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Bifunctional poly(acrylamide) hydrogels through orthogonal coupling chemistries

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Abstract

Biomaterials for cell culture allowing simple and quantitative presentation of instructive cues enable rationalization of the interplay between cells and their surrounding microenvironment. Poly(acrylamide) (PAAm) hydrogels are popular 2D-model substrates for this purpose. However, quantitative and reproducible biofunctionalization of PAAm hydrogels with multiple ligands in a trustable, controlled and independent fashion is not trivial.

Here, we describe a method for bifunctional modification of PAAm hydrogels with thiol- and amine- containing biomolecules with controlled densities in an independent, orthogonal manner. We developed copolymer networks of AAm with 9% acrylic acid and 2% N-(4-(5-(methylsulfonyl)-1,3,4-oxadiazol-2-yl)phenyl)acrylamide. The covalent binding of thiol- and amine-containing chromophores at tunable concentrations was demonstrated and quantified by UV spectroscopy. The morphology, mechanical properties and homogeneity of the copolymerized hydrogels were characterized by scanning electron microscopy, dynamic mechanical analysis, and confocal microscopy studies. Our copolymer hydrogels were bifunctionalized with polylysine and a laminin-mimetic peptide using the specific chemistries. We analyzed the effect of binding protocol of the two components in the maturation of cultured post-mitotic cortical neurons. Our substrates supported neuronal attachment, proliferation, and neuronal differentiation. We found that neurons cultured on our hydrogels bifunctionalized with ligand-specific chemistries in a sequential fashion exhibited higher maturation at comparable culture times than using a simultaneous bifunctionalization strategy, displaying a higher number of neurites, branches and dendritic filopodia. These results demonstrate the relevance of

quantitative and optimized coupling chemistries for the performance of simple biomaterials and with sensitive cell types.

1. Introduction

Cell fate is tightly regulated *in vivo* by the coordinated presentation of biochemical cues (tethered ligands and soluble factors) and biophysical cues (like matrix elasticity) over multiple time and length scales.[1, 2] The cooperative effect of such multiple factors in difficult to address on standard tissue culture plates, with poor control over tethered proteins and mechanical properties very different than those of the natural matrix. New materials allowing for a simple and quantitative orchestration of multiple factors are needed to progress in our understanding of the interplay between cells and their surrounding environment.[3]

During the last decade, researchers have recognized the potential of combining different cues on a single model platform to map and instruct cell behavior.[4] Poly(acrylamide) (PAAm) hydrogels have become very popular among biology laboratories as 2D model substrates with defined biochemical and mechanical properties. These hydrogels are cost effective, easy to prepare, reproducible and available in a wide range of stiffness (elastic moduli ~0.1-100kPa).[5-8]

Biofunctionalization of PAAm hydrogels with bioactive ligands (cell adhesive proteins or peptides, growth factors, etc.) in a controlled and functional fashion is not trivial due to the low reactivity of the amide side groups. PAAm hydrogels are frequently functionalized by physical coating of proteins on the surface, either by incubation with the protein solution of by transferring the protein from a pre-functionalized glass coverslip during gel preparation.[9] The latter method typically leads to a higher protein density on the gel surface, and can be further extended to create protein micropatterns.[9, 10] Although these methods have been successfully applied for mechanobiology studies, reproducibility and long-term stability of the coated material during the cell culture experiment is always a critical issue in physically coated PAAm. Covalent binding strategies for functionalization involve hydrogel activation with hydrazine,

periodate, UV or γ-ray, or the use of the photo-reactive bifunctional linker sulfo-SANPAH.[11-13] These coupling strategies involve toxic chemicals, irradiation steps and poor selectivity for binding site, however typically show low reproducibility. Copolymerization of PAAm hydrogels with reactive monomers has also been explored.[6, 14] In this case, the resulting copolymer is expected to keep the biocompatibility and antifouling of PAAm hydrogels, to be incorporated randomly and to do not disturb PAAm polymerization kinetics and final properties. Recently, we reported methylsulfonyl-functionalized PAAm hydrogels by copolymerizing benzothiazole- and oxadiazole- derived methylsulfonyl comonomers with acrylamide (AAm). These hydrogels allowed quantitative covalent coupling of thiol-containing ligands at physiological conditions and low reaction times (minutes)[15] to form a stable bioconjugate. Our strategy outperformed the non-selective sulfo-SANPAH mediated coupling[8, 16, 17] regarding functional and reproducible loading of ligands in a controlled density.

PAAm hydrogels have been used as suitable soft substrates to culture neuronal cells. [18-21] PAAm hydrogels of variable compliance and functionalized with adhesive factors have been applied to differentiate neurons,[18] as platforms for mechanosensing studies of astrocytes,[22] or to modulate the formation and activity of cortical neuronal networks.[23] In order to support attachment of neuronal cells on PAAm hydrogels, either charged ligands like poly-D-lysine (PDL)[24] or integrin-responsive ligands from extracellular matrix (ECM) molecules such as laminin or collagen[18, 25, 26] are typically immobilized on the hydrogel. PDL is a positively charged, non-specific ligand, which is expected to facilitate cell adhesion by electrostatic interactions with the cells membrane.[22, 23] Laminin is a well-known ECM protein with specific sites for neuronal attachment, and is required for proliferation and migration. Incorporation of

PDL or Laminin on PAAm hydrogels has been performed by physisorption[19] or by covalent binding mediated by hydrazinolysis.[12]

Here, we describe a clean and specific method for bifunctional modification of PAAm hydrogels with thiol- and amine-containing biomolecules with controlled densities in an independent, orthogonal manner. By copolymerizing AAm with methylsulfonyl (MS) and acrylic acid (AA) comonomers, we obtain PAAm-AA-MS hydrogels with defined mechanical properties and independently tunable concentrations of two different ligands. The copolymerization did not affect physical properties of PAAm hydrogels such as stiffness, transparency and biocompatibility. The quantitative and selective covalent binding on hydrogels was explored by UV spectroscopy, using amine- and thiol-containing chromophores as probes. With this strategy, we specifically and covalently immobilized polylysine and a laminin-mimetic peptide, and analyzed the effect of binding the two components by different protocols on the maturation of cultured post-mitotic cortical neurons. We found that neurons cultured for 5 days on hydrogels specifically bifunctionalized exhibited high maturation levels, displaying significantly higher number of neurites, branches and dendritic filopodia than cells on hydrogels which were non-specifically functionalized.

The approach presented here is robust, reproducible, and extensible to the study of the differentiation of other types of progenitor cells, since PAAm hydrogels of diverse mechanical properties can be independently loaded with thiol- and amine-containing ligands of very different molecular weight such as short peptides, ECM proteins or biopolymers.

2. Experimental Section

Materials and Methods

2.1. Materials

Methylsulfonyl co-monomer N-(4-(5-(methylsulfonyl)-1,3,4-oxadiazol-2-yl)phenyl)acrylamide (MS) was synthesized as previously reported.[15] The RGD peptides containing chromophores for the quantitative determination of ligand density within the hydrogels were synthesized by using previously reported protocols: cyclo[RGD(DMNPB)fK],[27] cyclo[RGD(DMNPB)fC],[28] cyclo[RGD(coum)fK],[29] and cyclo[RGD(coum)fC. IK-19 peptide (sequence CSRARKQAASIKVAVSADR) and poly-D-lysine (PDL) were obtained from Alfa Aesar. Laminin HiLyte was obtained from Cytoskeleton (Denvor, USA), streptavidin-SH and Thiol-PEG-fluorescein 1kDa from Nanocs Inc (NY, USA), and Atto425-biotin from Sigma Aldrich (Germany). All other chemicals were purchased from Sigma Aldrich and used as received. UV/VIS Spectra were recorded with a Varian Cary 4000 UV/VIS spectrometer (Varian Inc. Palo Alto, USA).

2.2. Preparation of PAAm hydrogel films

PAAm-AA-MS hydrogels were prepared following adapted reported procedures.[6, 15, 30] Briefly, acrylamide (18 wt%) was dissolved in phosphate-buffered saline (PBS) (1 mL), acrylic acid (AA) (1.8 wt%), and N,N-methylene-bis-acrylamide (0.2 wt%) were added and the pH of solution was adjusted to pH 8 by using 0.1M NaOH aq. solution. Methylsulfonyl comonomer (MS) (0.4 wt%) was dissolved in dimethylformamide (DMF) (125 μL) and mixed with the monomer solution. The solution was degassed to remove oxygen and the free radical initiator ammonium persulfate (10% solution, 1/100 of total volume) and *N,N,N′,N′*-tetramethylethylenediamine catalyst (1/1000 of total volume) were added. 10 μL drops of the polymer solution were placed on Sigmacote-coated glass slides and covered with 3-acryloxypropyl-trimethoxysilane (APM) functionalized coverslips. The hydrogel formed

between both glass slides and anchored to APM-coated slides. After 5 min, the hydrogel bound to the APM-coated coverslip was separated from the Sigmacote slide and kept in cold deionized water until further use. Hydrogels prepared this way had a storage modulus (G') of 23 kPa as obtained from dynamic mechanical analysis (DMA) measurement. Control PAAm hydrogels (lacking either AA or MS comonomers) were also prepared by the same method. The hydrogels were dried in a stream of nitrogen before UV measurement. Hydrogels are denoted in the following as PAAm-AA for Poly(acrylamide-*co*-acrylic acid) and PAAm-AA-MS for Poly(acrylamide-*co*-acrylic acid-*co*-methylsulfonyl). The complete characterization of the hydrogels, including measurement of swelling ratio, storage modulus (by DMA), and surface morphology by scanning electron microscopy (SEM) was performed following reported protocols[15] and is fully described in the Supporting Information.

2.3. Peptide coupling to PAAm-AA-MS hydrogels

Coupling of thiol-peptides to hydrogels was done by incubating the hydrogels with a peptide solution of different concentrations (0.25, 0.5, 0.75, 1.0, 1.5, and 2 mg/mL) in PBS for 1 h at room temperature. 20 μL of the peptide solution were placed on a Parafilm surface and covered with the hydrogel. After coupling, the hydrogel was washed with water and with 0.05 M aq. acetic acid solution (pH = 3) to rinse out non-specifically adsorbed peptide, followed by drying under nitrogen stream and measurement of UV absorbance.[15]

Coupling of amine-peptides required prior activation of the -COOH groups of the hydrogel. Incubation of the hydrogel in aqueous solution of 0.2 M N-(3-dimethylaminopropyl)-N′ ethylcarbodiimide hydrochloride (EDC), 0.1 M N-hydroxysuccinimide (NHS), 0.1 M 2-(N-morpho)-ethanesulfonic acid (MES) and 0.5 M NaCl for 15 min, was followed by incubation with peptide solutions of different concentrations (0.25 - 2 mg/mL) in PBS (pH 7.4) for 1 h. The

hydrogel was washed with water and 0.05 M aq. acetic acid solution (pH = 3) to rinse out non-specifically adsorbed peptide, followed by measurement of UV absorbance in dry state.

Orthogonal sequential coupling of thiol- and amine-containing peptides: It was carried out by using cRGD(coum)fC and cRGD(DMNPB)fK(1mgmL^{-1}) as probes. For testing thiol/amine coupling pathway, hydrogel was functionalized with cRGD(coum)fC followed by washing and measurement of UV absorbance. Subsequently, on the same hydrogel, carboxylic groups were activated by EDC/NHS followed by coupling with cRGD(DMNPB)fK and UV measurement. The amine/thiol route was also tested by first coupling amine peptides using EDC/NHS activation and followed by coupling of thiol peptide.

Simultaneous orthogonal coupling of thiol- and amine- peptides: To this end, PAAm-AA-MS hydrogel was first activated with EDC/NHS and then incubated with a 1:1 mixture of 1mg/mL cRGD(coum)fC and cRGD(DMNPB) aqueous solution for 1h, followed by washing and measurement of UV spectra.

2.4. Determination of ligand binding efficiency to hydrogels

The concentration *C* of ligand coupled to hydrogels was estimated from the UV absorbance *A* using the Lambert-Beer law

$$C = \frac{A}{l\varepsilon}$$

where the path length *l*= swollen thickness of the hydrogel, ε_{max} from *coum* chromophore (20000M^{-1}cm^{-1} at λ_{max} 390 nm).[29] The binding efficiency was the ratio between initial concentration of the incubation solution and the final concentration on hydrogel.

2.5. Functionalization of hydrogels with PDL and IK-19

For preparation of substrates modified with of PDL alone (0.01 mgmL^{-1}) and *simultaneous* IK-19/PDL (0.1/0.01 mgmL^{-1} premixed solution), PAAm-AA-MS hydrogels (G'=23 kPa) were first

activated by 0.2 M EDC, 0.1 M NHS, 0.1 M 2-(N-morpho)-ethanesulfonic acid and 0.5 M NaCl for 15 min, followed by coupling with peptide(s) solution. PAAm-AA-MS hydrogels functionalized with IK-19 alone (0.01 $mgmL^{-1}$) were modified by incubating the hydrogel for 1 h at room temperature with peptide solution, followed by washing and sterilization with ethanol.

Hydrogels functionalized with PDL and IK-19 by the *sequential* procedure were prepared as follows. PAAm-AA-MS hydrogels were first activated with EDC/NHS as explained above, followed by coupling with PDL solution (0.01 $mgmL^{-1}$) and rinsing. Subsequently, the modified hydrogel was incubated with IK-19 solution (0.01 $mgmL^{-1}$) for 1 h at room temperature, rinsed and sterilized with ethanol. All peptide solutions were prepared in PBS.

2.6. Functionalization of glass substrates with an IK-19/PDL mixture (0.1/0.01 $mgmL^{-1}$*) as control*

Glass substrates were incubated with 1% 3-aminopropyl-triethoxysilane solution (in 95% ethanol) for 15 min followed by washing with ethanol and baking for 30 min at 90 ºC. The glasses were activated with 8% aq. glutaraldehyde solution for 6 h, washed with water and incubated overnight with IK-19/PDL (0.1/0.01 $mgmL^{-1}$) solution. Glass substrates were washed with water and sterilized with ethanol.

2.7. Cell culture

Cerebral cortex from E14.5 of C57BL/6 mice was digested in 0.5% trypsin EDTA (GIBCO) for 15 min at 37 ºC. Then trypsin was inactivated by the plating medium (DMEM (GIBCO) and 10% FBS (Hyclone)) and gently triturated with a 5-ml disposable pipette to get single cells. Centrifugation at 1000 rpm was performed for 5 min. Cell pellets were re-suspended in 1 ml differentiating medium (low glucose DMEM/F12 (GIBCO) and 2% B27(Invitrogen)) and cell

number was counted. The hydrogel samples having different bound biomolecules were placed in 24-well cell culture plates with 2.6 x $10^4/cm^2$ cells. Cell growth was followed by regular intervals and cells were fixed after 5 days.

2.8. Immunostaining

Post-mitotic cortical neurons (CNs) were fixed with 4% Paraformaldehyde (PFA) solution for 10 min, washed 3 times with PBS pH 7.4, blocked and permeabilized with 2% bovine serum albumin (BSA) and 0.2% Triton in PBS for 45 min at room temperature. The cells were incubated at room temperature with (1:700 DCX (guinea pig, Neuronal lineage marker, dianova) and 1:1000 SMI-312 (Anti Pan-axonal Neurofilament mouse IgG1, Biolegend)) for 2h. Cells were washed 3 times with PBS followed by incubation for 2h at room temperature with (1:800 Cy3 anti-guinea pig and 1:1000 alexa-488 anti-mouse). The nucleus was stained by DAPI and mounted by following standard protocols. Images were taken with Zeiss Axio Observer microscope at 0.22 and 0.42 μm and per px. Time lapse videos were recorded in order to calculate cell viability and proliferation. The length of axon was measured using FIJI software. The number of branches and process and spines were quantified manually.

2.9 Statistical Analysis

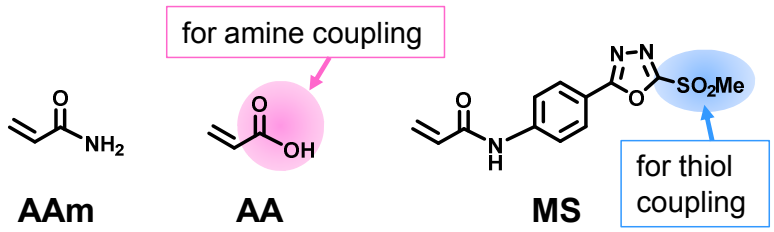
Data were expressed as mean ± standard deviation. For each condition, a minimum of three independent experiments were performed. Sample size was bigger than 50 measurements for all experiments. In all cases a value of $\alpha < 0.05$ was used for statistical significance. A one-way ANOVA with a Tukey test of the variance was used to determine the statistical significance between groups. In all cases we have compared the *sequential* coupling condition against all others, and significance difference was set to * $\alpha< 0.05$, ** $\alpha< 0.01$, *** $\alpha< 0.001$.

3. Results and Discussion

PAAm-AA and PAAm-AA-MS hydrogels were prepared by free radical copolymerization of acrylamide (AAm), acrylic acid (AA) and methylsulfonyl acrylate (MS) as comonomers, and bis-acrylamide (bisAAm) as crosslinker. MS and AA were incorporated in 2.2 and 8.8 wt% to the monomer mixture respectively. The amount of MS comonomer used is limited by solubility reasons, while an AA percentage close to 10% has proved successful elsewhere.[6] These functional groups allow specific bioconjugation of thiol- and amine- containing biomolecules to the hydrogel.

The introduction of a comonomer in a radical polymerization reaction can influence the polymerization kinetics and the final properties of the PAAm hydrogel. In order to test the effect of the comonomer in PAAm, we first copolymerized PAAm with AA and MS mixtures to form linear copolymers and compare their properties with the homopolymer PAAm (Table 1). Linear PAAm-AA copolymers with 8.8% AA showed similar molecular weight and polydispersity to PAAm. PAAm-AA derived hydrogels (with crosslinker) showed higher swelling and slightly lower storage modulus than PAAm parent hydrogels. SEM studies of freeze dried samples showed no significance differences in the morphology of homo- vs copolymer (see Supporting Information). Similar studies on PAAm-MS copolymers (2.2 wt% MS) showed no noticeable differences in molecular weight, swelling, storage modulus or morphology to PAAm.[15] When the three comonomers were mixed to prepare PAAm-AA-MS, the resulting copolymer showed swelling properties similar to PAAm-AA. In summary, MS and AA comonomers are incorporated in the AAm chain without affecting the polymerization reaction. The copolymerization of AA leads to higher water uptake in the hydrogel as a consequence of the ionic character of the -COOH side group of AA.

Table 1. Chemical structure of PAAm-AA-MS constituting comonomers, molecular weight and polydispersity of linear (co)polymers, and swelling ratio and Storage Modulus of derived hydrogels.



Polymer	Linear		Hydrogel	
	Mn	Polydispersity (PD)	Swelling Ratio [mass water/mass polymer]	Storage Modulus G' [kPa]
PAAm-AA	79320	2.43	23.0±0.2	25.6±1.3
PAAm-AA-MS	60867	2.56	21.0±0.5	22.5±2.3
PAAm-MS	69852 [a]	2.38 [a]	6.3±0.3	22.9±2.1
PAAm[15]	75194 [a]	2.30 [a]	5.6±0.5	24.4±1.1

[a]: values taken from ref [15]

We subsequently investigated the specific and selective conjugation of thiol- and amine-containing bioligands to PAAm-AA-MS hydrogels. In order to quantify ligand concentration in the hydrogel, we used ligands containing a chromophore and measured UV spectroscopy on the modified hydrogels. We selected cell-adhesive cyclic RGD peptides containing 7-(N,N-diethylamino)-4-(hydroxymethyl)-coumarin (*coum*) group as chromophore. Coum presents a

major absorption UV band at around 390 nm.[29] The cyclic peptides c[RGD(coum)fC] and c[RGD(coum)fK] present either cysteine or lysine as probes for thiol and amine coupling. Coupling of increasing concentrations of c[RGD(coum)fC] to PAAm-AA-MS at room temperature for 1h caused an increase in UV absorbance at λ≈390 nm, indicating effective and concentration-dependent loading of the thiol-containing ligand on the hydrogel (**Figure 1**a). Coupling of c[RGD(coum)fK] to PAAm-AA-MS hydrogels at room temperature for 1h after EDC/NHS activation was also successful (Fig.1b), though achieved coupling densities were lower than for c[RGD(coum)fC] at comparable coupling conditions. Longer incubation times (up to 24h) lead to an increase in peptide loading in both cases (Fig.1c-d). This effect was more pronounced in amine coupling. Two scenarios might explain this result: the kinetics of thiol binding is faster than amine at our coupling conditions (Fig S4), or the binding of amine ligands to NHS-activated carboxylic groups is hindered because of the observed decreased swelling of PAAm-AA-MS gels after NHS activation (Table S1).[31] Thiol coupling to PAAm-MS hydrogels had an efficiency of >90% after 1h,[15] whereas thiol coupling to PAAm-AA-MS hydrogels was found to be slower (Fig. 1c) and less efficient. The reason for the negative effect of the AA units in the coupling efficiency of thiols to PAAm-AA-MS hydrogels remains unclear at this point. Note that both ligands were coupled at pH 7.4. Other working pHs might lead to higher coupled ligand densities and will need to be optimized for each target ligand.[32]

To prove the chemoselectivity in the binding reaction, control experiments were performed. PAAm-AA hydrogels (lacking MS) did not show any absorbance after EDC/NHS activation and incubation with thiol-containing ligand. Similarly, no amine coupling was seen on PAAm-AA-MS hydrogels without EDC/NHS activation (Figure S3, Supporting Information). Overall, these

results show that thiol and amine ligands can be specifically bound to our hydrogels in controllable densities.

We analyzed and compared the swelling properties of PAAm-AA-MS hydrogels in PBS after the different coupling steps by gravimetry. EDC/NHS treatment of PAAm-AA-MS hydrogels resulted in a reduced swelling to values comparable to PAAm-MS gels (lacking AA). The esterification of carboxylic groups during EDC/NHS activation step reduced the density of free ionizable -COOH side groups in the hydrogel and lead to lower swelling ratios. NHS activated PAAm-AA-MS gels maintained in PBS for 24h recovered 35% on the initial swelling before activation, presumably as a consequence of partial hydrolysis of NHS groups and recovery of -COOH groups. Control PAAm-AA-MS gels first equilibrated in MES buffer (without EDC/NHS) and then equilibrated in PBS recovered 73% of the initial swelling (Table S1). This result indicates that the pH change from PBS to MES buffer might cause partial, irreversible collapse of the hydrogel. We also conducted confocal microscopy measurements of the thickness of fluorescently labeled hydrogel films and compared them with the swelling analysis of bulk samples (Table S2 and Figure S6, Supporting Information). NHS-activated PAAm-AA-MS thin films showed a 3-fold decrease in thickness, from 205 μm to 63 μm, after NHS activation, in agreement with the measurements on bulk samples. The fluorescence signal was homogeneously distributed along the gel thickness, indicating that bound fluorescent ligands were uniformly distributed across the hydrogel.

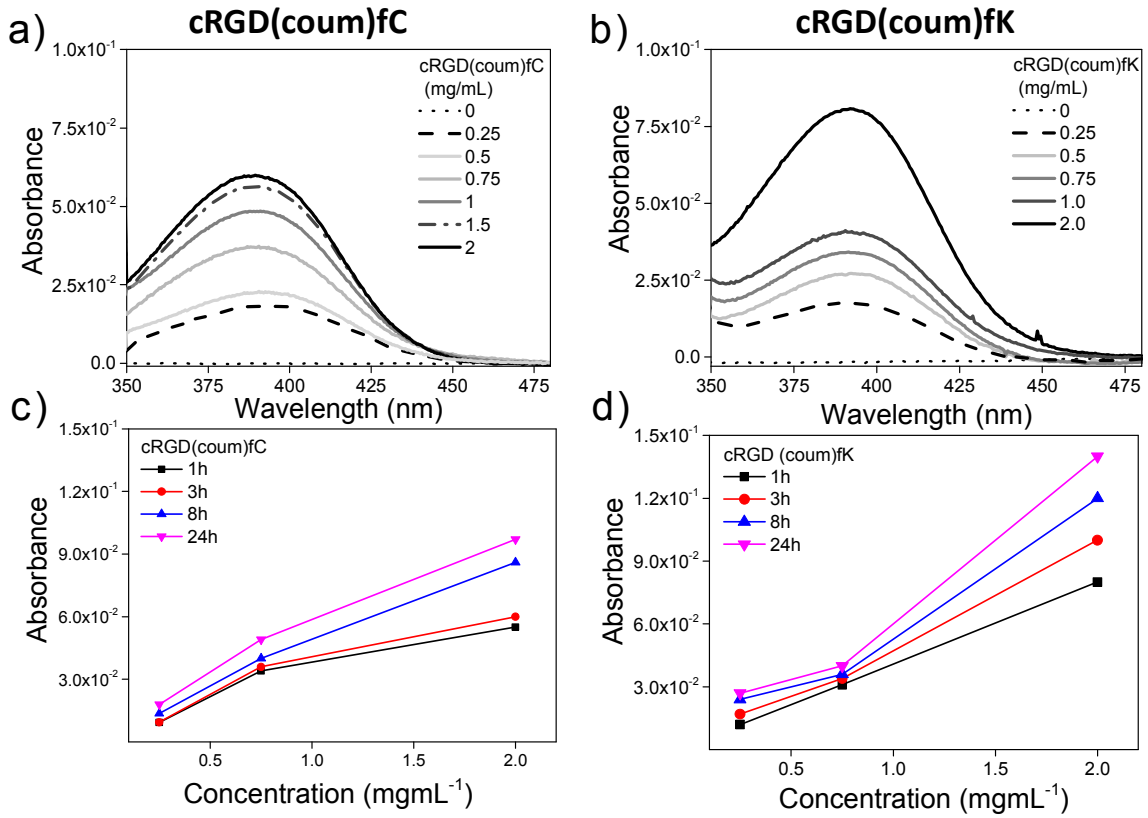


Figure 1. UV spectra of PAAm-AA-MS hydrogels after coupling cRGD(coum)fC (left) or cRGD(coum)fK (right) at increasing peptide solution concentrations at room temperature. a-b) coupling for 1h, c-d) coupling at increasing coupling times (1 to 24h, λ_{max}= 390 nm). Note that there are 9% of –COOH groups for amine binding vs 2% of MS groups for thiol binding on the hydrogels.

We then explored the orthogonal conjugation of thiol and amine biomolecules to the same hydrogel in two sequential incubation steps (**Figure 2**). In order to follow individual binding to the two different comonomers, we used RGD peptides containing two different chromophores: cRGD(coum)fC (λ_{max} ca 390 nm, ε_{390}= 20000$M^{-1}cm^{-1}$) and cRGD(DMNPB)fK, (λ_{max} ca 360 nm, ε_{360}= 4100$M^{-1}cm^{-1}$). Two different sequential procedures were performed. In one case, thiol was coupled first followed by amine coupling (Fig.2, orange curve). In the second case, the ligands

were coupled in the inverse order (green curve on Fig.2). After incubation and washing, the modified hydrogels showed a clear UV absorption band at λ_{max} 380-385 nm, resulting from the overlap of the two chromophores and demonstrating that both peptides can be effectively linked to the same bifunctional hydrogel. The coupling density depended on the order of the coupling sequence. The first coupling step was ca twice more efficient than the second. The possibility of simultaneous biofunctionalization was also tested by incubating EDC/NHS activated hydrogels with a 1:1 pre-mixed solution of both peptides (Fig.2, violet curve). In this case, thiol coupling efficiency was similar to the one observed in the thiol-amine sequence, but amine coupling efficiency dropped significantly.

In summary, these results demonstrate that PAAm-AA-MS hydrogels allow effective and selective functionalization with amine- and thiol-containing molecules at tunable concentrations by changing the incubation conditions and protocols. The modified hydrogels retain the low unspecific binding of PAAm hydrogels.

We also tested the bifunctionalization of our gels with larger proteins. We first bound Hilyte-labeled laminin (Mw~ 850 kDa) by amine coupling. Absorption at λ_{max} 590 nm proved successful coupling (see Figure S5 in the Supporting Information). Subsequently, we bound streptavidin (75 kDa) through thiol coupling, followed by incubation with biotin-Atto (0.71 kDa), which resulted in a noticeable increase in absorption at λ_{max} 425 nm. This result shows that our gels can support functionalization with larger ligands such as ECM components. We have recently reported the binding of rhodamine-labeled fibronectin (Mw~440 kDa) to PAAm-MS gels.[15]

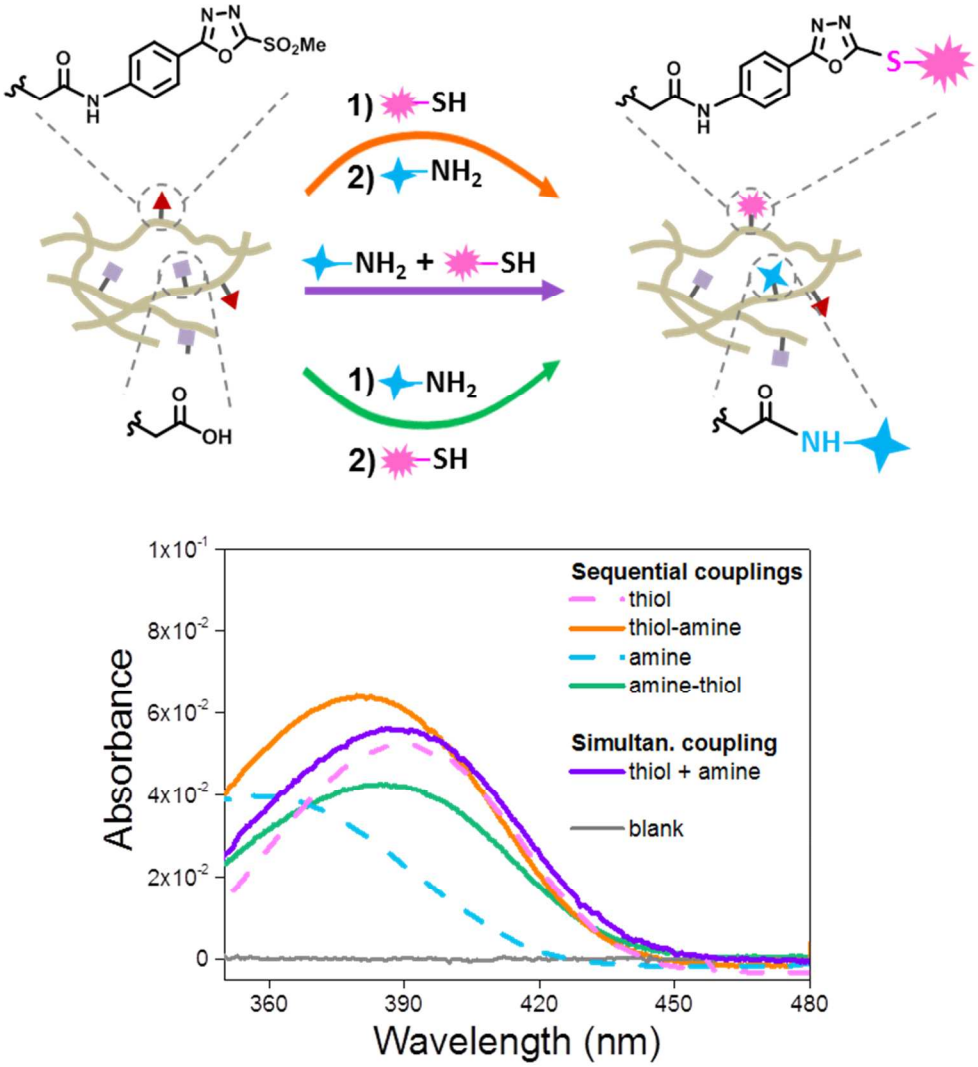


Figure 2. Reaction scheme and UV spectra of PAAm-AA-MS hydrogels after sequential incubation first with thiol- and then with amine- containing molecules at 1 mg/mL concentration for 1h at room temperature (orange curve) or in the inverse order (green curve). The simultaneous coupling of the two molecules was also tested (violet curve). The thiol probe was cRGD(coum)fC (λ_{max} ca 390 nm) and the amine probe was cRGD(DMNPB)fK (λ_{max} ca 360 nm).

Having demonstrated the ability of our substrates to couple thiol- or amine-presenting molecules orthogonally, we subsequently tested their performance as cell culture platforms

presenting two adhesive molecules. As a proof of principle, we decided to study the maturation of postmitotic cortical newborn neurons on PDL and laminin adhesive coatings. PDL is used in neuronal cultures for attachment of neurons through electrostatic interactions. Laminin is an abundant ECM protein in brain tissue; it improves spreading and growth of cultured neurons[33] and promotes extension of neurites[34]. These are desirable features for enhancing neuron maturation. The commercially available IK-19 peptide (sequence CSRARKQAASIKVAVSADR) is a short mimetic peptide of adhesive site on laminin alpha chain. This peptide stimulates neurite growth, branching and maturation by interaction with integrin receptors such as $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_6\beta_1$[35] and is frequently used for neuronal cultures as replacement for laminin. In our studies, we used the cysteine containing IK-19 peptide to be coupled to the MS comonomer of PAAm-AA-MS hydrogels, and we coupled PDL to the EDC/NHS-activated AA comonomer.

We functionalized PAAm-AA-MS hydrogels in different ways (see **Table 2**): with PDL, with IK-19, with both components sequentially or with a mixture of both components (PDL+IK-19). For comparison, cells were also seeded on standard glass substrates modified by a mixture of PDL+IK-19 (denoted as *mixture glass*) by using glutaraldehyde as bifunctional cross-linker, which reacts with amine on both functionalized glass and on the molecules. For this study we chose to work with hydrogels of G´= 23 kPa, since they are easier to handle and quantify than softer hydrogels (~0.2 kPa) and yet stiff enough[36] to decouple the effect of stiffness over neuronal cell fate and allow for direct comparison with classical glass substrates.

Table 2. Details of the bifunctionalization of PAAm-AA-MS hydrogels with PDL and IK-19 peptide.

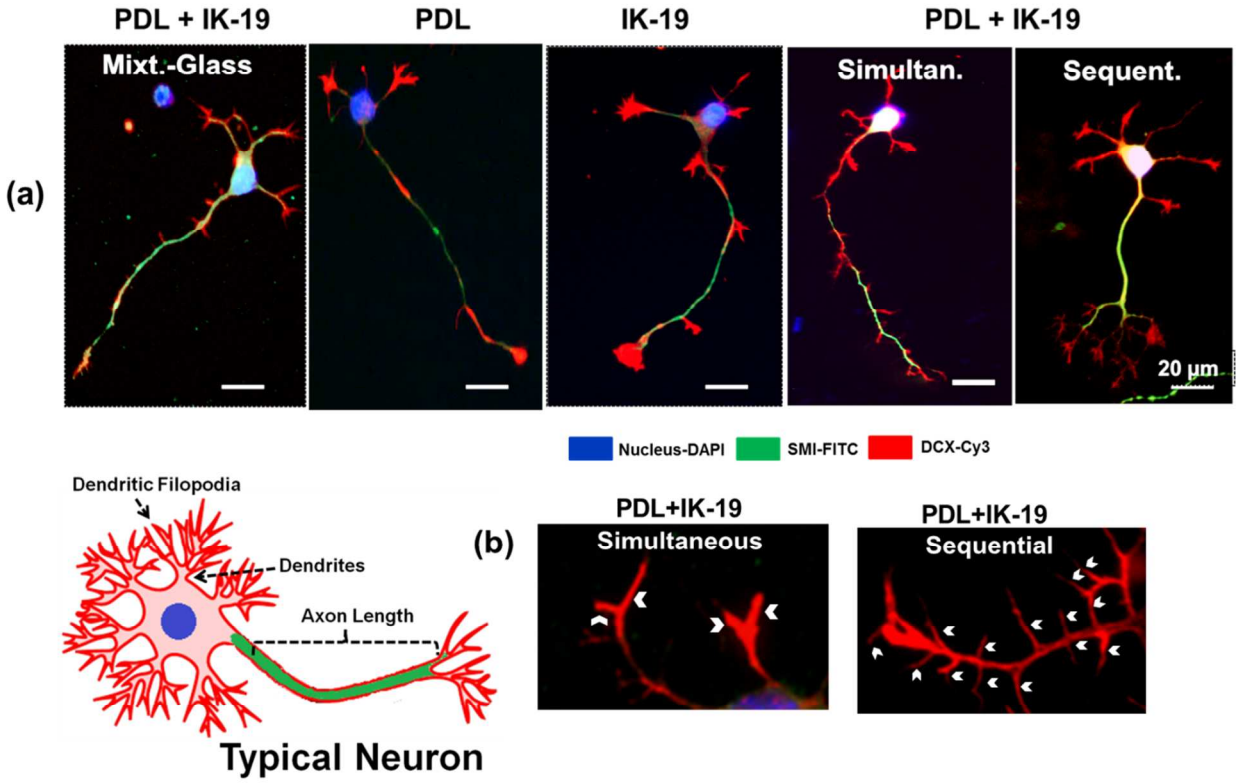
Substrate	**Bifunctionalization Strategy**			**Experiment denoted as**
		coupled molecule(s)		
	pre-activation	PDL	IK-19	
PAAm-AA-MS hydrogels	EDC/NHS	Yes	no	*PDL*
	none	No	yes	*IK-19*
	EDC/NHS	yes both molecules at once	yes	*Simultaneous*
	EDC/NHS	yes (first)	yes (second)	*Sequential*
Glass (control)	silanization and glutaraldehyde	yes both molecules at once	yes	*Mixture Glass*

Post-mitotic cortical neurons (CNs) from mouse embryo (E-14.5) were seeded on the functionalized hydrogels and cultured for 5 days. No significant differences in cell viability were detected among the different conditions after 24h of culture. However, cells cultured on our copolymer hydrogels showed a significant improvement of cell survival (23%) after 5 days (Figure S7). We quantified cell maturation by measuring the average number of neurites, the axonal length, the neurite branching and the developed dendritic filopodia after 5 days. All functionalized PAAm-AA-MS hydrogels supported attachment of CNs and promoted the growth of neurites, branches and dendritic filopodia after 5 days (**Figure 3a**). No significant differences in the axonal length were found among the different substrates. The presence of IK-19 increased the number of neurites and branches. *Simultaneous* coupling of and IK-19 and PDL did not improve neurite formation, branching or formation of dendritic filopodia (see SI). However,

bifunctionalization of PAAm-AA-MS hydrogels by *sequential* coupling of PDL and IK-19 using our selective coupling strategy, lead to a significant enhancement of dendritic filopodia formation (3-fold) (Figure 3b, 3c). Dendritic filopodia are the morphological precursors of neuronal spines,[37] which are required for neuronal synapses and enhance neuronal complexity.[38]

These results show that the coupling strategy for biofunctionalization of PAAm hydrogels has a relevant impact on cell culture. We attribute the improved performance of sequential PDL/IK-19 coupling for neuronal maturation to two reasons: a) the coupling of short peptide is not hampered by the big PDL molecule, leading to higher IK-19 densities, and b) the bound IK-19 retains a higher activity due to the chemoselective immobilization through its terminal thiol group. Note that IK-19 peptide contains two lysine rests in the chain, one of them at the IKVAV epitope, and thus its non-selective immobilization via those amine side groups during *simultaneous* coupling may be responsible for an impaired activity of the short laminin peptide. This highlights the value of our orthogonal coupling regarding bioactivity retention of chemoselectively bound ligands.

It is generally accepted that neuronal growth and differentiation is favored on soft substrates with elasticity modulus in sub-kPa range.[20] We therefore extended our study to 0.17kPa hydrogels bifunctionalized with PDL+IK-19 components (Figure S8). As expected, softer substrates supported neuron growth and differentiation, showing slightly increased axon length (~13%) and number of neurites (~17%) compared to stiffer hydrogels. We found significantly longer axons and higher number of neurites for cells cultured on *sequential* substrates in comparison to *simultaneous* coupling, but no significant differences in number of branches. These results show that our strategy is robust and easily extensible to PAAm hydrogels with diverse mechanical properties.


PDL + IK-19
PDL
IK-19
PDL + IK-19
Mixt.-Glass
Simultan.
Sequent.
(a)
20 μm
Nucleus-DAPI
SMI-FITC
DCX-Cy3
Dendritic Filopodia
Dendrites
Axon Length
Typical Neuron
(b)
PDL+IK-19
Simultaneous
PDL+IK-19
Sequential

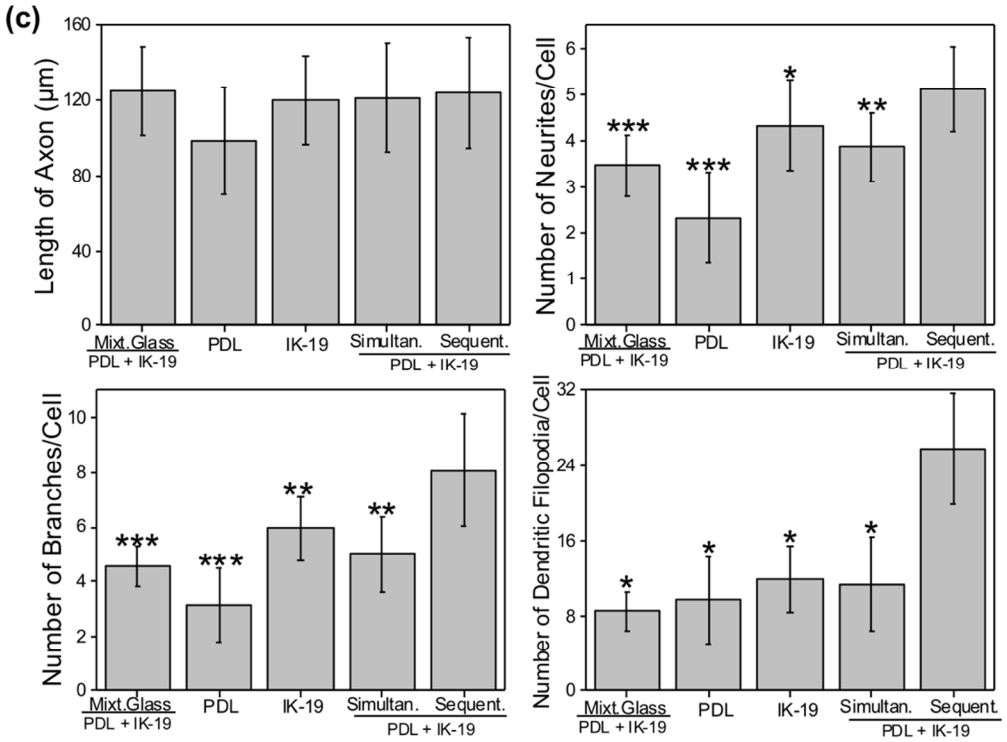

(c)
Length of Axon (μm)
Number of Neurites/Cell
Number of Branches/Cell
Number of Dendritic Filopodia/Cell
Mixt.Glass
PDL + IK-19
PDL
IK-19
Simultan.
Sequent.
PDL + IK-19

Figure 3. Post-mitotic cortical neurons (CNs) from mouse embryo (E-14.5) culture after 5 days seeding on PAAm-AA-MS hydrogels. (a) Representative pictures of single neuron developed under different scenarios. (b) High magnification images depicting distributions of dendritic filopodia on neuronal process. Arrows show the type of counted filopodia. (c) Quantification of axonal length, amount of neurites, branches and dendritic filopodia developed in newborn neurons after 5 DIV. Significant differences showed in the graphics are compared against *sequential* substrate.

4. Conclusions

We have demonstrated that bifunctional PAAm-AA-MS hydrogels can couple molecules containing amine and thiol groups with high chemical selectivity, efficiency and functionality. These properties allowed enhanced maturation of neuronal cultures on PAAm hydrogels. This hydrogel platform can be expanded to other adherent molecules. Our approach is robust, reproducible, and extensible to the study of adhesive factors for the differentiation of other types of progenitor cells. PAAm-AA-MS hydrogels of diverse mechanical properties can be independently loaded with thiol- and amine-containing ligands of very different molecular weight such as short peptides, ECM proteins or biopolymers.

Associated content

Supporting Information

Detailed physicochemical characterization of hydrogels and supporting UV spectroscopy, swelling ratio, confocal microscopy, and cell culture results.

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Notes

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Bifunctional poly(acrylamide) hydrogels through orthogonal coupling chemistries

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