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Supporting Material

Surface creasing instability of soft polyacrylamide cell culture substrates

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SUPPLEMENTARY MATERIAL

Materials and Methods

Materials for pAAm Substrate Synthesis

Acrylamide (AAm), poly(ethylene glycol) 1000 monomethyl ether monomethacrylate (pEGMA), acrylic acid (AAc), acetic acid, N,N'-methylenebis(acrylamide) (Bis; Chemzymes ultrapure grade), and N,N,N',N'-tetramethylethylenediamine (TEMED) were purchased from Polysciences, Inc. (Warrington, PA). N-hydroxysulfosuccinimide (sulfo-NHS), 2-(N-morpholino) ethanesulfonic acid, 0.9 % sodium chloride buffer (MES; "BupH MES Buffered Saline Pack"), and sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1- carboxylate (sulfo-SMCC) were acquired from Pierce (Rockford, IL). QTX ([3-(3,4-Dimethyl-9-oxo-9H-thioxanthen-2-yloxy)-2-hydroxypropyl] trimethylammonium chloride), ammonium persulfate, dichlorodimethylsilane, sodium acrylate (NaAc), poly-L-lysine hydrobromide

(PLL, Mw = 150 ~ 300 kg/mol), fluorescein isothiocyanate (FITC), triethylamine (TEA), anhydrous dimethyl sulfoxide (DMSO), and phosphate buffered saline (PBS, 10 mM phosphate buffer in 138 mM aqueous NaCl, pH 7.4) were obtained from Sigma-Aldrich (St. Louis, MO).

Methacryloxypropyltrimethoxysilane (MTMS) and allyltrichlorosilane (ATC) were obtained from Gelest (Morrisville, PA). Diamino-poly(ethylene glycol) [$PEG(NH_2)_2$; 3400 g/mol, chromatographically pure] was purchased from Nektar (Huntsville, AL). RGD peptide was synthesized by American Peptide Co. (Sunnyvale, CA) and characterized using mass spectrometry and high performance liquid chromatography (purities > 95%; based on the integrin-binding sequence from rat bone sialoprotein: **bsp-RGD(15)** peptide, Acetyl-CGGNGEP**RGD**TYRAY-NH_2). All other chemicals used were reagent grade and used as purchased without further purification. All glassware was cleaned with a 2% solution of Contrad 70 (Deacon Laboratories, Inc.; Cat # 04-355) in water for 2 hours, rinsed copiously with water and baked dry. All water used in this study was ultra pure ASTM Type I reagent grade water (18.2 MΩ-cm, pyrogen free, endotoxin < 0.03 EU/m) (1). All reactions were carried out at room temperature unless otherwise stated.

pAAm surface preparation

Glass coverslips (18 mm diameter; Fisher Scientific, Santa Clara, CA) were cleaned by submersion in ultrapure water (UPW) and sonicated for 30 min in UPW (Branson model 5510, 40 kHz, 469 W, 117 V). This sonication step was repeated with acetone and then with UPW again. After cleaning, the samples were dried (N_2) and cleaned with an O_2 plasma (March Plasmod; Concord, CA) at 1 Torr and 125 Watts for 5 min to remove any adsorbed surface species.

To activate glass substrates for synthesis, glass coverslips were treated with an organosilane to functionalize the surface for polymerization. Samples were covered in a 1% (v/v) methacryloxypropyltrimethoxysilane, 94% (v/v) methanol (MeOH), and 5% (v/v) water solution containing 5 μL of acetic acid per 100 mL of solution. For some samples, methacryloxypropyltrichlorosilane, 0.4% (v/v) in ethanol was used instead. Samples were then rinsed 3x in MeOH and baked for 30 min at 110°C. This activation method is comparable to previously published glutaraldehyde activation methods (2).

A series of pAAm gels of varying composition were first prepared, as summarized in Table 1 of the main text; for each composition, two chemically identical gels were polymerized between glass coverslips. Solutions of AAm and varying amounts of Bis were made in water. Polymerization was initiated by adding 10-20 μL of *N,N,N',N'*-tetramethylethylenediamine (TEMED) and 100 μL of 10% (w/v) ammonium persulfate (AP) per 10 mL of de-gassed aqueous pre-gel reactant solution. 60 μL of this solution was pipetted immediately onto the coverslips, and covered with glass "coverdisks" placed face down on top of the monomer/crosslinker solution to ultimately create a smooth surface for cell culture. These glass coverdisks consisted of 18 mm diameter coverslips, were treated with dichlorodimethylsilane for 1 min, and then wiped clean with Kimwipes prior to use. For some samples, dichlorodimethylsilane treatment of the releasing coverslips was not used. Samples were allowed to polymerize for ~10-30 min, then the coverdisks were removed and samples were stored in water overnight.

Unconstrained pAAm swelling and post-swelling attachment

The linear extent of swelling λ_f was determined in one of two ways. In the first method, bulk gels (1 mm thick) were prepared by pouring 5 mL of the pre-gel solution containing AAm, Bis, TEMED and AP into a 60-mm-diameter polystyrene petri dish. The resulting gel was freed from the petri dish and swelled in deionized water for greater than 24 h, with the swelling ratio taken as the ratio of the gel diameter after swelling to that immediately post polymerization.

In the second method, a 5 μL volume of a diluted aqueous suspension of fluorescent polystyrene beads (3 μm diameter, Polysciences: Warrington, PA) was added to each 200 μL pre-gel solution prior to polymerization, yielding a final content of ~ 100 beads/μL. Polymerization was performed between two glass coverslips as above but without using the methacryloxypropyltrimethoxysilane adhesion-promoter and instead by using dichlorodimethylsilane on both coverslips. Following polymerization, the glass slides were separated; and upon swelling the gel delaminated from the substrate thus expanding freely in three-dimensions. Unconstrained gels were transferred to a swelling solution and agitated for at least one hour with three changes of solution to extract non-cross-linked components and allow the degree of swelling to equilibrate. Either phosphate-buffered saline (PBS) or deionized water was used as the swelling medium with no measured difference, consistent with the nonionic nature of the pAAm gels. Fluorescence images of a collection of beads (typically ~ 10) near the center of the gel were taken prior to swelling and after equilibration with a low magnification (10x) microscope objective. The ratio of distances of each bead from the center of mass was determined in the two cases, with the average taken as the linear degree of swelling and the uncertainty as the standard deviation. This method yielded good agreement with the first method, but generally smaller uncertainties and better sample-to-sample reproducibility.

For post-swelling attachment to substrates, unconstrained gels were manually transferred after equilibration onto glutaraldehyde-activated coverslips (2) and allowed to attach for 30 min.

Cell culture surface preparation

A variable-modulus interpenetrating polymer network (vmIPN) was grafted to the pAAm-containing glass coverslip using a two-step sequential photopolymerization developed from previously published protocols (3, 4). The first polymerization step of the vmIPN synthesis consisted of creating the pAAm gel as described above. Then, an interpenetrating polymer network (IPN) was created by polymerizing a second layer of poly(ethylene glycol) (pEG) (~4 nm thick) within the top few nm of the first AAm layer. A solution of 0.02 g/mL poly(ethylene glycol)-1000-monomethylether (pEGMA), 0.0162 mL/mL AAc, and 0.01 g/mL Bis was initiated with 0.005 g/mL QTX in a degassed solution of 97:3 (v/v) water:isopropanol (IPA; Fisher Scientific, Santa Clara, CA) for 60 min using an ultraviolet light transilluminator table (model TFL-40; Ultra-Violet Products, Upland, CA). The power of the table was measured at 2.3 mW/cm^2 using a radiometer (International Light, Inc., Massachusetts) with a 352-377 nm band-pass filter. This resulted in an IPN with a high density of pEG at the surface (5).

Details regarding peptide grafting to the IPN can be can be found elsewhere (3, 4), but are described briefly below. Following the formation of the IPN, the samples were

equilibrated with buffer (>30 min, MES, 0.5 M, pH 7). To functionalize the IPN with biological ligands, 3400 M_w $PEG(NH_2)_2$ spacer chains were grafted to the AAc sites via a carbodiimide reaction (60 min, 0.5 M MES, pH 7, 0.150 g/mL $PEG(NH_2)_2$, 0.005 g/mL EDC, 0.0025 g/mL Sulfo-NHS). After the reaction, the solution was aspirated and the samples were rinsed 2x with 0.1 M MES buffer (pH 7.0) followed by 2x rinse with 50 mM sodium borate buffer (pH 7.5). At this point, the samples were sterilized by UV irradiation in a biosafety hood for 30 minutes. All following subsequent solutions were sterile filtered through a 0.22 μm filter. To couple bioactive molecules to the $PEG(NH_2)_2$-modified IPN, the heterobifunctional cross-linker, sulfo-SMCC, was reacted with the free amine on the $PEG(NH_2)_2$ chains (0.0005 g/mL Sulfo-SMCC, pH 7.5, borate buffer, 30 min). The solution was then aspirated, and the samples were rinsed 2x with borate buffer followed by 2x with peptide-coupling buffer (sodium phosphate, 0.1 M, pH 6.6). Finally, the peptide [bsp-RGD(15)] was coupled (20 μM) to the maleimide (sulfo-SMCC) at the peptide N-terminal free thiol. Following the reaction overnight, the solution was aspirated and the samples were rinsed 4-5 times with coupling buffer, sonicated (UPW, 5 min), rinsed (UPW), and stored in PBS with 5000 U-μg/mL PennStrep (5000 U penicillin and 5000 μg streptomycin per milliliter) at 4°C.

Laminin cell culture surfaces were coated with poly-ornithine and saturated with mouse laminin I from the Engelbreth-Holm-Swarm sarcoma (Invitrogen, Carlsbad, CA) as described previously (6).

Fluorescent staining of the gel surface

10 mg of PLL was fluorescently labeled with 0.78 mg of FITC (corresponding to labeling 3 % of lysine units) in 1 mL of anhydrous DMSO containing 20 μl of TEA for 2 hours at room temperature. The solution was dialyzed (14 kDa molecular weight cutoff, Spectra/Por) against PBS for 1 day and deionized water for 2 days and finally lyophilized to yield fluorescein tagged PLL (f-PLL). AAm gel (10 wt% AAm, 0.01 wt% NaAc and 0.02 wt% Bis) was polymerized on the functionalized glass coverslip as described above. Prior to swelling the gel in water, 100 μl of f-PLL solution (8 mg of polymer in 100 μl of deionized water) was pipetted onto the gel surface and waited for 10 seconds to allow electrostatic adsorption of f-PLL on the near-surface region of the gel. Subsequently, the gel surface was thoroughly washed with deionized water to remove non-adsorbed f-PLL. The gel formed creases on its surface upon swelling in deionized water, and then the fluorescently stained surface was imaged by confocal fluorescence microscopy.

Rheological materials characterization

During the AAm polymerization step of the IPN synthesis, 5 mL of reactant solutions of AAm, Bis, TEMED, and AP was poured into a 60 mm diameter polystyrene dish. The resulting bulk gels (~1 mm thick) were swollen in water overnight and then trimmed and loaded into a rheometer. Oscillatory shear measurements of pAAm gels were performed on a rheometer (MCR300, Anton Paar, Ashland, VA) with 50 mm parallel plates at a gap height of 0.5-3.0 mm. A humidity chamber was placed around the sample to prevent dehydration. The lower plate temperature was regulated with a Peltier heating element connected to a recirculating water bath. Frequency sweeps from 0.001-

10 Hz at 5% strain were performed first at 25°C and then at 37°C. No significant difference in complex modulus was seen between the two temperatures. *G'* is the complex storage modulus at 1 Hz as measured by the rheometer. Results from atomic force measurements on vmIPNs and rheological measurements on the bulk polyacrylamide gels showed good agreement (5). Furthermore, the modulus of the polyacrylamide layer and the full vmIPN were not statistically different (5). Samples containing 10 wt % AAm and Bis contents of 0.02 wt %, 0.04 wt %, and 0.10 wt % were instead measured using an AR 20000ex shear rheometer (TA instruments) equipped with a 40-mm-diameter aluminum parallel plate and solvent trap to avoid evaporation. Shear moduli were estimated from the values of storage modulus (G') in the plateau region from frequency sweeps over the range of 100 rad/s to 0.1 rad/s at a constant stress of 1 Pa.

Neural stem cell culture

Neural stem cells were isolated from the hippocampi of adult female Fischer 344 rats as previously described (7) and used between 10 and 30 passages of culturing. Cells were seeded (25,000 cells/cm^2 for differentiation) onto peptide-modified vmIPNs or laminin-modified culture wells and incubated (37˚C, 5% CO_2) in serum-free media consisting of Dulbecco's Modified Eagle Medium (F-12/DMEM, high glucose, with L-glutamine, with pyridoxine hydrochloride, without sodium pyruvate; Invitrogen, Carlsbad, CA) with N-2 supplement (Invitrogen, Carlsbad, CA). This medium was supplemented with soluble factors to induce differentiation: 1 μM retinoic acid with 1 v/v% fetal bovine serum (Invitrogen, Carlsbad, CA) for mixed glial and neuronal differentiation. Wells were rinsed every 48 hrs with fresh media. Differentiation of these exact cells on IPNs grafted to polystyrene have been published already (4) and compared to laminin-coated polystyrene. Three biological replicate wells (n=3) were used per condition.

Immunofluorescence assays

For immunofluorescence staining, cells were cultured for 5-6 days then fixed with 4% paraformaldehyde (Fisher Scientific, Santa Clara, CA), blocked for 1 hour with 2% goat serum (Sigma-Aldrich, St. Louis, MO), permeabilized with 0.3% Triton X-100 (Calbiochem, San Diego, CA), and incubated for 36 hours with primary antibodies rabbit anti-nestin (1:500 dilution; AbCam, Cambridge, MA), mouse anti-β-tubulin III (1:500 dilution; Sigma-Aldrich, St. Louis, MO), and guinea pig anti-glial fibrillary acidic protein (GFAP; 1:1,000 dilution; Advanced Immunochemical, Long Beach, California). The primary antibody solution was then removed, and the cells were rinsed and incubated for 1.5 hours with Alexa Fluor® secondary antibodies 488 goat anti-rabbit IgG, 546 goat anti-mouse IgG, and 647 goat anti-guinea pig IgG at a dilution of 1:500 (all from Invitrogen, Carlsbad, CA). Nuclei were stained with DAPI (Invitrogen, Carlsbad, CA). Images were collected on a Nikon Eclipse TE2000-E and a Zeiss META 510 laser scanning confocal microscope.

Results

TABLE S1. Complete modulus and swelling data for samples in Figure 3B.

AAm content w_m (wt %)	Bis content w_x (wt %)	G' (Pa)*	λ_f*	Surface-attached gel
3.0 %	0.025 %	12	1.79	Creased
	0.051 %	80	1.46	Flat
	0.075 %	130	1.39	Flat
	0.10 %	130	1.33	Flat
	0.20 %	160	1.18	Flat
5.0 %	0.025 %	140	1.66	Creased
	0.051 %	57	1.41	Flat
	0.075 %	440	1.34	Flat
	0.10 %	670	1.27	Flat
	0.20 %	1140	1.16	Flat
10.0 %	0.020 %	400	1.51	Creased
	0.040 %	820	1.44	Flat
	0.10 %	2250	1.28	Flat
	0.15 %	5460	1.18	Flat
	0.30 %	9580	1.11	Flat

*Typical estimated uncertainties are ± 10 Pa for G' and ± 0.04 for λ_f.

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