on the surface, such that the grooves became microchannels. An $AC_{10}(VN)C_{10}$Acys(L11C) solution (3.5%, pH 11.8) was introduced into these microchannels through an inlet fabricated in the PDMS device. After the solution dried out, the PDMS was peeled off and the coverslip was placed in a 6-well cell culture plate.

3T3 fibroblast cells were seeded into the cell culture plate in serum-free DMEM medium supplemented with 1% (v/v) penicillin/streptomycin at a density of 40,000 cells/cm^2, and incubated for 1 hr. The medium was aspirated away and the sample was washed with PBS twice, followed by imaging using a phase contrast microscopy equipped with a CCD video camera (Sony, Model DXC-151A).

3. Results and discussion

The ability to form robust artificial protein hydrogels in open systems (discussed in Chapter IV and V) opens the way to biomedical applications. Therefore, we examined their toxicity to mammalian cells. 3T3 fibroblast cells were cultured in DMEM/serum medium in cell culture dishes. Addition of the AC_{10}Atrp protein (up to 4.3 mg/mL) into the cell culture medium yielded no difference in viability, as revealed from WST-1 viability assay (Figure 6.2). These results suggest that artificial proteins containing leucine zipper domains are not toxic to mammalian cells at concentrations up to 4.3 mg/mL.

We further successfully incorporated the RGD cell-binding domain of vitronectin into the midblock backbone of a leucine zipper hydrogel characteristic of long-term stability in open solutions. The DNA sequence of the new protein $AC_{10}(VN)C_{10}$Acys(L11C) was verified, and the expected molar mass of the protein was confirmed by mass spectral analysis. Solutions of $AC_{10}(VN)C_{10}$Acys(L11C) form gels in pH 7.6, 100 mM phosphate buffer or DMEM cell culture medium at concentrations above ca. 2.5%. They become viscous liquids at high pH where the secondary structure of the leucine zipper domain is denatured, exhibiting reversible gelation in response to pH.

The effects of the incorporated RGD domain on cell morphology and survival were assessed. To demonstrate that biological determinants incorporated into artificial protein hydrogels can play important roles in regulating cell behavior, serum-free DMEM/Ham's F-12 medium was used to avoid the fibronectin component in serum that

could overshadow the effect of the RGD domain in the artificial protein. Anchorage-dependent epithelial cells spread well and adopted an elongated morphology on $AC_{10}(VN)C_{10}$Acys(L11C) hydrogel films that contained the RGD cell-binding domain, but remained round on AC_{10}Acys(L11C) hydrogel films that did not contain the RGD domain (Figure 6.3). The Apo-ONE caspase-3/7 assay revealed significant apoptosis in cells cultured on AC_{10}Acys(L11C) films after 42 hrs (Figure 6.4). The apoptosis level in cells cultured on $AC_{10}(VN)C_{10}$Acys(L11C) films was slightly greater than that in negative control samples on fibronectin treated surfaces, and was ca. half of that in cells cultured on AC_{10}Acys(L11C) films. The same results were shown when an alternative positive control was prepared by inducing apoptosis in cells cultured on leucine zipper hydrogel films using anisomycin[49]. These results demonstrate that cell-adhesion ligands incorporated in leucine zipper hydrogels play a key role in regulating cell behavior when these materials are used as cell culture substrates.

When 3T3 fibroblast cells were encapsulated in an $AC_{10}(VN)C_{10}$Acys(L11C) gel without control of the gel dimensions, most cells died within 12 hrs, as revealed from fluorescence live-dead assay. Cell death most likely results from oxygen supply limitation, which has been recognized as an important factor responsible for cell death in many encapsulation experiments[10,50]. In vivo, most cells exist within 100 μm of a capillary[51]. Therefore, the thickness of hydrogel samples encapsulating cells are usually controlled on the order of 100 μm to assure enough cell viability[10,50,52]. To encapsulate cells in bigger gels, other method such as simultaneous angiogenesis[53] or microfabrication of blood vessels[54] must be incorporated. To assess our material without complicating it by this issue, the thickness of the sample was controlled at ca. 300 μm as

described in the section of materials and methods. More than 95% of the cells such encapsulated remained viable after 12 hrs of culture (Figure 6.5), suggesting that these leucine zipper hydrogels have proper permeability for transport of oxygen, essential nutrients, and metabolic waste. Studies beyond 12 hrs have not been carried out. Future investigation is needed to address whether the encapsulated cells exhibit normal phenotype. Nevertheless, preliminary studies suggest that these hydrogels may be promising scaffolds for 3-dimensional cell culture.

Reversible gelation in response to environmental stimuli allows these hydrogels to be readily micropatterned. Gels were immobilized on PEG-modified aminated surfaces through electrostatic attraction between the negatively charged glutamic acid residues in the protein materials and the positively charged unreacted amine groups on the surface. Retention of the original micropatterns in open solutions is assured by the long-term stability of the materials and their anisotropic swelling behavior when immobilized on aminated surfaces (discussed in Chapter V). Micropatterning of an $AC_{10}(VN)C_{10}$Acys(L11C) hydrogel on a PEG-modified glass surface limits cell adhesion to the hydrogel region (Figure 6.6), demonstrating the potential of using these hydrogels to micropattern cells. The demonstrated amenability to microfabrication makes these materials attractive for tissue engineering research.

4. Conclusions

Artificial proteins containing leucine zipper domains showed no evidence of toxicity to mammalian cells. An RGD cell-binding domain incorporated into the midblock backbone plays an important role in regulating cell morphology and survival.

Encapsulated 3T3 fibroblast cells remained viable inside the hydrogel for at least 12 hours, suggesting that these materials have proper permeability for transferring oxygen, nutrients, and metabolic waste. Their amenability to microfabrication makes leucine zipper hydrogels attractive for tissue engineering research. Preliminary cell culture results demonstrate that appropriately engineered leucine-zipper hydrogels are promising biomaterials for cell culture and tissue engineering.

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NheI

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AA   TTC  GCT  AGC  GTC  GAC  ACG  GCT  GAG  TGC  AAG
       G  CGA  TCG  CAG  CTG  TGC  CGA  CTC  ACG  TTC

                         R    G    D
CCC  CAA  GTG  ACT  CGC  GGG  GAT  GTG  TTC  ACT  ATG
GGG  GTT  CAC  TGA  GCG  CCC  CTA  CAC  AAG  TGA  TAC

CCG  GAG  GAT  GAG  TAC  ACG  GTC  GGT  GCC  CTC  GAG
GGC  CTC  CTA  CTC  ATG  TGC  CAG  CCA  CGG  GAG  CTC
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SpeI

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ACT  AGT  A
TGA  TCA  TTC  GA
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(a)

MRGSHHHHHHGSDDDDKWASGDLENEVAQCEREVRSLEDEAAELEQKVSRLKNEIEDLKAEI GDHVAPRDTSYRDPMG[AGAGAGPEG]$_{10}$ARMPTSVDTAQCKPQVTRGDVFTMTSYRDPMG[A GAGAGPEG]$_{10}$ARMPTSGDLENEVAQCEREVRSLEDEAAELEQKVSRLKNEIEDLKAEIGDHVA PRDTSMGGC

Abbreviation for domains:

A(L11C): SGDLENEVAQCEREVRSLEDEAAELEQKVSRLKNEIEDLKAE

C_{10}: [AGAGAGPEG]$_{10}$

VN: VDTAQCKPQVTRGDVFTM

His-tag: HHHHHH

(b)

Figure 6.1. Incorporation of the RGD cell-binding domain of vitronectin into leucine zipper hydrogels (a) DNA encoding the vitronectin cell-binding domain (VN); (b) the amino acid sequence of $AC_{10}(VN)C_{10}$Acys(L11C).

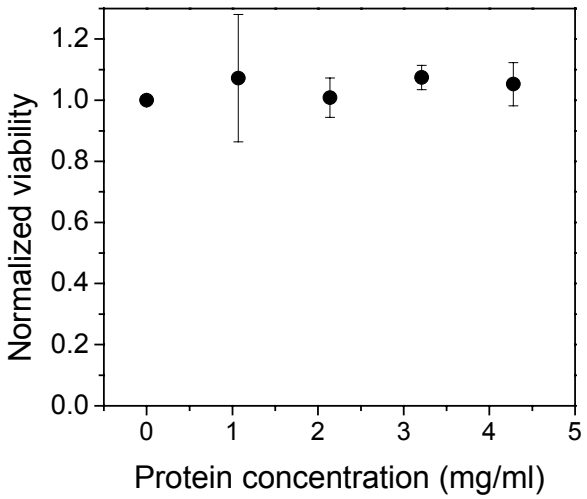


Figure 6.2. WST-1 assay for 3T3 fibroblast cells cultured in the presence of artificial protein $AC_{10}Atrp$. Signals are normalized to the control sample cultured in the absence of $AC_{10}Atrp$. Cells were cultured in DMEM/serum medium in a humidified incubator at 37 °C and 5% CO_2 for 24 hrs before the assay.

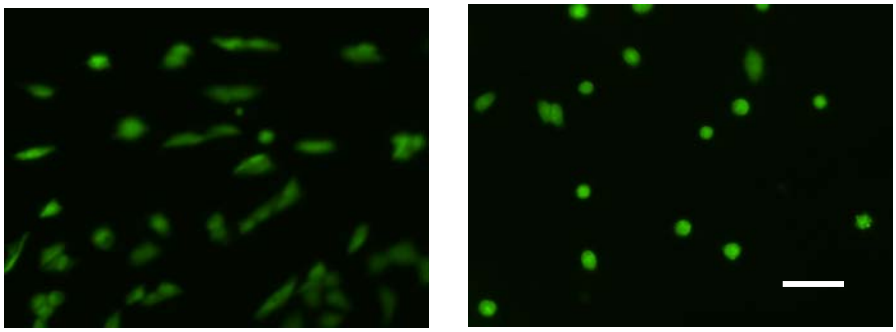
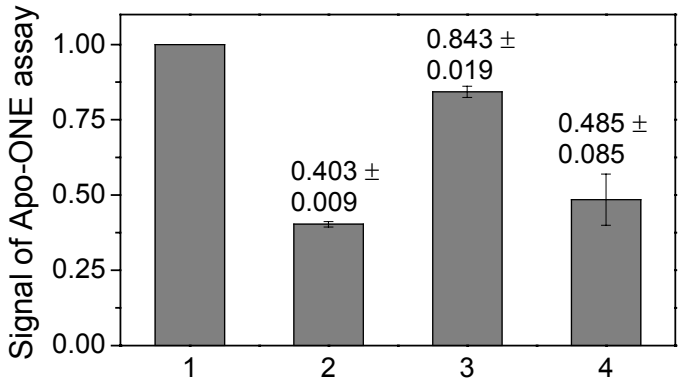


Figure 6.3. MCF10A cells cultured on (a) an $AC_{10}(VN)C_{10}$Acys(L11C) film; and (b) an AC_{10}Acys(L11C) film in serum-free DMEM/Ham's F-12 medium for 6 hrs. The cells were labeled with BCECF AM. The scale bar represents 50 μm.



1: PolyHEMA surface

2: Fibronectin surface

3: AC_{10}Acys(L11C) film

4: $AC_{10}(VN)C_{10}$Acys(L11C) film

Figure 6.4. Apo-ONE caspase-3/7 assay for MCF10A cells cultured on leucine zipper hydrogel films, polyHEMA treated surfaces (positive control), and fibronectin treated surfaces (negative control). Signals are normalized to the positive control. Cells were cultured for 42 hrs in serum-free DMEM/Ham's F-12 medium before the assay.

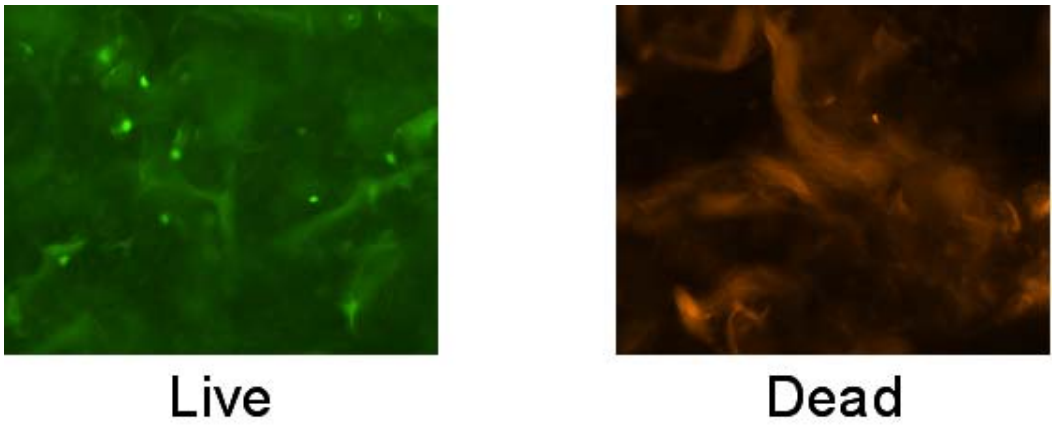


Figure 6.5. Fluorescence live-dead assay for 3T3 fibroblast cells encapsulated in an $AC_{10}(VN)C_{10}$Acys(L11C) gel and cultured in DMEM/serum medium for 12 hrs. The two images were taken from the same frame.

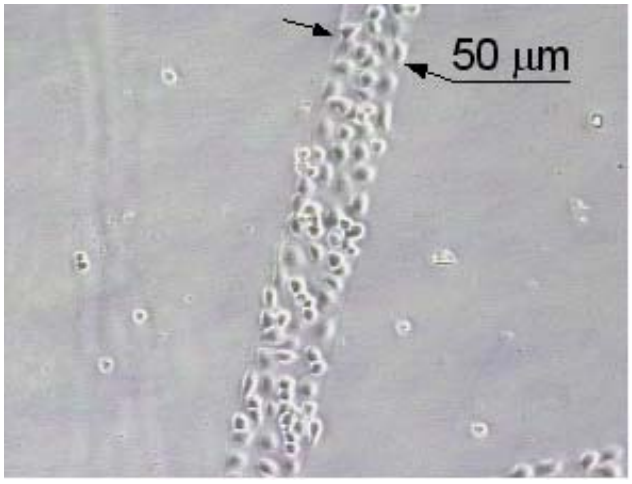


Figure 6.6. Micropatterning of a hydrogel containing the RGD domain on a PEG-modified glass surface limited cell adhesion to the hydrogel region. 3T3 fibroblast cells were seeded in serum-free DMEM medium and incubated in a humidified incubator at 37 °C and 5% CO_2 for 1 hr before the medium was aspirated away and the sample was washed with PBS.