Hydrogels

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Bioconjugating Thiols to Poly(acrylamide) Gels for Cell Culture Using Methylsulfonyl Co-monomers

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Abstract: *Poly(acrylamide) P(AAm) gels have become relevant model substrates to study cell response to the mechanical and biochemical properties of the cellular microenvironment. However, current bioconjugation strategies to functionalize P(AAm) gels, mainly using photoinduced arylazide coupling, show poor specificity and hinder conclusive studies of material properties and cellular responses. We describe methylsulfonyl-containing P(AAm) hydrogels for cell culture. These gels allow easy, specific and functional covalent coupling of thiol containing bioligands in tunable concentrations under physiological conditions, while retaining the same swelling, porosity, cytocompatibility, and low protein adsorption of P(AAm) gels. These materials allow quantitative and standardized studies of cell-materials interactions with P(AAm) gels.*

Poly(acrylamide) P(AAm) gels have become relevant model substrates to study cell responses to the biochemical and mechanical properties of the cellular microenvironment.[1] P(AAm) gels can be easily prepared with inexpensive lab supplies. They are compatible with high-resolution microscopy and allow cell–force measurements through traction–force microscopy and micromanipulation. P(AAm) gels with defined stiffness and porosity are easily obtained by polymerizing acrylamide with variable amounts of bis-acrylamide crosslinker. The resulting gels show negligible interactions with proteins, and are used for ligand-specific studies of cell-materials communication after functionalization with cell adhesive proteins or peptides. Biofunctionalization of P(AAm) gels for cell culture is typically achieved by covalent coupling of the ligand to the gel using the photoreactive linker sulfo-SANPAH (sulfosuccinimidyl-6-(4′-azido-2′-nitrophenylamino) hexanoate).[1] Upon irradiation, the aryl-azide group of sulfo-SANPAH is activated to highly reactive intermediates that insert sulfo-SANPAH into the P(AAm) backbone through non-specific chemical pathways.[2] The lateral NHS-ester of the P(AAm)-attached sulfo-SANPAH is expected to react with amine groups on the bioligands. Although widespread, the sulfo-SANPAH linker offers very poor control over the immobilization sites, both at the material and at the bioligand. Photoinduced arylazide reactions lack specificity (that is, they react with almost any type of bond) and amine groups are ubiquitous in biomolecules. This can lead to nonfunctional immobilization of ligands and, consequently, to poor reproducibility in the following cell experiments, a frequent experience among sulfo-SANPAH users, or discrepancies in the data among different labs.[1a,3] Alternative solutions for specific and easy biofunctionalization of P(AAm) gels are required, though difficult to realize owing to the low chemical reactivity of the amide side-group of P(AAm).

Functionalization of biomaterials by thiol-derivatized ligands allows significantly higher site-specificity than by amine groups. Moreover, thiol labeling is widely represented among commercially available bioligands. To couple thiol-functionalized molecules to P(AAm), thiol-reactive monomers are required as part of the P(AAm) chain. Different chemistries are established for thiol bioconjugation: thiol–maleimide, thiol–ene, thiol–yne, thiol–vinylsulfone, and thiol–pyridyl disulfide.[4] However, most of these thiol-reactive groups contain unsaturated bonds which interfere with the radical polymerization of the AAm matrix when included into a co-monomer structure. Recently, methylsulfonyl heteroaromatic derivatives have been reported as promising reagents for thiol bioconjugation by nucleophilic aromatic substitution (Figure 1).[5] The coupling reaction is fast (a few minutes), it occurs with high yields under mild conditions (PBS buffer, room temperature, no UV light), and the adduct formed is more stable to hydrolysis than thiol–maleimide adducts.[5] Methylsulfonyl derivatives are not expected to interfere with the radical polymerization and, therefore, they are excellent candidates as co-monomers for thiol coupling to P(AAm). Herein, we describe methylsulfonyl-derivatized P(AAm) hydrogels for cell culture, able to be specifically functionalized with thiol-containing biomolecules by simple copolymerization of <2 mol% of phenyl oxadiazole methylsulfone acrylate **1** or benzothiazol methylsulfone acrylate **2** with the AAm monomer and *N*,*N*-methylene-bis-acrylamide crosslinker. The gels P(AAm-*co*-**1**) and P(AAm-*co*-**2**) (Figure 1) allow quantitative, specific, reproducible, and stable covalent coupling of thiol-containing bioligands in tunable concentrations under physiological conditions, and their specific recognition by cells in culture. Furthermore, the introduction of <2 mol% co-monomer in the gel composition does not modify the physical properties (swelling, mechanical properties, low protein absorption, morphology)

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Supporting information for this article, including detailed information on the synthesis of the monomers, sample preparation, and measuring procedures, is available on the WWW under http://dx.doi.org/10.1002/anie.201509986.

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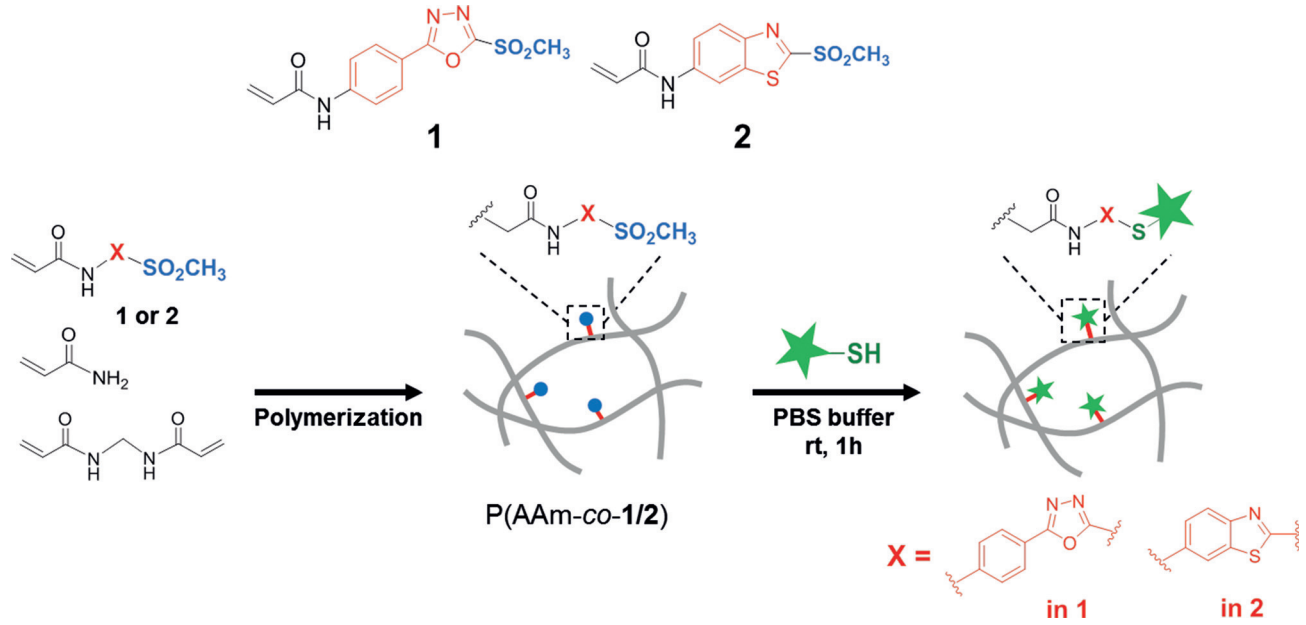

Figure 1. P(AAm)-methylsulfone hydrogels P(AAm-*co*-**1**) and P(AAm-*co*-**2**) used for thiol-bioconjugation.

of the P(AAm) homopolymer. Cell studies demonstrate the superiority of the methylsulfonyl P(AAm) gels for quantitative and standardized studies of cell-materials interactions, and the inherent limitations of sulfo-SANPAH coupling for such studies.

We selected the phenyl oxadiazole methylsulfone and benzothiazol methylsulfone as thiol-reactive groups (Figure 1). According to reported work, these units show the fastest reaction kinetics and form the most stable adducts with thiols.[5a] The acrylate monomers **1** and **2** containing the methylsulfone derivatives as side groups were synthesized in gram scale with high purity and good yields starting from inexpensive chemicals (Figure 2; Supporting Information).

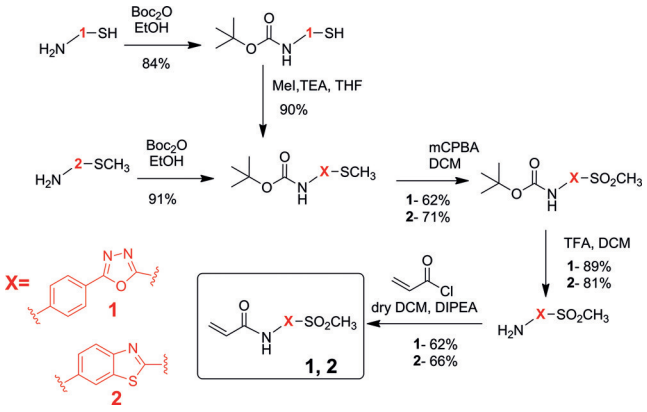

Figure 2. Reaction pathway for the synthesis of methylsulfone acrylates.

The monomers showed acceptable solubility in water (2 mg mL^{-1}) and remained stable over months in the fridge as a solid or in DMF solution. Mixtures of AAm and 2 mol% of monomers **1** or **2** were copolymerized to obtain P(AAm-*co*-**1**) and P(AAm-*co*-**2**) copolymers. GPC analysis of linear polymerization products showed comparable values of molecular weight and polydispersity in P(AAm) and the copolymers (Supporting Information, Table S1), demonstrating that methylsulfone monomers do not interfere with the radical polymerization reaction. The insertion of the comonomers **1** and **2** in the acrylic network was corroborated by the UV absorbance bands of the corresponding chromophore in the UV spectra of thin films of P(AAm-*co*-**1**) and P(AAm-*co*-**2**) gels prepared with bisacrylamide crosslinker (Figure 3 A).

We evaluated the swelling degree, stiffness, and porosity of the P(AAm-*co*-**1**) and P(AAm-*co*-**2**) gels and compared them with P(AAm) gels. No significant differences were observed in any of these parameters between P(AAm) and the methylsulfonyl copolymers (Table S1). SEM imaging of the cryo-dried hydrogels showed comparable ultrastructure and pore size distribution in the homo- and copolymers (Figure S7).[1a] These results demonstrate that the introduction of the comonomers **1** or **2** in the P(AAm) chain does not affect the morphology and hydration of the PAAm backbone, which are relevant parameters for the cell culture experiments.[1a]

The reactivity of the methylsulfone side groups in the gel was tested by incubating the gels with the peptide c[RGD-(DMNPB)fC].[6] This molecule contains the cell adhesive sequence c[RGDfC] with a thiol group at the Cys residue for coupling to the gel, and the 3-(4,5-dimethoxy-2-nitrophenyl)-2-butyl ester (DMNPB) chromophore for quantification of the coupling density by UV spectroscopy. Coupling was performed in PBS for 1 h at room temperature. UV analysis of the P(AAm-*co*-**1**) and P(AAm-*co*-**2**) gels after incubation with c[RGD(DMNPB)fC] showed the absorbance band at 350 nm of the DMNPB chromophore (Figure 3 B, C), indicating successful coupling of the c[RGD(DMNPB)fC] molecule to the gels. Variation of the concentration of c[RGD-(DMNPB)fC] in the incubation solution allowed fine tuning of the peptide loading in the gel. The calculated coupling

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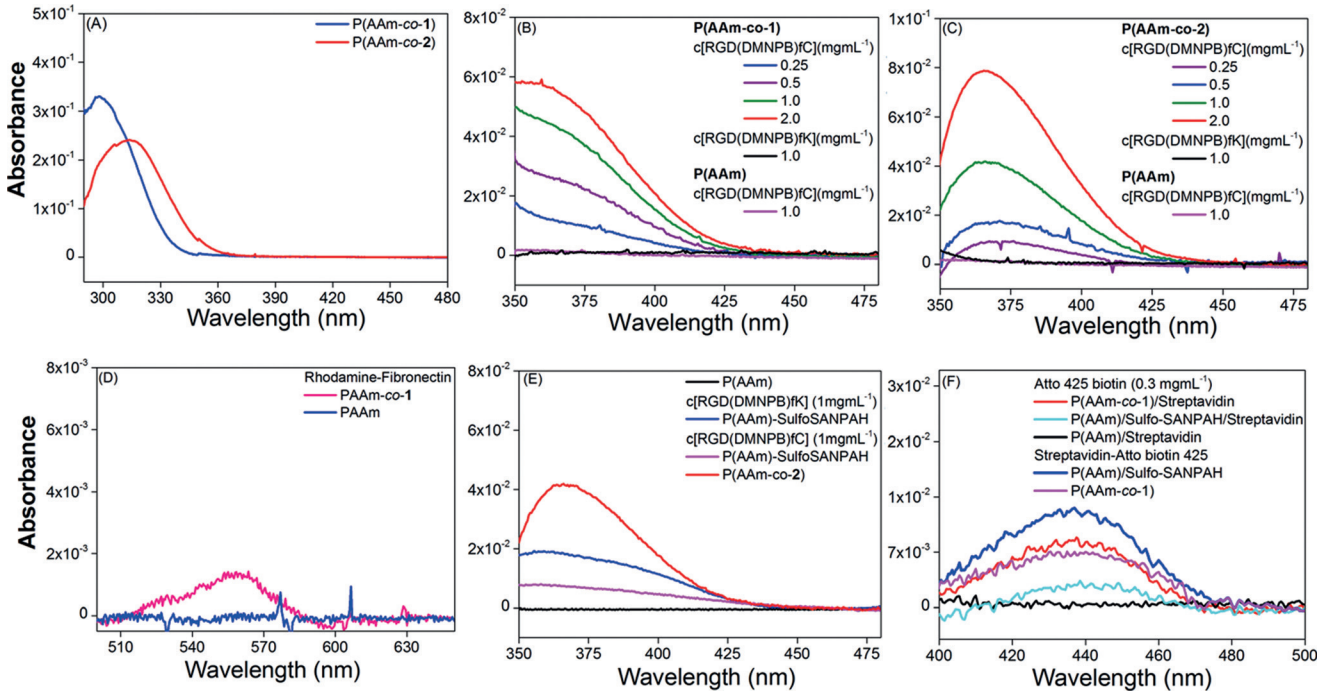

Figure 3. UV/Vis spectra of films of A) P(AAm-co-**1**) and P(AAm-*co*-**2**) gels (30 μm dry thickness, 70 μm swollen thickness) supported on a quartz slide. B) P(AAm-*co*-**1**) and C) P(AAm-*co*-**2**) gels after incubation with increasing concentrations of c[RGD(DMNPB)fC] solutions (0.25–2 mg mL^{-1}) during 1 h and washing, control experiments with P(AAm) gel and c[RGD(DMNPB)fK] peptide at 1 mg mL^{-1}; D) P(AAm-*co*-**1**) gel after incubation with Rhodamine labeled fibronectin (1 mg mL^{-1}); E) P(AAm)/sulfo-SANPAH and P(AAm-*co*-**2**) gels incubated with c[RGD(DMNPB)fC] and c[RGD(DMNPB)fK] respectively for 1 h and washing; F) P(AAm)/sulfo-SANPAH and P(AAm-*co*-**1**) gels first functionalized with streptavidin followed by Atto 425 biotin complexation, or gels after coupling of preformed streptavidin-Atto-biotin 425 complex.

efficiency of the peptide in the gel was >90%, similar to reported coupling yields in solution,[5] indicating that the gel architecture did not hinder the chemical reaction. Incubation times longer than 1 hour did not lead to higher coupling densities (Figure S9). The maximum coupling efficiency was observed at pH 7.4 (Figure S10), in agreement with the reported reactivity of methylsulfones in solution.[5a] No drop in the UV absorbance of the chromophore was observed after keeping the gels in water for 6 weeks, corroborating the high stability of the adduct. Stability tests in cell culture medium in the presence of serum for 10 days also demonstrated better stability of the methylsulfone adducts versus sulfo-SANPAH- or EDC/NHS-based coupling strategies.

Several control experiments were performed to demonstrate the specificity of the binding step. P(AAm) gels incubated with c[RGD(DMNPB)fC] did not show any absorbance (Figure 3B,C), demonstrating that immobilization of the thiol derivative was solely mediated by the presence of the methylsulfone monomers in the copolymer gels. P(AAm-*co*-**1**) and P(AAm-*co*-**2**) gels incubated with c[RGD(DMNPB)fK], where the Cys residue was substituted by Lys, showed no absorbance (Figure 3B,C), indicating that the coupling reaction is specific for thiol groups and orthogonal to the presence of amines. Altogether, these results demonstrate that the methylsulfone functionalized gels P(AAm-*co*-**1**) and P(AAm-*co*-**2**) allow effective, specific covalent binding of thiol peptides to P(AAm) gels under mild conditions (PBS, room temperature, 1 h), and represent ideal materials for cell studies.

We also tested the possibility of immobilizing proteins via Cys residues to the P(AAm-*co*-**1**) gels using Rhodamine labeled Fibronectin. This protein has four cysteine residues and is widely used in cell cultures on P(AAm) to mediate cellular attachment to the hydrogel.[1a,7] A clear absorbance maximum at 560 nm (λ_{max} of Rhodamine) was observed after fibronectin coupling and washing to the P(AAm-*co*-**1**) gel (Figure 3D). The control experiment with P(AAm) showed no absorbance (Figure 3D), indicating specific immobilization of Cys-containing proteins to the new gels.

The cytocompatibility and the ability of the P(AAm-*co*-**1**) and P(AAm-*co*-**2**) gels to support cell growth was also tested. Gels functionalized with c(RGDfC) or with fibronectin were incubated with HeLa cells. Cells attached and spread on c(RGDfC) and fibronectin functionalized gels (Figure S11A,B), and remained viable (Figure S12). Control experiments on gels without modification did not show cell attachment (Figure S11C,D), indicating that cell attachment was solely mediated by the coupled adhesive peptides or FN, and not by the gel itself or by non-specific absorption of serum proteins to the gel.

We then compared the performance of our new gels with the widely used P(AAm)/sulfo-SANPAH gels in terms of specific and functional biofunctionalization, and the impact of these parameters in cell experiments.[1a,8] We incubated P(AAm-*co*-**1**) with c[RGDfC] and P(AAm)/sulfo-SANPAH with c[RGDfK] at the same concentration (1 mg mL^{-1}). A three times higher density of c[RGD(DMNPB)fC] was immobilized on the methylsulfone gels than c[RGD-

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(DMNPB)fK] on P(AAm)/sulfo-SANPAH gels (Figure 3E). To test coupling specificity, the thiol-terminated peptide c[RGD(DMNPB)fC] was incubated with the P(AAm)/sulfo-SANPAH-activated gel. The thiol-terminated peptide coupled to P(AAm)/sulfo-SANPAH almost at the same concentration as the amine-terminated c[RGD-(DMNPB)fK], demonstrating lack of any specificity of the sulfo-SANPAH immobilization. Note that coupling in P-(AAm-*co*-**1**) and P(AAm-*co*-**2**) gels was selective for the thiol group and orthogonal to the rest of functionalities (Figure 3B,C). Moreover, coupling of thiols to methylsulfone gels was complete in one hour and a single step; whereas sulfo-SANPAH coupling required 24 hours to achieve maximum coupling, followed by several incubation and washing steps. Altogether, these factors demonstrate the superiority of methylsulfone gels versus P(AAm) gels for bioconjugation in terms of coupling yield, selectivity, and simplicity of the coupling procedure.

An important consequence of nonspecific ligand coupling to a material is the loss of functionality of the immobilized ligand. We tested the functionality of immobilized proteins on P(AAm)/sulfo-SANPAH and P(AAm-*co*-**1**) gels using the streptavidin/biotin complex (Figure 3F). The gels were incubated overnight with streptavidin followed by washing and incubation with Atto 425-biotin (0.3 mg mL^{-1}). The UV spectra showed a clear absorbance band of Atto 425-biotin on the P(AAm-*co*-**1**) gel, and almost no absorbance on the P(AAm)/sulfo-SANPAH gel. Control experiments with Atto 425-biotin did not show any absorbance on any of the gels. The lower biotin content on P(AAm)/sulfo-SANPAH gels was surprising considering that Streptavidin has only 4 thiol groups for coupling to P(AAm-*co*-**1**) gels versus 25 amine groups for coupling to the P(AAm)/sulfo-SANPAH gels. Two scenarios are possible: a very low coupling efficiency of P(AAm)/sulfo-SANPAH gels or a very low functionality of streptavidin coupled to P(AAm)/sulfo-SANPAH gels. To differentiate between these two scenarios, an immobilization experiment with a premixed solution of streptavidin and biotin was performed. Under these conditions, the streptavidin/biotin complex is formed before the streptavidin is coupled to the gel and the detected absorbance should be correlated with the amount of immobilized protein. On P(AAm-*co*-**1**) gels, the same absorbance value was detected under both incubation conditions, indicating that streptavidin was fully functional after coupling to the gel. Interestingly, a significantly higher absorbance was detected on the P-(AAm)/sulfo-SANPAH gel, indicating that streptavidin coupled by sulfo-SANPAH loses its activity, whereas streptavidin immobilized on P(AAm-*co*-**1**) remains fully functional and is able to recognize and complex biotin.

Finally, we tested the impact of non-functional ligand immobilization on P(AAm) gels in cell behavior. We performed cell adhesion studies on P(AAm)/sulfo-SANPAH gels functionalized with c[RGDfK] and P(AAm-*co*-**1**) gels functionalized with c[RGDfC] at equal density of RGD ligand (ca. 8 μM, determined by UV spectroscopy; Figure S13). Spreading of HeLa cells was characterized at different times after cell seeding. Cells on the P(AAm)/sulfo-SANPAH/c[RGDfK] were poorly spread after 6 hour incubation. Spreading improved at longer incubation times. In contrast, cells on the P(AAm-*co*-**1**)/c[RGDfC] gel readily spread after 6 h of peptide incubation, and the spreading area was significantly larger than on the P(AAm)/sulfo-SANPAH/c[RGDfK] gel after 24 hours. The slower spreading kinetics and the lower final spreading area can only be explained by the lower density of functional RGD on the P(AAm)/sulfo-SANPAH gels, since both gels present equal density of immobilized ligand and comparable gel properties (stiffness, swelling). As a consequence of lower availability of functional adhesive sites, cells on P(AAm)/sulfo-SANPAH/c[RGDfK] and p(AAm-*co*-**1**)/c[RGDfC] gels displayed significant differences in the formation and distribution of the stress fibers of the cytoskeleton (Figure 4).[9] Taking into account the demonstrated relevance of the adhesive and tensional state of the

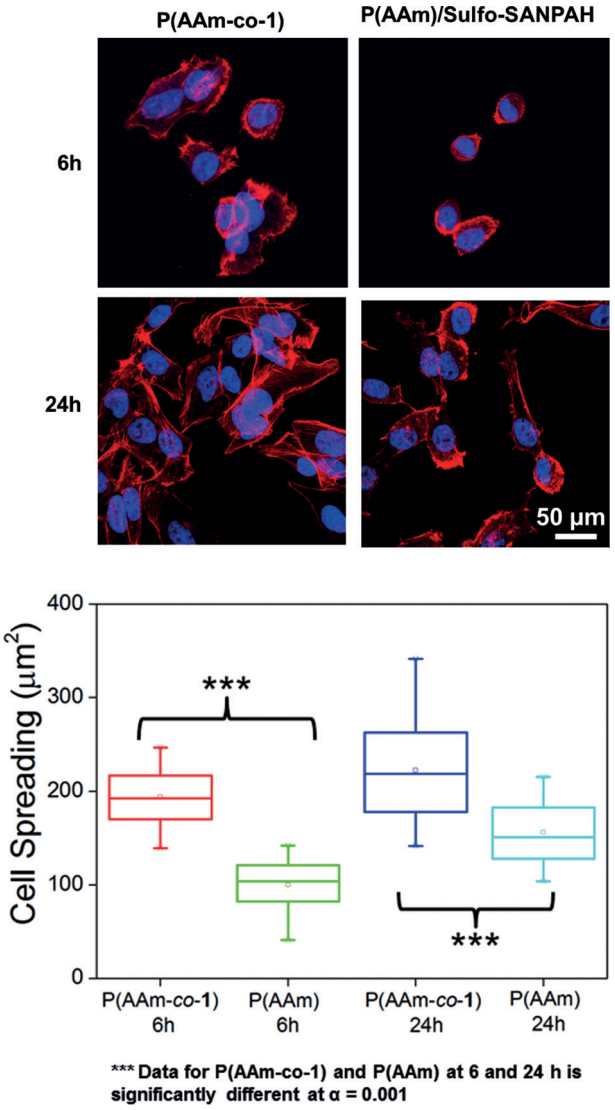

Figure 4. Confocal fluorescence images of HeLa cells cultured on P(AAm-*co*-**1**) or P(AAm)/sulfo-SANPAH gels functionalized with the same ammount of adhesive peptide (ca 8 μM c[RGDfC] or c[RGDfK] respectively) for 6 or 24 hours. The statistical analysis using one-way ANOVA shows significant differences between samples at alpha = 0.001 level and Tukey contrast.[10]

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cell in cellular decisions, the use of selective ligand coupling methods for P(AAm) gels is an unavoidable need for conclusive studies of cell-materials interactions.[1a,3]

In conclusion, the methylsulfone-derivatized hydrogels P(AAm-*co*-**1**) and P(AAm-*co*-**2**) are ideal models for quantitative and standardized studies of cell-materials interactions and outperform the currently used P(AAm)/sulfo-SANPAH system. They can effectively, specifically, and functionally conjugate to thiol-containing biomolecules, while retaining all desirable properties of classical P(AAm)gels required for cell culture (transparency, swelling, protein repellence, tunable stiffness). These materials will finally allow conclusive and reproducible experimentation in relevant open questions at the interface between cell biology and biomaterials science, in particular the interplay between ligand density and stiffness in cell attachment and derived cellular functions.

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Keywords: cell culture · chemoselectivity · poly(acrylamide) gels · protein coupling · thiol bioconjugation

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