Facile and Versatile Method for Micropatterning Poly(acrylamide) Hydrogels Using Photocleavable Comonomers

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ABSTRACT: We here present a micropatterning strategy to introduce small molecules and ligands on patterns of arbitrary shapes on the surface of poly(acrylamide)-based hydrogels. The main advantages of the presented approach are the ease of use, the lack of need to prefabricate photomasks, the use of mild UV light and biocompatible bioconjugation chemistries, and the capacity to pattern low-molecular-weight ligands, such as peptides, peptidomimetics, or DNA fragments. To achieve the above, a monomer containing a caged amine (NVOC group) was co-polymerized in the hydrogel network; upon UV light illumination using a commercially available setup, primary amines were locally deprotected and served as reactive groups for further functionalization. Cell patterning on various cell adhesive ligands was demonstrated, with cells responding to a combination of pattern shape and substrate elasticity. The approach is compatible with standard traction force microscopy (TFM) experimentation and can further be extended to reference-free TFM.

KEYWORDS: mechanotransduction, integrin ligands, cell–material interactions, photopatterning, traction force microscopy

INTRODUCTION

Understanding how cells sense and respond to the physical and mechanical properties of their insoluble microenvironment, i.e., their extracellular matrix (ECM), is a major challenge of mechanobiology research. Among different approaches, the ex vivo interrogation of cells on artificial substrates with controlled biophysical and biochemical properties has proven to be a powerful tool to test hypotheses and gain mechanistic insight into mechanosensing and mechanotransduction of living cells. Upon adhesion on a compliant substrate, cells exert traction forces at the sites of attachment, where multiprotein complexes, termed focal adhesions (FAs), assemble.1,2 Cell-surface receptors of the integrin family at FAs transmit intracellularly generated forces produced by actomyosin contractility to substrate-immobilized extracellular ligands.3 The amplitude and dynamics of these forces depend on substrate viscoelasticity, and in turn determine the tension and force loading rate experienced by mechanosensing proteins present at FAs.4 At the same time, cell shape and size additionally control the magnitude and orientation of traction forces, through control of adhesion geometry and actin cytoskeleton organization.4,5

Efforts to control cell morphology have revealed how cell confinement affects cell growth,6 gene transcription,7 and differentiation.8 Overall, both cell shape and substrate mechanics control cell physiology, and hence the need to control independently these parameters when designing cell culture substrates. An attractive approach to achieve this goal is to pattern adhesive ligands on viscoelastic substrates so that cells conform to the designed patterns.9,10

Hydrogels based on synthetic or natural non-ECM polymers offer the advantage of decoupling ligand presentation from mechanical properties, due to their tunable stiffness and biologically inert background, on which adhesive ligands can be incorporated.11–15 Various systems and cross-linking chemistries have been developed to this end, including but not

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limited to poly(ethylene glycol), alginate, hyaluronan, poly(N-isopropylacrylamide), and poly(hydroxyethyl methacrylate) hydrogels.\textsuperscript{13−15} To spatially pattern adhesive ligands on the hydrogel surface,\textsuperscript{16,17} light has proven an excellent means due to a range of developed photocleavable groups and the precision photopatterning allows.\textsuperscript{18,19} Current methodologies for hydrogels photopatterning rely primarily on soft lithography and can be classified into two main categories. The first, most common strategy is based on localized activation of reactive groups or exposure of protein-adsorbing surfaces on the gel surface and subsequent, selective attachment of ligands.\textsuperscript{20−28} Within this category, additionally falls the spatially controlled uncaging of...
adhesive ligands, which does not require a second reaction step, but prior synthesis and incorporation of caged ligands.\textsuperscript{29,30} The second strategy is based on hydrogel formation on a substrate prepatterned with the ECM protein of choice.\textsuperscript{4,31,32}

Among the developed hydrogel systems, polyacrylamide (pAAm) gels remain the most popular choice for cell mechanobiology studies due to their ease of fabrication and established use in traction force microscopy (TFM) studies following decoration with fiducial markers.\textsuperscript{22,33–35} Despite inherent limitations, such as lack of physiological structure and viscoelastic character, pAAm hydrogels offer a reproducible, robust, and stable system that can be readily functionalized with high-molecular-weight adhesive ligands.\textsuperscript{35,36} To pattern such ligands, a variety of micropatterning techniques were developed, or adapted for pAAm,\textsuperscript{37} published protocols for the spatial patterning of this material still suffer from a few drawbacks: (i) a new pattern requires the design and fabrication of a new mask, an expensive and time-consuming process; (ii) current approaches do not allow efficient patterning of small (1–3 kDa) peptide ligands, (iii) aligning multiple, sequential patterns is difficult; and (iv) flexibility and control over ligand presentation remains limited. Consequently, there is room for improved techniques that are more versatile, easy-to-perform, and accessible.

Here, we introduce a facile and versatile method to pattern pAAm hydrogels using UV light, through the co-polymerization of monomers containing light-sensitive caged amine groups. The generated primary amines can be subsequently used to immobilize cell ligands throughout various conjugation methods. The caged comonomer was designed such that the cleavage of the caged amine (caged AEMA; Figure 2A) would expose primary amines for further functionalization as schematically shown in Figure 3A. Due to low aqueous solubility, caged AEMA was dissolved in DMF instead of water. Moreover, preliminary studies showed that a higher amount of radical initiation was required for polymerization due to the radical scavenging properties of the nitro group.\textsuperscript{31} The nitrophenyl group absorbs light in the UV range; quantification of comonomer incorporation through absorbance measurements following extensive washing of the gel revealed a monotonic increase in caged AEMA incorporation (Figure 1C). The caged comonomer was quantitatively incorporated, except for the highest concentration tested (3 mM), where approximately 70% of the initial caged AEMA was present in the final hydrogel (Figure 1C). The mechanical properties of the resulting hydrogels were influenced by the presence of the caged comonomer, depending on the cross-linking ratio: for the stiffer hydrogels (0.3% Bis), the obtained Young’s moduli were similar to controls, whereas for hydrogels with lower cross-linking, a reduction in stiffness was observed. For the lower cross-linking concentration (0.03% Bis), the hydrogels obtained were too soft to handle and measure with the AFM setup used.

Light-Induced Uncaging Enables Spatial Patterning. The caged comonomer was designed such that the cleavage of the carbamate group upon UV irradiation would expose primary amines for further functionalization as schematically shown in Figure 2A. To determine the light exposure required to deprotect the amines of gel-incorporated caged AEMA, circular patterns of 10–50 μm in diameter on the hydrogel surface were exposed to UV light (375 nm) for varying times using a PRIMO micropatterning system. Deprotected amines were then reacted with an NHS-coupled fluorescent dye (AlexaFluor 568). A linear increase in fluorescence intensity within patterned areas was observed up to a laser dose of 250 mJ/mm², followed by a plateau (Figure 2B, C). The plateau value was the same as the one for hydrogels containing AEMA at the same concentration, indicating complete uncaging of caged AEMA at illuminated regions. Nonpatterned areas exhibited similar fluorescence to the negative controls (no AEMA incorporated), demonstrating a lack of uncaging from ambient light in nonilluminated regions. Based on these results, a dose of 500 mJ/mm² was selected for further experiments.

The PRIMO micropatterning system with its associated software also enabled the facile preparation of virtual masks, and thus the patterning of custom designs (Figure 2D), as well as the formation of gradients of immobilized fluorescent labels, through the use of virtual masks with a gradient in gray value (Figure 2E). Patterning of two different dyes in complex, predesigned patterns was also achieved with sequential light exposure, fluorescent labeling, and alignment using the fluorescence of the first pattern (Figure 2F).

Exposure to UV light and deprotection of caged AEMA did not affect the stiffness of the hydrogel surface layer, as determined by AFM indentation measurements. The Young’s modulus was the same within and around patterns, as these were identified by fluorescent labeling with NHS-AF568 (Figure 2G). Of note, reaction with NHS-AF568 labeled the deprotected amines throughout the hydrogel bulk following the UV light path used during patterning (Figure S2). In principle, localized patterning with the hydrogels presented here is also feasible in the z-direction using two-photon microscopy.\textsuperscript{32,33}

Hydrogel Functionalization. Having validated the presence of reactive amines on patterned regions (Figure 2), three different chemistries were examined with the aim of introducing spatially patterned functional molecules on the surface of
poly(acrylamide) hydrogels (Figure 3). First, deprotected amines in hydrogels were reacted with a heterobifunctional NHS-biotin linker to exploit the robust biotin−streptavidin interaction to immobilize ligands on surfaces. Subsequent incubation with fluorescently labeled streptavidin (100 μg/mL) verified the applicability of this approach to surface pattern hydrogels (Figure 3A). In this case, Atto565-Streptavidin was only present on the hydrogel surface, due to the size-dependent exclusion of the protein from diffusion inside the hydrogel (Figure S2). Second, click chemistry was performed following reaction of patterned hydrogels with a heterobifunctional NHS-PEG-Alkyne linker: an azide-functionalized fluorophore (AlexaFluor488) was used to validate this approach (Figure 3B). Inversely, the azide was immobilized on the patterned hydrogel using a heterobifunctional NHS-PEG-Azide linker and patterned regions were labeled with alkyne-functionalized AlexaFluor 555 (Figure 3C). Third, a bifunctional NHS-PEG-Maleimide linker was first introduced to react the NHS esters with the amines, followed by coupling to maleimides of a short thiol-containing DNA fragment, labeled with fluorescein (Figure 3D). Due to maleimide degradation in aqueous solutions, these two steps should be performed in quick succession. The above results highlight the versatility of patterning this type of hydrogels with commonly used bioconjugation chemical strategies.

**Cell Patterning on Hydrogels.** An unmet challenge for the majority of existing patterning methods for pAAm hydrogels is the patterned immobilization of short peptide or peptidomimetic ligands. Techniques to pattern such low-molecular-weight ligands have been reported for other hydrogel systems; here, we demonstrated as a proof of principle, the patterning in lines of integrin peptide or peptidomimetic ligands on pAAm hydrogels, and visualized cell adhesion using optical microscopy. Primary human dermal fibroblasts (pHDF) adhered selectively to patterned hydrogels functionalized using click chemistry with cyclic peptides containing the RGD peptide motif (cyclic RGDfK; Figure 4A). pHDF elongated and migrated along the lines, often exhibiting high aspect ratios of >10 (Movie S1). Small peptidomimetic integrin-selective ligands were also immobilized using the maleimide-thiol coupling strategy. pHDF adhered selectively to patterned areas with α5β1-selective integrin ligands (Figure 4B), while Chinese hamster ovary cells overexpressing the αIIbβ3 integrin (CHO-A5) recognized αIIbβ3-selective integrin ligands patterned in lines (Figure 4C). Immobilization of larger ECM proteins, such as fibronectin (FN), was also successful using the biotin-avidin linkage to confine cells in defined patterns (Figure 4D). The above results

![Figure 3](https://example.com/figure3.png)
demonstrate the versatility of the presented system to pattern various ligands and the generality in application through the use of different cell types.

Effects of Pattern Shape on Cell Behavior. We next examined how the shape of micropatterned ligands affects cell mechanosensing. pHDF fibroblasts were seeded on 7 kPa hydrogels exhibiting crossbow patterns of two different sizes functionalized with cyclic RGDK-containing peptides using click chemistry, or the same hydrogels homogeneously coated with fibronectin and the commonly used sulfo-SANPAH crosslinker. pHDF fibroblasts adhered selectively to patterns, adopting a polarized shape (Figure 5A,B). Notably, the cell size distribution was narrower on patterned substrates compared to the homogeneously coated hydrogel, demonstrating an advantage of cell patterning (Figure 5B). On the larger crossbows (50 μm in height and width), fibroblasts assembled distinct focal adhesions at their periphery as well as discernable actin stress fibers (Figure 5A). In contrast, on the smaller-sized crossbows (30 μm in height and width), fibroblasts assembled much smaller adhesions and did not exhibit an organized actin cytoskeleton (Figure 5A). Fibroblast height was also determined by pattern/cell shape; cells on the larger patterns were flatter compared to those confined to a smaller area (Figure S3).

Next, the localization of yes-associated protein 1 (YAP), a mechanosensitive transcriptional regulator that shuttles between the nucleus and cytoplasm depending on cell shape and actomyosin contractility, was examined. Previous work has demonstrated that confinement of cell size by reducing accessible adhesive area on rigid glass, or culturing of cells on soft substrates, leads to YAP nuclear export.1 In particular, the transition from cytosolic to nuclear YAP in isolated cells occurs in the 1−10 kPa range, with mostly nuclear YAP present on FN- or collagen-coated pAAm hydrogels with a Young’s modulus of 10 kPa.49,50 However, the actual threshold for nuclear accumulation/export of YAP depends not only on stiffness but also on other parameters such as ligand density.39,51 Here, we examined how YAP localization is influenced by the spread area on 10 kPa hydrogels. The nuclear/cytoplasmic YAP ratio was significantly higher for fibroblasts seeded on the larger patterns (Figure 5C,D). Fibroblasts adhering to homogeneously FN-coated hydrogels of the same stiffness showed mostly nuclear YAP localization as expected49,50 (Figure 5C,D), highlighting the effect of pattern shape on mechanotransduction. In sum, the above results demonstrate how control over the size of adhesive patterns on soft elastic hydrogels can be used to study cell behavior and open the way for studying the combinatorial effects of substrate stiffness, cell shape, and ligand type. Of particular interest is the investigation of the relative contributions of substrate stiffness and cell area on YAP mechanotransduction to test recently developed models.52 Moreover, while the effect of ligand density was not investigated here, the possibility to control the concentration of caged comonomer (Figure 1) and light dose (Figure 2B) can be employed to independently control the number of adhesive ligands as well.

Figure 4. Cell patterning on poly(acrylamide) hydrogels. Line patterns were prepared on 5% Am/0.1% Bis pAAm hydrogels incorporating 3 mM caged AEMA. Patterns were functionalized with indicated integrin ligands. Primary human dermal fibroblast adhered selectively to patterns of immobilized cyclic RGDK peptides (A) and α5β1 integrin-selective integrin peptidomimetics (B). (A) Confocal microscopy image of fixed and phalloidin-stained pHDF cells. Phalloidin stains filamentous actin; nuclei are stained in blue. (B) Phase contrast microscopy image of live pHDF cells on hydrogel patterned with α5β1 integrin-selective peptidomimetics. (C) CHO-AS cells adhered to patterned α5β1 integrin-selective ligands as exemplified with the epifluorescence microscopy image of phalloidin-stained cells presented (nuclei stained in blue). (D) Phase contrast microscopy image of live pHDF recognizing line patterns of fibronectin immobilized through biotin−streptavidin chemistry. Scale bar (A,D): 100 μm, B: 75 μm, (C): 50 μm.

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Figure 5. Size of cell adhesive patterns on hydrogels affects cell mechanosensing. (A) Confocal microscopy images of pHDF cells on hydrogels patterned with cyclic RGDfK peptides (small and large crossbows) or homogeneously coated FN. pHDF were fixed 4 h after seeding and stained with tetramethylrhodamine (TRITC) phalloidin, which stains filamentous actin (F-actin), and against pY, which stains focal adhesions. (B) Quantification of cell area of pHDF adhering to patterned, or FN-coated hydrogels. Each data point corresponds to a single cell, from two independent experiments. (C) Confocal microscopy images of yes-associated protein 1 (YAP) immunostained pHDF cells 4 h after seeding. (D) Quantification of nuclear-to-cytoplasmic ratio of YAP in pHDF adhering to patterned, or FN-coated hydrogels. Each data point corresponds to a single cell, from two independent experiments. Data in (B) and (D) were compared using one-way analysis of variance (ANOVA) \((n = 9, 10, \text{ and } 20 \text{ for small xbows, large xbows, and Gel FN, respectively}); P values are shown. Scale bars 20 μm.

Figure 6. Applicability of patterned poly(acrylamide) hydrogels for traction force microscopy applications. (A) Confocal microscopy image of fluorescent beads on the upper layer of the hydrogel, merged with the transmission image showing a live pHDF fibroblast adhering on a line pattern (schematically overlaid in gray). The hydrogel used had a Young’s modulus of 2 kPa and was functionalized with \(\alpha_5\beta_1\) integrin-selective peptidomimetics. (B) Particle image velocimetry (PIV) analysis results from bead displacements observed before and after the removal of the cell shown in (A). The magnitude of the color-coded vectors is given in pixels. (C) Confocal microscopy image of a patterned and AlexaFluor 568-labeled hydrogel. A square grid pattern of 1 μm circles spaced 3 μm apart was used as the virtual mask. (D) Merged confocal microscopy image of a live REF-YFP-PAX cell with a fluorescent pattern on a 7 kPa hydrogel. (E) Substrate deformation field calculated from PIV analysis of panel (D). Cell outlines (white) in (D, E) are a guide to the eye. Scale bar: 20 μm; inset: 5 μm.
Traction Force Microscopy on Patterned Hydrogels.

One major application of pAAm hydrogels remains traction force microscopy (TFM) for the estimation of cell-generated forces, despite the development of more physiologically relevant viscoelastic substrates or three-dimensional (3D) culture systems. In TFM, fluorescent beads embedded in the elastic substrate serve as fiducial markers that report on substrate deformations induced by cells. The confinement of cells within adhesive patterns on top of TFM substrates allows the estimation of traction forces as a function of cell shape, as well as the averaging over many cells to obtain more reliable conclusions. Existing protocols however rely on immobilization of large ECM proteins. To test if our patterning approach was compatible with TFM, hydrogels were functionalized with established chemistries and characterized by indentation measurements using a Nano-Wizard III atomic force microscope (AFM; JPK Instruments AG, Germany), mounted on an optical microscope (Zeiss Axiovert200). Cantilevers with a spherical, borosilicate glass probe 5 μm in diameter (sQube) and a spring constant between 0.45 and 0.60 N/m were used. The exact value of the spring constant was determined using the thermal noise calibration method prior to measurements. Force-distance (F–d) curves were obtained from immobilized gels with a cantilever speed of 1.0 μm/s in phosphate-buffered saline (PBS) at room temperature. Young’s moduli were calculated by fitting the F–d curves using the software provided by JPK and the Hertz model for a spherical indenter.

EXPERIMENTAL SECTION

Materials. A list of commercially available reagents and antibodies used in this study are presented in the Supporting Information (Tables S1 and S2). Integrin-selective peptidomimetic ligands against integrins α,β1 and α,β3 containing a thiourea (S) short DNA sequence (5′-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3′) coupled to a thiol at the 5′ end and fluorescein to the 3′ end was purchased from Biomers.net. The cyclic peptide RGDK functionalized with an azide at the ε-amino of lysine was purchased from PSL laboratories (Heidelberg, Germany).

NVOC-protected aminoethylmethacrylamide (Caged AEMA) was prepared using a previously described protocol. Briefly, 2-aminomethylmethacrylamide hydrochloride (100 mg, 0.6 mmol, 1 equiv) was mixed with Na2CO3 (63.58 mg, 0.6 mmol, 1 equiv) in 16 mL of H2O. Then, an equimolar amount of 4,5-dimethoxy-2-nitrobenzyl chloroformate (165.38 mg, 0.6 mmol, 1 equiv) was dissolved in 16 mL of dioxane and was slowly added to the aqueous solution with vigorous stirring. After stirring at room temperature for a period of 1 h, the reaction was diluted with 15 mL of dichloromethane, followed by the addition of 10 mL of 1 M H2SO4 for acidification.

The organic phase was collected, and the aqueous phase was extracted with dichloromethane (3 × 15 mL). The combined organic extracts were dried over anhydrous MgSO4 and concentrated in vacuo to give 142 mg (66%) of a yellowish solid, which was used without further purification. The product was conserved, at −20 °C, protected from light.

Hydrogel Preparation. Poly(acrylamide) hydrogels were prepared using radical polymerization according to a published protocol. The weight percent of acrylamide monomer was kept constant at 5%, while the weight percent of the cross-linker bis-acrylamide was varied between 0.03 and 0.3%. Comonomers were introduced in the precursor solution at defined concentrations. 2-Aminoethylmethacrylamide (AEMA) was introduced as an aqueous solution, while caged AEMA was introduced in DMF. Fluorescent beads, 200 nm in diameter, were added (1:100 stock solution) to the precursor mixture prior to gelation. After mixing, 1 μL of tetramethylethylenediamine (TEMED) and 15 μL of an APTES solution in ethanol (10% v/v), in which 10 μL of a 1 M KH2PO4 solution was added, was introduced in DMF. Fluorescent probes, 200 nm in diameter, were added (1:100 stock solution) to the precursor mixture prior to gelation. After mixing, 1 μL of tetramethylethylenediamine (TEMED) and 15 μL of freshly prepared solution of ammonium persulfate (APS; 100 mg/mL) were added and the precursor solution was vortexed and pipetted between a hydrophobic glass coverslip, treated with Rain-X for 10 min, and a glass coverslip treated with (3-aminopropyl)triethoxysilane (APTES). For silanization, the coverslips were immersed for 1 h in 100 mL of an APTES solution in ethanol (10% v/v), in which 10 μL of water and 10 μL of triethyamine were added. Then, the coverslips were rinsed with ethanol, then water, dried, and placed in an oven at 120 °C for 1 h. The hydrogel thickness was controlled using spacers between the two coverslips. Gelation was left to proceed for at least 30 min at room temperature. Gels attached to the APTES-treated coverslips were recovered after removing the hydrophobic coverslip and were washed in excess of water five times to remove unreacted monomers and initiators.

Mechanical Characterization. Hydrogels were mechanically characterized by indentation measurements using a Nano-Wizard III atomic force microscope (AFM; JPK Instruments AG, Germany), mounted on an optical microscope (Zeiss Axiovert200). Cantilevers with a spherical, borosilicate glass probe 5 μm in diameter (sQube) and a spring constant between 0.45 and 0.60 N/m were used. The exact value of the spring constant was determined using the thermal noise calibration method prior to measurements. Force–distance (F–d) curves were obtained from immobilized gels with a cantilever speed of 1.0 μm/s in phosphate-buffered saline (PBS) at room temperature. Young’s moduli were calculated by fitting the F–d curves using the software provided by JPK and the Hertz model for a spherical indenter.
Hydrogel Functionalization. Micropatterned hydrogels were equilibrated in 10 mM PBS prior to the reaction of deprotected AEMA dosed was selected. After illumination, hydrogels were washed with water. (100 µL) was placed on top of the hydrogel and incubated overnight at 4 °C. Hydrogels were washed three times with PBS prior to cell seeding. Cell Culture. Primary human dermal fibroblasts (pHDF) were purchased from ATCC (Cat # PCS-201-010). Chinese hamster ovary cells overexpressing the α5β1 integrin (CHO-A5) were a kind gift from the lab of Prof. M. Ginsberg (UCSD). REFS2 stably transfected with paxillin fused to a yellow fluorescent protein (REFPAX) was a gift from Prof. B. Geiger (Weizmann Institute). All cells were cultured as sub-confluent monolayers at 37 °C and 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies; Prod. #10938), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). pHDF cultures were used until passage 15. Cell cultures were checked regularly for the absence of mycoplasma. Immunofluorescence and Microscopy Imaging. Cells were fixed on hydrogels after washing once with PBS and incubating with a 4% paraformaldehyde (PFA) PBS solution for 20 min at room temperature. After washing with PBS, the cells were permeabilized with 0.1% Triton X-100 for 5 min, followed by blocking with 1% bovine serum albumin (BSA) in PBS for 1 h. The cells were incubated with primary antibodies (diluted in 1% BSA) for 1 h at room temperature, washed four times with PBS, and incubated with corresponding secondary antibodies for another 1 h at room temperature. AlexaFluor488, was performed in HEPES buffered (100 mM, pH 7.4). The hydrogel was then irradiated with a portable UV light lamp for 5 min and rinsed first with water and then with PBS. Finally, a dibenzoctin solution in PBS (40 µg/mL) was placed on top of the hydrogel and incubated overnight at 4 °C. Hydrogels were washed three times with PBS prior to cell seeding.

Hydrogels were alternatively functionalized using the standard approach with sulfo(succinimidyl-6-[4′-azido-2′-nitrophenylamino]-hexanoate (sulfo(SANPAH)). Briefly, hydrogels were washed with PBS before applying a 1 mg/mL sulfo(SANPAH) solution in HEPES buffer (100 mM, pH 7.4) and then irrigated with a portable UV light lamp for 5 min and rinsed first with water and then with PBS. Finally, a dibenzoctin solution in PBS (40 µg/mL) was placed on top of the hydrogel and incubated overnight at 4 °C. Hydrogels were washed three times with PBS prior to cell seeding.

C. Hydrogels were washed three times for 5 min with water prior to functionalization and were protected from light.

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