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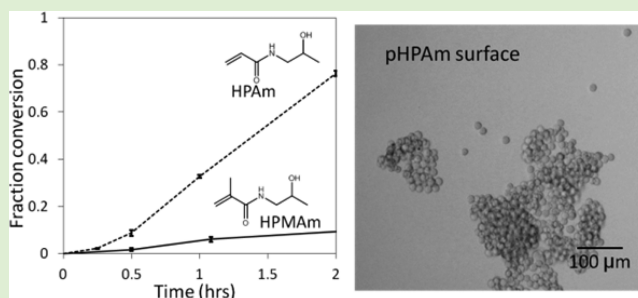
# Inhibition of Protein and Cell Attachment on Materials Generated from *N*-(2-Hydroxypropyl) Acrylamide

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## S Supporting Information

**ABSTRACT:** Effective control over biointerfacial interactions is essential for a broad range of biomedical applications. At this point in time, only a relatively small range of radically polymerizable monomers have been described that are able to generate low fouling polymer materials and surfaces. The most important examples that have been successfully used in the context of the reduction of nonspecific protein adsorption and subsequent cell attachment include PEG-based monomers such as poly(ethylene glycol) methacrylate (PEGMA), zwitterionic monomers such as 2-methacryloyloxyethyl phosphorylcholine and noncharged monomers such as acrylamide and *N*-(2-hydroxypropyl) methacrylamide (HPMAm). However, issues such as oxidative degradation and poor polymerization characteristics limit the applicability of most of these candidates. Here we have synthesized the monomer *N*-(2-hydroxypropyl) acrylamide (HPAm), examined its polymerization kinetics and evaluated its suitability for RAFT mediated polymerization in comparison to HPMAm. We also synthesized hydrogels using HPMAm and HPAm and evaluated the ability of HPAm polymers to occlude protein adsorption and cell attachment. In RAFT-controlled polymerization, much faster (8×) polymerization was observed for HPAm relative to HPMAm and better control was achieved over the molecular weight distribution. The performance of hydrogels prepared from HPAm in the prevention of protein adsorption and cellular attachment was equivalent to or better than that observed for materials made from HPMAm and PEG. These results open the door for HPAm based polymers in applications where effective control over biointerfacial interactions is required.



## INTRODUCTION

The challenges in the development of effective biomaterials are multifaceted. Not only must a functional biomedical material possess the appropriate mechanical properties (strength and flexibility so that the material does not break under physiological stress, hardness so that it does not wear too quickly, etc.), but it must also interact appropriately with the biochemical environment in which it is placed. For a bone graft this may mean a material that encourages cellular integration while for an artificial heart valve or ocular lens, this may mean a nonadhesive material to which cells fail to attach.

Cellular attachment to a material can be via specific interactions, such as for materials that are synthesized to present defined integrin binding sites to promote cellular adhesion. More commonly, however, cellular attachment to materials occurs as a result of nonspecific adsorption of serum proteins on the surface of a material, presenting a myriad of cell adhesion motifs.<sup>1–3</sup> Minimizing the adsorption of proteins from biological fluids therefore reduces cell attachment in many cases.

While synthetic polymers that interact very little with biological macromolecules and cells are understandably a popular foundation for bioinert materials, they are also often a crucial component of bioactive materials.<sup>4–7</sup> A material that is

intrinsically bioactive may cause a host of cascading biological responses, many of which are undesirable. By employing polymers which have low protein adsorption and cell attachment properties, however, materials can be designed that act as a nonreactive platform or “blank slate.” Discrete biochemical signals and functional groups can subsequently be added to elicit specific desired biological responses to the material while preventing undesired responses that may otherwise be generated as a result of nonspecific protein adsorption.<sup>4–7</sup>

While a number of low protein and cell attachment polymers have been identified, the stand-out performers all have disadvantages which limit their broad application. Those polymers belonging to the class of naturally derived materials, including polypeptides and polysaccharides, lack long-term stability under physiological conditions. Similarly, polymers containing esters (e.g., polyesters, polyacrylates, and polymethacrylates) are subject to hydrolytic degradation, which has been implicated in the clinical failure of hydroxyethyl acrylate copolymer hydrogel implants.<sup>8,9</sup> Poly(ethylene glycol) (PEG)

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is by far the most popular low-fouling polymer employed in academic research and industry, having very low protein interactions.<sup>7,10–12</sup> Another reason that PEG is so widely used is the chemical versatility of the end groups and the regular molecular weights of the polymers themselves; anionic polymerization of ethylene oxide results in telechelic polymers of extremely low dispersities. While PEG has been used (among other purposes) as a polymer surface coating,<sup>12–15</sup> a hydrogel platform<sup>16,17</sup> and attached to therapeutic molecules to improve pharmacokinetic properties,<sup>18–20</sup> the ether groups in the polymer chains are observed to degrade oxidatively in biologically relevant conditions,<sup>11,21,22</sup> thus, limiting their use in long-term applications. Moreover, concern has been raised in recent years that PEG is more immunologically active than previously understood.<sup>23</sup>

Recently, several poly zwitterionic polymers have shown great promise in the development of low protein adsorption materials. Phosphorylcholine methacrylate polymers, mimicking the head groups of lipids composing the outer membrane of red blood cells, have shown great resistance to protein adsorption.<sup>24–26</sup> However, the phosphoester group is susceptible to hydrolysis<sup>27</sup> and the hygroscopic character of the compound makes it a difficult material to synthesize and handle.<sup>28</sup> Sulfobetaine-containing monomers are easier to handle and more hydrolytically stable, but, in at least one study, have not shown the same performance as the phosphorylcholine monomers.<sup>29</sup> Of the zwitterionic polymers, carboxybetaine monomers appear now to have the best combination of performance and stability.<sup>28,30</sup> Most zwitterionic monomers, however, fall into the categories of acrylates and methacrylates, so long-term hydrolytic stability is an ongoing issue.

Pioneered by Jindrich Kopecek and his research group, polymers made from *N*-(2-hydroxypropyl) methacrylamide (HPMAm), Figure 1, have been employed in biomaterial and

mediated polymerization of HPMA, telechelic, or diend-functional polymers of lower dispersities can be obtained, making pHPMA competitive with PEGs where polymers with more uniform, defined molecular weights, and reactive end-groups are required.

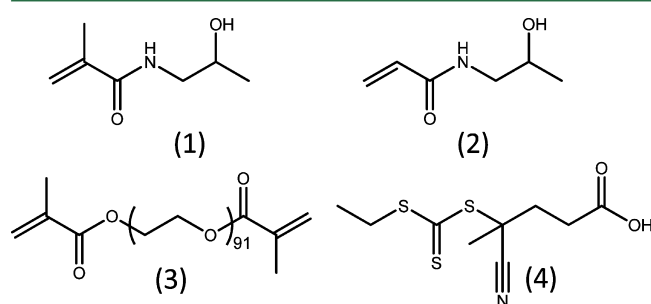
Yet, for some applications, the HPMAm monomer has limitations that preclude the effective application of its otherwise desirable polymer. Characteristic of methacrylamides, HPMAm exhibits low polymerization rates relative to (meth)acrylates and acrylamides. This shortcoming is particularly noted in nonaqueous solution polymerizations and limits both the ultimate chain length and the speed with which polymerizations can be performed.<sup>38,39</sup> Additionally, for RAFT mediated polymerization, the variety of appropriate RAFT agents is limited relative to monomers of other polymerizable groups. To address these issues, an acrylamide analogue of HPMAm was synthesized, *N*-(2-hydroxypropyl) acrylamide or HPAm (Figure 1). Despite its structural similarity, this acrylamide analogue has, as of yet, not been explored in many of those biomaterial applications for which HPMAm excels. Relative rates of polymerization were determined as was the molecular weight and dispersities of polymers resulting from RAFT-mediated polymerization (see Figure 1 for structure of RAFT agent). Potential for application in low protein attachment and low cell attachment materials surfaces was evaluated by measuring adsorption of europium labeled fibronectin in fetal bovine serum and by seeding adherent cells on hydrogels formed from HPAm and HPMAm compared against hydrogels of PEGDMA as well as tissue culture polystyrene and commercial low-attachment culture plates as adsorption/attachment controls (Figure 1).

## EXPERIMENTAL SECTION

Key compounds used in this work are shown in Figure 1. Unless otherwise noted, all reactants and initiators were purchased from Sigma-Aldrich and used as received.

**Synthesis of HPMAm (1).** HPMAm was synthesized by reacting 10 g (65 mmol) of methacrylic anhydride in diethyl ether (50 mL) with an equimolar quantity of amino-2-propanol (4.9 g, Acros) in 25:1 mixture ether/dichloromethane (50 mL) at room temperature. In a 500 mL round-bottom flask, amino-2-propanol in diethyl ether was stirred while dichloromethane was added dropwise until the complete dissolution of the amine was obtained. The methacrylic anhydride solution was added in a thin stream causing a vigorous reaction that resulted in boiling of the solution in the reaction flask (for this reason a large capacity flask was used). Following addition of the anhydride solution the reaction was stirred for 1 h at room temperature. The reaction mixture was transferred to a smaller flask and then cooled to  $-20^{\circ}\text{C}$  overnight to facilitate crystallization of the product. Solid HPMA was collected via filtration and recrystallized from 4:1 diethyl ether/dichloromethane. The yield was 65%. This scheme was applied in lieu of the reaction of methacryloyl chloride<sup>31</sup> because of the less stringent transport restrictions of methacrylic anhydride and because of the ease of product recovery. Product identity and purity was confirmed by HNMR and atmospheric pressure chemical ionization mass spectroscopy (APCI MS; Figures S1 and S2 in Supporting Information). Chemical shifts in deuterated chloroform: 1.16 d (3H), 1.92 m (3H), 3.12 m (1H), 3.38 br (1H), 3.45 m (1H), 3.90 m (1H), 5.30 m (1H), 5.68 m (1H), 6.49 br (1H).

**Synthesis of HPAm (2).** Good yield was obtained by adding 5 g of acryloyl chloride (55 mmol, Merck) dropwise in dry ethyl acetate (40 mL) to a stirred solution of 2-fold excess amino-2-propanol (8.3 g) in dry ethyl acetate (50 mL) at a temperature of  $-10^{\circ}\text{C}$  under nitrogen. By repeated synthesis attempts, it was determined that reduced reaction time, relative to analogous protocols used for HPMAm synthesis,<sup>31</sup> improved yield. Following completion of the addition of



**Figure 1.** Key compounds used in this study: (1) *N*-(2-hydroxypropyl) methacrylamide (HPMAm) (2) *N*-(2-hydroxypropyl) acrylamide (HPAm), (3) poly(ethylene glycol) dimethacrylate  $M_w = 4000$  (PEGDMA), and (4) cyano-4-[(ethylsulfanylthiocarbonyl)sulfanyl]pentanoic acid (RAFT agent).

biotechnological applications as an alternative to PEG.<sup>11,31,32</sup> The amide bond of acrylamides and methacrylamides is considerably more stable to hydrolysis than are the esters of analogous (meth)acrylates. Rodriguez-Emmenegger et al. report that the protein adsorption observed with pHPMAm surfaces was below surface plasmon resonance (SPR) detection limits, making the polymer a prime candidate for the generation of low fouling biomaterial surfaces<sup>33,34</sup> as well as for the modification of therapeutic agents to improve their pharmacokinetic properties.<sup>11,32,35–37</sup> Additionally, by employing RAFT-

the acryloyl chloride (30 min) the reaction mixture had separated into two phases: a thin solution containing the majority of the recoverable product and a viscous phase containing some product as well as polymerized product and amine salt byproducts. The thin liquid phase was passed through a 2 cm plug of silica. The viscous phase was dissolved in water (50 mL) and extracted with ethyl acetate (50 mL). The organic phase from the extraction was then passed through the same silica plug which was then eluted with more ethyl acetate (200 mL). Ethyl acetate phases were combined and solvent removed via rotary evaporation. The remaining viscous liquid product was placed in a glass vial and stored at  $-20^{\circ}\text{C}$  whereupon it crystallized. The yield was 50%. Product identity and purity was confirmed by  $^1\text{H}$  NMR and APCI MS (Figures S3 and S4 in Supporting Information). Chemical shifts in deuterated water: 1.06 d (3H), 3.14 m (1H), 3.21 br (1H), 3.84 m (1H), 5.66 dd (1H), 6.08 m (1H), 6.17 dd (1H).

**Synthesis of PEGDMA.** A total of 5 g of poly(ethylene glycol),  $M_w = 4000$ , was reacted with 3 equiv methacryloyl anhydride in 50 mL of anhydrous dichloromethane with 0.2 equiv dimethylaminopyridine as catalyst. After 18 h, the PEGDMA was precipitated in cold diethyl ether, redissolved in dichloromethane, washed with 5% sodium bicarbonate solution, and brine and then reprecipitated in cold diethyl ether. Residual solvent was removed via vacuum. Degree of substitution was determined to be greater than 80% via HNMR in deuterated chloroform.

**Synthesis of the RAFT Agent.** The RAFT agent was synthesized via a previously published protocol<sup>40,41</sup> but with the substitution mercaptoethane for 1-dodecanethiol and of potassium *tert*-butoxide for sodium hydride.

**Polymerization Kinetics.** A total of 1 g of each monomer was dissolved in deuterated DMSO to a concentration of 1.75 M. Small volumes (small enough to not significantly dilute monomer) of concentrated solutions of RAFT agent and azobis(isobutyronitrile) (AIBN) in deuterated DMSO were added to a final concentration of 5.0 and 0.50 mM, respectively. Monomer solutions were deoxygenated by three cycles of freeze–pump–thaw, whereupon the contents were transferred, under nitrogen, to individual sealed vials with a volume of 200  $\mu\text{L}$  each. All vials were placed in shaking incubator at  $80^{\circ}\text{C}$  and 100 rpm and removed at various time points. These polymerization mixture samples were further diluted with deuterated DMSO and  $^1\text{H}$  NMR spectra were obtained using a Bruker 400 MHz NMR spectrometer. Monomer conversion to polymer was determined by the integration of the alkene-associated proton peaks (at 5.5–6.5 ppm for the acrylamide and 5.3–5.7 ppm for the methacrylamide) relative to the total amide proton peak (at  $\sim 6.5$  ppm).

**GPC-MALS.** Molecular weights and dispersities were determined by gel permeation chromatography and multiangle light scattering (GPC-MALS) on a Shimadzu Chromatography System (LC-20AD) with Ultrahydrogel 2000 and 120 columns connected in series. A column temperature of  $40^{\circ}\text{C}$  was maintained and 0.10 M sodium nitrate was used as the mobile phase. The column outlet was connected to a Dawn Heleos II light scattering detector and an Optilab REX refractive index detector (both from Wyatt Technology Corporation) and data was analyzed with ASTRA V software (Wyatt).

**Hydrogel Formation.** Hydrogels were made by the copolymerization of monomers (concentration 2 M) with *N,N*-methylenebis-(acrylamide) (40 mM) in acetate buffer (pH = 5.4). Polymerizations were initiated at  $50^{\circ}\text{C}$  using 2,2'-azobis(2-methylpropionamide)-dihydrochloride at concentrations of 40 mM for 10 h for HPMAM gels and at a concentration of 10 mM for 3 h for HPAm. Gels were formed between untreated glass microscope slides sealed with a 1 mm thick rubber gasket. Following polymerization, gels were placed in PBS to remove any unreacted reagents and to equilibrate gels for biological testing. After a minimum of 18 h, 10 mm discs were cut out using a biopsy punch for use in protein adsorption and cell culture experiments. PEGDMA hydrogels were synthesized similarly to that as described previously.<sup>42</sup> Briefly, an aqueous solution of 20% PEGDMA with 0.02% photoinitiator LAP (synthesized according to previous reports<sup>42</sup>) was irradiated between glass microscope slides for 5 min at 10 mW/cm<sup>2</sup>, 365 nm UV light.

**Contact Angle Measurements.** Static contact angle measurements were performed by depositing 5  $\mu\text{L}$  droplets of 0.2  $\mu\text{m}$  filtered deionized water onto surfaces of hydrogels. Gels were placed on a glass slide on top of moistened tissue in a closed container to maintain humidity. Containers were opened only briefly for imaging with a KSV Cam 200 tensiometer.

**Europium Labeling and Protein Adsorption Quantification.** The labeling of fibronectin with europium and subsequent adsorption assay were performed as described previously<sup>43</sup> but with fibronectin rather than human serum albumin. Briefly, 1 nmole fibronectin from human serum (Sigma) in 100  $\mu\text{L}$  of 0.1 M bicarbonate buffer (pH 9.3) was treated with a 15-fold excess of Eu-labeling reagent (Delfia Eu–N1 ITC chelate, Perkin Elmer) at  $4^{\circ}\text{C}$  for 18 h. Labeled fibronectin was purified via dialysis.

Adsorption was performed with 10% FBS (fetal bovine serum) in PBS (phosphate buffered saline) supplemented with 0.3  $\mu\text{g}/\text{mL}$  Eu-labeled fibronectin (approximately 1/100 of the endogenous fibronectin component of FBS). Gels and surfaces were incubated with supplemented FBS solution at  $20^{\circ}\text{C}$  for 18 h then washed 7 $\times$  with PBS. After the last wash, Eu(III)-liberating Enhancement Solution (Perkin Elmer) was added and allowed to cover surfaces for 45 min. Following this short incubation, 0.1 mL of the Eu-complex solution was removed from each surface and transferred to a white 96-well plate for time-resolved fluorescence assay on a PHERAstar instrument with excitation and measured emission at 337 and 620 nm, respectively, as per Perkin Elmer product instructions. Concentrations of Eu(III) were determined via comparison with standards. Raw measurements were corrected for volume of enhancement solution and surface area of substrates. Total fibronectin adsorption was calculated based on the ratio of labeled to unlabeled fibronectin in 10% FBS.

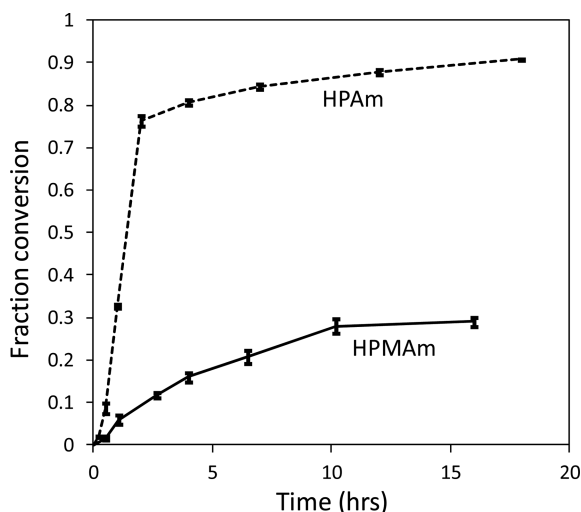
**Cell Culture Experiments.** Hydrogels were incubated with 2 $\times$  antibiotic antimycotic solution (anti–anti, Gibco) in PBS for 3 h at  $37^{\circ}\text{C}$  to minimize risk of microbial and fungal contamination. After the anti–anti solution was removed, L929 mouse fibroblast cells in media supplemented with 10% fetal bovine serum (FBS), and 1 $\times$  anti–anti, were seeded at a density of 20000 cells/cm<sup>2</sup>. Photomicrographs of the sample surfaces (i.e., attached and unattached cells) were taken after 24 h incubation. As controls, cells were also seeded on tissue culture treated polystyrene (TCPS, Nunclon delta surface, Nunc) and ultra-low attachment surface (ULA, Corning) under the same conditions, except for the use of anti–anti.

**MTS Assays.** To quantify the number of metabolically active cells adhered to and removed from hydrogels' surfaces, a colorimetric MTS assay was performed using Promega's CellTiter 96 aqueous non-radioactive cell proliferation assay. This assay measures the enzymatic conversion of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS), which corresponds to the number of metabolically active cells. At 24 h after cellular seeding, the surface of each gel, as well as the ULA and TCPS surfaces, was photographed and then lightly rinsed with the media already present in its respective well. That media from each well (approximately 750  $\mu\text{L}$ ) was transferred to a fresh well in a TCPS plate, whereupon concentrated solutions of MTS and phenazine methosulfate (PMS) were added to each well to meet the recommended working concentrations (0.4 and 0.01 mg/mL respectively). To the gels was then added 1 mL of fresh media containing the assay's constitutive compounds already at the recommended concentrations. Following 3 h incubation at  $37^{\circ}\text{C}$ , the absorbance at 490 nm for each well was measured against media containing assay components but no cells. Statistical significance was determined by Student's *t* test.

## RESULTS AND DISCUSSION

As hypothesized, the polymerization of HPAm proceeded much faster than that of HPMAM, as may be seen in Figure 2. After 16 h, the conversion of HPAm exceeded 90%, while that obtained for HPMAM remained just over 30%. To a large extent, the low conversion of HPMAM after 16 h resulted from





**Figure 2.** Conversion plotted against time of HPAm (solid line) and HPAm (dashed line) under identical polymerization conditions: 1.75 M monomer, 5.0 mM RAFT agent, and 0.50 mM AIBN in DMSO at 80 °C. Measured conversions indicated by standard error bars. Lines are drawn to guide the eye. Error bars represent standard deviation at each time point.

the low residual concentration of AIBN, which at this point would be almost entirely exhausted. But examining just the first 2 h of polymerization, where the depletion of AIBN was not limiting and the consumption of monomers was linear, the polymerization rate of HPAm exceeded that of HPMAM by a factor of 8. The rate of polymerization is described by eq 1, where  $k_p$  is the polymerization rate constant,  $[M]$  is the concentration of monomer,  $[I]$  is the concentration of initiator,  $k_d$  is the disassociation rate constant of the initiator,  $k_t$  is the rate constant of termination, and  $f$  is the initiator efficiency.<sup>44</sup>

$$R_p = k_p[M] \left( \frac{fk_d[I]}{k_t} \right)^{1/2} \quad (1)$$

Polymerization conditions were chosen so as to keep as many variables in eq 1 equivalent among polymerizations, and therefore, based on the relative rates of monomer consumption observed in these experiments,  $k_{pAm} k_{tAm}^{-1/2} = 8k_{pMAM} k_{tMAM}^{-1/2}$ , where  $k_{pAm}$  and  $k_{tAm}$  are the polymerization and termination rate constants, respectively, for HPAm and  $k_{pMAM}$  and  $k_{tMAM}$  are the corresponding rate constants for the HPMAM.

Many applications in the development of biomedical materials require polymers of defined and narrowly dispersed molecular weights that are of a particular architecture. For this reason, the amenability of HPAm to RAFT-controlled polymerization was evaluated against HPMAM under identical reaction conditions. GPC-MALS was used to determine the molecular weights and dispersities of the polymers resulting

from the kinetics experiments. The results obtained are summarized in Table 1. The concentrations of monomer, initiator, and RAFT agent ideally would lead to a pHPMAM with a  $M_n$  of 50 kg mol<sup>-1</sup> and slightly lower for the pHPAm because of the lower molecular weight of the monomer. The target molecular weights for the polymers were calculated via eq 2, assuming complete conversion.<sup>45</sup>

$$\bar{M}_n = \frac{[M]_0 - [M]_t}{[T]_0} m_M + m_T \quad (2)$$

Here  $[M]_0$  and  $[M]_t$  are the initial and final molar concentrations of monomer, respectively.  $[T]_0$  is the initial concentration of RAFT agent and  $m_M$  and  $m_T$  are the molecular weights of the monomer and RAFT agent, respectively.

The 50 kg mol<sup>-1</sup> target was chosen as others had reported difficulty in obtaining RAFT-controlled polymers of this molecular weight with HPMA in organic solvents.<sup>38,39</sup> After a 20 h reaction, the  $M_n$  for pHPMAM was less than half the target value, a consequence of low monomer conversion (~30%). The  $M_n$  of the pHPAm obtained, on the other hand, was very close to the target  $M_n$  value. Additionally, the molecular weight dispersity achieved for the pHPAm synthesized was significantly closer to unity than that obtained for pHPMAM. Theoretically, polymerization mediated with an appropriate RAFT agent should result in lower or equivalent dispersity at lower conversions than at higher conversions. The exact reason for the higher dispersity obtained for the pHPMAM is not known. It should be noted that conversion,  $M_n$  and dispersity could possibly be improved for pHPMAM with optimization of the polymerization conditions used (solvent, initiator, RAFT agent, temperature). McCormick and co-workers, for example, report obtaining high molecular weights and low dispersities<sup>46</sup> for RAFT-mediated polymerizations of HPMAm in acetate buffer. Similar conditions, however, yielded inferior results in our experience. The use of HPAm resulted in reactions that demonstrate desirable kinetics while being amenable to RAFT control without time-consuming optimization procedures.

One feature of RAFT-mediated polymerization is that not every RAFT agent will provide the desired control over every monomer. For example, some RAFT agents are more suitable for methacrylate monomers. The degree of control that a particular RAFT agent facilitates is determined by the substituents, the “Z” and “R” groups, of the central thiocarbonylthio group. In this study we explicitly chose a RAFT agent, a trithiocarbonate with a cyanovaleric acid substituent, which is compatible with and has been used previously for the controlled polymerization of HPMAm.<sup>47–49</sup> It should be noted, however, that in addition to increased polymerization rates, one advantage of acrylamide monomers relative to methacrylamide monomers is the greater variety of RAFT agents that can provide control of polymerization.

**Table 1.** Summary of GPC-MALS Results for Polymers Resulting from Kinetics Experiment<sup>a</sup>

	$M_n$ (target) kg mol <sup>-1</sup>	$M_n$ (theoretical) kg mol <sup>-1</sup>	conversion %	$M_n$ (measured) kg mol <sup>-1</sup>	PDI
pHPMAM	50	15	29 ± 1	21 ± 2.1	1.4 ± 0.10
pHPAm	45	41	91 ± 1	41 ± 2.8	1.1 ± 0.06




<sup>a</sup>Based on the concentration of initiator and RAFT agent, target  $M_n$  is the value that would be obtained if reactions proceeded without nonidealities and conversions were complete. Theoretical  $M_n$  refers to the value that is calculated based on the concentration of initiator and RAFT agent and the observed, rather than absolute, conversion. Measured  $M_n$  and PDI were determined via GPC-MALS.

Polymers of HPMAM have demonstrated outstanding characteristics in biomedical research but have been limited by relatively low molecular weights and slow polymerization. The improvement of polymerization kinetics and control with HPAm relative to HPMAM allow the monomer greater versatility in its application. Radical damage during grafting to therapeutic proteins, for example, may be mitigated by a lower initiator concentration. Growing polymers off of RAFT-functionalized surfaces may be performed in less time and with a greater polymer surface density. Polymers with pHPAm blocks of high degrees of polymerization may be generated to obtain otherwise elusive desired properties. It must be demonstrated, however, that pHPAm shares the same advantageous qualities of pHPMAM. To that end, materials made with HPAm were compared directly with those made from HPMAM to determine whether the biological interfacial reactions were similar.

One area in which pHPMAM has shown great utility is in the generation of surfaces which have low protein adsorption properties. Such surfaces ostensibly allow the fabrication of so-called, “stealth” biomaterials that can be implanted with potentially little foreign body response. To explore the suitability of pHPAm for the preparation of similar materials, hydrogels of both pHPMAM and pHPAm, cross-linked via *N,N*-methylenebis(acrylamide), were synthesized and the surface wettability and protein adsorption to the surface of each assessed. The effect of the cross-linker on these properties was assumed to be negligible because of the small relative proportion in composition (approximately 2% by weight) and the chemical group similarity with HPAm and HPMAM. Each hydrogel was compared against PEGDMA hydrogels and ultralow attachment coated polystyrene (Corning; ULA) and tissue culture treated polystyrene (TCPS) in cell attachment assays.

Certain property requirements are generally considered important in polymer coatings used in low adhesion applications: very hydrophilic and composed of materials containing no hydrogen bond donors.<sup>10,50</sup> Surfaces grafted with HPAM polymers, however, not only present hydrogen bond donor sites but also exhibit only moderate wettability. This was observed in the fabricated hydrogels where sessile contact angles at room temperature were measured to be 45°, Table 2.

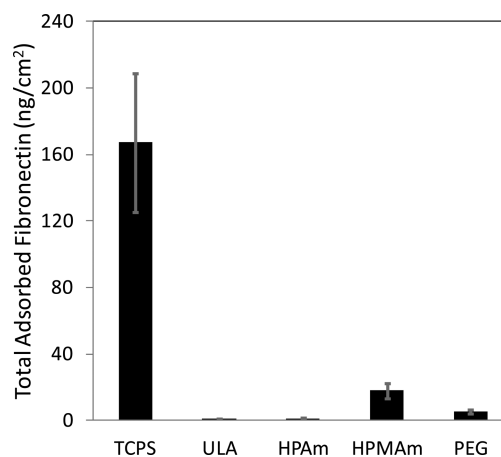
**Table 2. Contact Angles and Representative Images for Hydrogels**

Hydrogel	Contact angle	Representative image
HPMAM	45 ± 2°	
HPAm	44 ± 3°	
PEGDMA	37 ± 2°	

Hydrogels of HPAm exhibited statistically similar contact angles (44°), indicating similar hydrophilicity on the surface of the materials, while PEGDMA hydrogels were determined to be only slightly more hydrophilic (Table 2). These results are consistent with measurements made by authors of another study on brush-polymer coatings of pHPMAM and pPEGMA<sup>33</sup> and support their observations that the mechanisms for the suppression of protein adsorption are poorly understood.

Because surfaces coated with pHPMAM brushes have been reported to show very low protein adsorption, it was

hypothesized that materials made from HPAm would show similarly low protein adsorption properties. Protein adsorption to surfaces was measured using a time-resolved fluorescence europium assay. Surfaces were incubated for 18 h with 10% fetal bovine serum in PBS supplemented with 0.30 µg/mL europium-labeled fibronectin (approximately 1% relative to the endogenous fibronectin in FBS). These conditions were chosen to approximate physiological environments. Following adsorption and subsequent washing, europium(III) was liberated from adsorbed protein via a dissociation “enhancement” solution. From the 620 nm wavelength emission of the released europium, surface-bound fibronectin was calculated. The sensitivity of this technique (detection of femtomole of Eu-labeled protein) is similar to that of <sup>125</sup>I radiolabeling. As Figure 3 indicates, total fibronectin adsorption on TCPS was

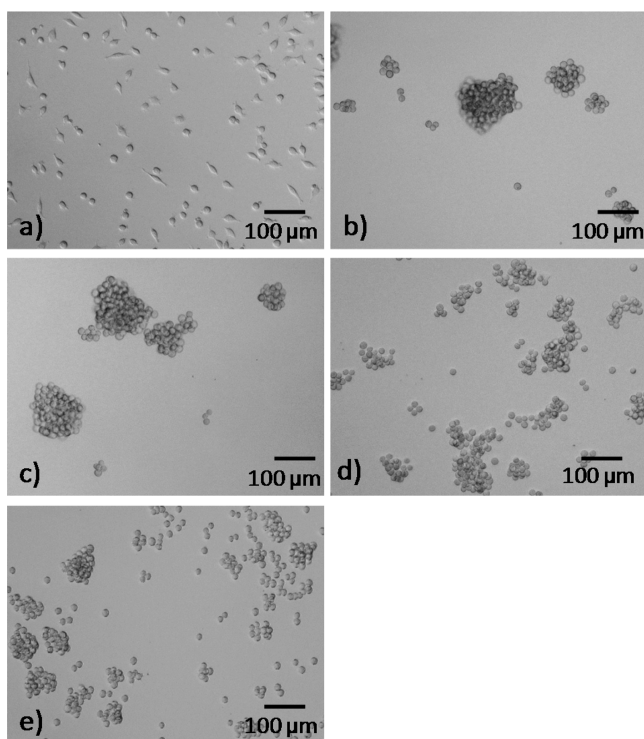


**Figure 3.** Binding of fibronectin to TCPS, ULA, pHPAm, pHPMAM, and PEGDMA hydrogels, *n* = 3. Error bars indicate confidence intervals ( $\alpha$  = 0.01).

approximately 160 ng/cm<sup>2</sup>. Adsorption on pHPAm hydrogels was very low—less than 1% of that measured for the fibronectin adsorption on TCPS and roughly equal to that of the ULA-treated polystyrene. Fibronectin adsorption was also very low on the PEG hydrogels, approximately 3% of that of TCPS. While adsorption was low on pHPMAM hydrogels, it was substantially higher than on the other hydrogels, roughly 10% relative to the TCPS control.

Typically, low protein adsorption on materials translates to low cell attachment as bound endogenous proteins are not present to provide adhesion sites for the cells. It is also notable that soluble polymers with low protein interaction are often conjugated to therapeutic agents to tune pharmacokinetic properties. Low interaction of proteins at pHPAm surfaces suggests the possible application of pHPAm as an alternative to PEGylation of bioactive compounds in a similar fashion to that demonstrated for pHPMAM.<sup>11,32,35–37</sup>

Presented in Figure 4 are the results obtained for cell attachment experiments on synthesized hydrogels as well as the positive and negative controls (TCPS and ULA surfaces, respectively). As can be seen in Figure 4a, TCPS supported substantial L929 cellular adhesion. No cells were observed floating freely when the plate was rocked gently. Moreover, many of the attached cells were flattened with a spread morphology and protrusions, suggesting significant integrin-mediated binding.



**Figure 4.** Cellular attachment of L292s after 24 h on (a) tissue culture polystyrene, (b) ultralow adhesion polystyrene, (c) HPAm hydrogels, (d) HPMAM hydrogels, and (e) PEG hydrogels.

Cells seeded on ULA, on the other hand, bound together in cellular clumps rather than to the surface, Figure 4b. Cells maintained a rounded morphology and floated freely in the media. A small minority of cells remain unattached to larger clumps, but they too exhibited a rounded morphology and failed to adhere to the plate.

Like those cells seeded on the ULA surface, L929 fibroblasts seeded on the pHPAm, pHPMAM, and PEGDMA hydrogel surfaces (Figure 4c–e) formed clumps that floated freely in the media and were not attached. The clumps formed by cells over the pHPAm hydrogel surface appeared, on average, to be larger, and fewer individual cells or clumps of 2–3 cells were observed.

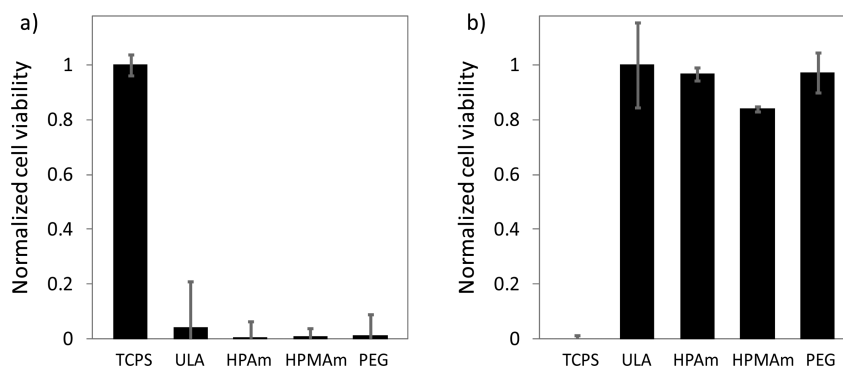
Following gentle rinsing, cells were removed from the ULA plate as well as from the pHPAm, pHPMAM, and PEGDMA gel surfaces almost entirely. The same rinsing procedure on the

TCPS samples resulted in no observable change in cellular density, attachment, or spreading.

To quantitatively verify observed cellular attachment, the metabolic activity of the adherent cells was determined via a MTS assay. This assay measures the enzymatic conversion of MTS to a colorimetric product, roughly proportional to the number of metabolically active cells present in or on a sample. Presented in Figure 5a is the absorbance measured from cells attached to TCPS, ULA, pHPAm, pHPMAM, and PEGDMA hydrogel surfaces following a gentle rinse to remove non-adherent cells. The absorbances values, proportional to the number of remaining cells, were normalized to that of the TCPS sample (the positive control). The total metabolic activity from cells attached to pHPAm, pHPMAM, and PEGDMA hydrogels was approximately 1% of that obtained for the TCPS surface. These results represent a statically robust difference ( $p < 10^{-7}$ ) for both pHPMAM and pHPAm surfaces compared to TCPS). There was no statistically significant difference among the pHPMAM, pHPAm, PEGDMA, and ULA surfaces. To verify that the low cell adhesion observed was a result of occlusion of cellular binding at the surface and not because of cell morbidity, metabolic assays were performed on the reserved media with which the plate wells and hydrogels were rinsed. These measurements were normalized to that obtained for the ULA plate. As can be seen in Figure 5b, the media from TCPS exhibited extremely low metabolic activity, because the viable cells remained adhered and few were removed by the gentle rinsing procedure. The reserved media from the ULA plate was, however, metabolically active as the rinsing procedure collected most cells from the surface. The metabolic assay confirms the viability of those cells as well. Similarly, cells removed from the pHPAm, pHPMAM, and PEGDMA hydrogels remained viable and metabolically active. There was no statistical difference between the reserved ULA media and the media from the hydrogels ( $\alpha = 0.01$ ), indicating little if any cell morbidity associated therewith.

## CONCLUSION

While HPAm has demonstrated efficacy as a polymer for the control of biointerfacial interactions, its slow polymerization kinetics limits its utility. Here we have demonstrated that HPAm provides a promising substitute for HPMAM for applications in low protein and cell attachment materials featuring equivalent performance, but improved polymerization



**Figure 5.** (a) Absorbance from MTS metabolic assay of cells remaining on surfaces following gentle rinsing. Values normalized to that of TCPS. (b) Absorbance from MTS metabolic assay of media collected from rinsing of cell-seeded surfaces. Values normalized to that of media removed from the ULA plate. Taken together, these data indicate that cells exhibited negligible mortality on and failed to adhere to HPAm, HPMAM and PEGDMA hydrogels similar to ultralow adhesion culture plates. Error bars represent confidence intervals ( $\alpha = 0.01$ ).



and control. The polymerization in DMSO was determined to be faster by almost an order of magnitude. In addition, we have demonstrated that higher molecular weights can be obtained and that more uniform dispersities can be achieved via controlled processes such as RAFT-mediated polymerization. Hydrogels formed from HPAm exhibited at least equivalent prevention of cell attachment compared to HPMAM-based materials, underlining the suitability of this monomer in applications where low biointerfacial interactions are required. Additionally, the low interaction of proteins with the polyHPAm material may portend its broader suitability in the development of polymer-conjugated therapeutics. HPAm is structurally identical to HPMAM, with the exception of a single methyl group. This small distinction translates to a remarkable difference in polymerization characteristics, but with no apparent deficit in properties, which are important in controlling biological interaction. This study establishes the monomer HPAm as one of relatively few monomers that can be used for the manufacture of low cell attachment materials and surfaces that are expected to be more stable under physiological conditions than (meth)acrylate polymers and other materials containing hydrolyzable moieties.

## ■ ASSOCIATED CONTENT

### Supporting Information

The NMR and MS spectra of synthesized monomers. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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