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# Modifying network chemistry in thiol-acrylate photopolymers through postpolymerization functionalization to control cell-material interactions

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**Abstract:** Thiol-acrylate photopolymers often contain pendant, unreacted thiol groups even following complete reaction of the acrylate functional groups. The results presented herein demonstrate a high throughput method for quantifying pendant thiol group concentrations using FTIR spectra of thiol-acrylate microspot arrays. Using this technique, more than 25% of the original thiol groups were detected as pendant groups in microspots made from monomer solutions containing at least 40 mol % thiol functional groups. Subsequent modification reactions allowed postpolymerization tailoring of the network chemistry. The extent of modification was controlled by the concentration

of the pendant thiols (ranging from 0.01 to 0.4M) and the duration of the modification reaction (0–10 min for photocoupling reactions, 0–24 h for Michael-type addition reactions). Further, when photocoupling was used to modify the networks, spatial and temporal control of the light exposure facilitated the formation of chemical patterns on the surface and throughout the material. © 2007 Wiley Periodicals, Inc. *J Biomed Mater Res* 86A: 23–30, 2008

**Key words:** thiol-acrylate; photopolymerization; chemical modification; RGD peptide; high-throughput; micropatterning

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## INTRODUCTION

When biomaterials are designed or selected for a particular biomedical application, a key consideration is how their biomaterial properties (such as their mechanics and hydrophobicity/hydrophilicity) impact their performance. This general statement applies to devices that historically have been used in applications such as joint replacements, bone fixation, dental implants, or soft contact lenses, as well as for degradable polymers currently being developed and investigated for numerous tissue engineering and drug delivery applications. Unfortunately, most biomaterial properties are strongly correlated with one another. This correlation often leads to devices with satisfactory mechanics, but inadequate

interaction between the biomaterial and the surrounding tissue.

A common approach, which is used to address these complexities, is to modify the device surface to maintain the mechanical properties while simultaneously enhancing its biocompatibility. In this manner, non-specific protein adsorption is limited and the desired cell-material interactions are induced.<sup>1,2</sup> Approaches for achieving this type of modification include immobilizing bioactive peptides onto solid substrates,<sup>2–4</sup> as well as attaching peptides, proteins, and antibodies onto synthetic polymer surfaces.<sup>1,5–10</sup> For applications requiring *in situ* forming materials, chemical modification is most often accomplished by including the modifier in the precursor solution, which results in bulk modification of the final material.<sup>6,7,11–16</sup>

Recently, there has been an emerging interest in degradable thiol-acrylate photopolymers as potential biomaterials. Previous research has identified a number of key attributes, which make these crosslinked polymer networks well suited for many biomaterial applications.<sup>17,18</sup> For example, thiol-acrylate networks are formed through photopolymer reactions where they are rapidly converted from liquid monomer to crosslinked polymer under physiological

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conditions.<sup>19–21</sup> This photopolymerization reaction provides spatial and temporal reaction control and occurs in the presence of tissue, cells, proteins, and even DNA.<sup>7,12,13,15,16,22–29</sup> Unlike many other materials formed through photopolymerization reactions, thiol-acrylates rapidly cure in the presence of little or no added initiators,<sup>17,30</sup> are curable to significant depths (more than 10 cm),<sup>17</sup> have reduced sensitivity to oxygen inhibition, and display delayed gelation. Variations in the monomer molecular weight, functionality, and functional group concentration combine to control network structure, which further influences mechanical properties and degradation behavior.<sup>17,18</sup>

Here, another important attribute of thiol-acrylate photopolymers is examined: the presence of pendant thiol functional groups that enable facile chemical modification of the network. Thiol-acrylate photopolymers are formed through a mixed step and chain growth reaction mechanism<sup>17,18,31</sup> which consumes multiple acrylate functional groups for each thiol group that reacts, creating materials where complete acrylate conversion rarely equates to complete thiol conversion. When multifunctional thiol monomers are used to form thiol-acrylate polymers, conversion of the monomer (i.e., attachment to the network) is high while the total thiol functional group conversion is significantly lower, leaving a high residual thiol concentration that is tethered to the network. A variety of chemistries can be utilized to modify these pendant groups post-gelation (e.g., functionalized acrylates, methacrylates, vinyl ethers, and allyl ethers will all react with the pendant thiol groups). Additionally, because chemical modification occurs after network formation is complete, patterned regions are easily created using standard photolithographic techniques.

In this paper, we first describe a high-throughput technique for characterizing pendant thiol functional groups by spotting microliter solutions of various monomer formulations onto glass microscope slides, subsequently polymerizing them, and extracting the soluble fraction from the resulting networks. After each step of this procedure, the concentration of thiol groups was determined from analysis of the specimen's FTIR spectra. Next, control over the concentration of attached modifier groups was demonstrated both by varying the reaction conditions present during post-gelation modification and by adjusting the concentration of thiol groups available for functionalization. Studying how these various polymerization and modification steps impact thiol concentration using arrays of polymer microdots allowed numerous polymer samples of known composition to be rapidly fabricated, purified, modified, and analyzed. Finally, photocoupling of both an acrylated dye and an acrylated peptide segment to

the pendant thiol groups was used to demonstrate how patterned regions are easily created on larger films of thiol-acrylate polymer using standard photolithographic procedures.

## MATERIALS AND METHODS

### Glass slide pretreatment

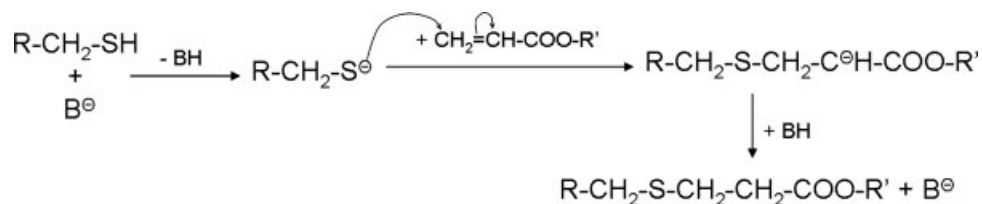
To prevent most of the spotted regions from detaching from the surface of the glass slides during subsequent solvent extraction steps, the slides were cleaned using PIRANA (1:3 v/v hydrogen peroxide (Fisher Scientific) and sulfuric acid (Mallenkrondt)) and then treated with a methacrylated self-assembled monolayer (SAM) (methacryloxypropyl trimethoxysilane, Gelest) prior to being spotted with the various monomer solutions. To apply the SAM, the slides and 40  $\mu\text{L}$  of the silane were placed into an argon-purged Teflon reaction chamber and heated to 60°C for a minimum of 2 h. The slides were used within 2 days of pretreatment. Standard microscope slides (25 mm  $\times$  75 mm) were used for all experiments.

### Monomer solutions

Monomer solutions containing five different ratios of thiol and acrylate functional groups were created: 0, 10, 30, 40, and 50 mol % thiol functional groups. (Note, throughout this manuscript samples will be referred to in terms of "mol % thiol functional groups." Each thiol monomer molecule contains four thiol functional groups and each acrylate monomer molecule contains two acrylate functional groups. Therefore, a monomer mixture that contains 50 mol % thiol functional groups could also be referred to as a mixture that contains 33.33 mol % thiol monomers.) To each of these mixtures, 3 wt % of the photoinitiator 1-[4-(2-hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propane-1-one (Irgacure 2959, I2959, a gift from Ciba) was added. The monomers used were a diacrylate (poly(ethylene glycol)-diacrylate,  $\overline{M}_n = 1260$  Da, Monomer-Polymer Dejac) and a tetrathiol (pentaerythritol tetrakis(3-mercaptopropionate), Aldrich). Each of the monomer mixtures were heated slightly to melt the diacrylate, diluted with 50 wt % methylene chloride (Fisher Scientific), and thoroughly mixed. These solutions were spotted (>5 nL per spot) using a BioRad ChipWriter Pro microspotter into 5  $\times$  10 arrays that varied in composition by column. Columns were spaced 2 mm and rows 1.5 mm apart. Four arrays were printed per glass slide.

### Polymerization and soluble fraction extraction

The glass slides containing monomer spots were heated to 45°C and purged with argon for approximately 3 min to evaporate any remaining solvent and melt the monomer mixtures before being placed under a UV lamp (BlakRay model B100 AP, peak intensity of 15 mW/cm<sup>2</sup>) for 10 min. The glass slide and arrays of polymer dots were then



**Scheme 1.** Michael-type addition mechanism for base-catalyzed thiol-acrylate coupling. B(−) is the deprotonated base and the arrows show the movement of electrons during the reaction from the thiolate ion (Michael-donor) to the carbon-carbon double bond of the acrylate (Michael-acceptor) and back to the base catalyst to form the Michael adduct.

submerged in a 50/50 v/v mixture of methylene chloride and acetone (Fisher Scientific) for 30 min to extract the sol fraction. The arrays were rinsed and completely dried at ambient conditions before being further processed.

### Postpolymerization modification

Modification of pendant thiol groups was accomplished using both photo-coupling and Michael-type addition<sup>32</sup> reactions. To demonstrate the reactivity of the pendant thiol groups under photopolymerization conditions, the arrays of polymer drops were coated with a thin film of tetraethylene glycol divinyl ether, covered with a glass cover slip, and exposed to UV light for varying amounts of time. The slides were soaked in acetone and methylene chloride to remove excess tetraethylene glycol divinyl ether. Similarly, fluorescein was coupled to the thiol groups through a Michael-type addition reaction by exposing spots to a solution of  $5 \times 10^{-5}$  M acrylated fluorescein in PBS (0.1M, pH 7.4) for varying times. The acrylated fluorescein was prepared as described by Sebra et al.<sup>33</sup> Excess acrylated fluorescein was removed by soaking the slides in deionized water for 24 h. A schematic of the reaction that occurs during thiol-acrylate Michael-type addition is shown in Scheme 1. During this base-catalyzed reaction the nucleophilic thiol acts as the Michael-donor, while the acrylate is the Michael-acceptor.<sup>34</sup>

### Thiol-group quantification

The thiol group concentrations in the monomer spots, polymer dots, and modified polymer dots were quantified using an FTIR microscope (Nicolet continuum, Nicolet). This equipment allows each individual monomer spot or polymer dot to be located (using the software-controlled motorized stage) and scanned using transmittance IR microscopy very quickly, making this technique amendable to high-throughput screening applications. Additionally, this technique is well suited for determining thiol functional group concentration since the IR signal absorbance by the glass slides, which is only significant below  $\sim 2200$   $\text{cm}^{-1}$ , does not interfere with the thiol peak of interest at  $2560$   $\text{cm}^{-1}$  and the polymer dots and monomer spots are thin enough that dispersion of the IR signal is insignificant.

To determine changes in thiol group concentration, changes in the peak area of this thiol S—H absorption

peak between monomer spots, polymer dots, and modified polymer dots were used. Variations in dot thickness were accounted for using an internal reference peak at  $4008$   $\text{cm}^{-1}$ . Molar concentrations were calculated using a thiol density of  $1.28$  g/mL, and the assumption that the PEG-acrylate monomer has the density of pure PEG ( $1.09$  g/mL).

### Photopatterning crosslinked thiol-acrylate polymers

To highlight the ability to chemically modify thiol-acrylate networks postpolymerization with a biologically-significant functionality, thin thiol-acrylate polymer films were patterned using a  $1.5 \times 10^{-4}$  M solution of acrylated PEG<sub>3400</sub>-RGDS<sup>7,16</sup> in deionized water (RGDS is arginine-glycine-aspartic acid, a well-known cell adhesion peptide sequence<sup>1-4,6,35</sup>). Again, the thiol-acrylate samples were polymerized, the soluble fraction removed by extraction, and the samples swollen in the acrylate-PEG-RGDS solution for a minimum of 15 min. The samples were then covered with a photomask and exposed to  $\sim 60$  mW/cm<sup>2</sup> of collimated UV light (365 nm) for 900 s, after which they were rinsed with deionized water (dH<sub>2</sub>O) and soaked in fresh dH<sub>2</sub>O for 6 h. Samples were sterilized overnight by exposure to UV light (254 nm), seeded with NIH 3T3 fibroblasts ( $20,000$  cells/cm<sup>2</sup>), and cultured in DMEM (high glucose Dulbecco's Modified Eagle Medium with 10% (v) fetal bovine serum, 0.2% (v) fungizone, 1% (v) penicillin/streptomycin, and 0.2% (v) gentamicin). The samples were incubated at  $37^\circ\text{C}$  in 5% CO<sub>2</sub> for 24 h before being imaged using an inverted microscope (Eclipse) in bright field.

### Synthesis procedures for the RGD-acrylate monomer

RGDS was synthesized using solid phase methods on an ABI 433A Peptide Synthesizer (Applied Biosystems, Foster City, CA) and following procedures for HBTU (2-(1H-benzotriazol-1,1,3,3-tetramethyluroniumhexafluorophosphate) activation coupling. The peptides, after UV-monitored synthesis, were cleaved from the solid support with a cocktail consisting of 5% phenol, 5% water, and 2.5% triisopropylsilane in trifluoroacetic acid (TFA). The peptide was then washed with copious amounts of ice-cold diethyl ether, redissolved in distilled water, and dialyzed (Spectrum, 500 MW cutoff) over 24 h with two exchanges of distilled water.

RGDS was coupled to acrylated-PEG following a previously reported method.<sup>11</sup> Briefly, RGDS was dissolved in sodium bicarbonate buffer (50 mM, pH 8.4). Acryloyl-PEG-N-hydroxysuccinimide (3400 Da, Nektar Therapeutics) was reacted with the peptide while stirring at room temperature for 2 h. The mixture was dialyzed (Spectrum, 1000 MW cutoff) in distilled water over 24 h with two distilled water exchanges. The dialyzed acryloyl-PEG-RGDS was lyophilized and stored at 4°C until use.

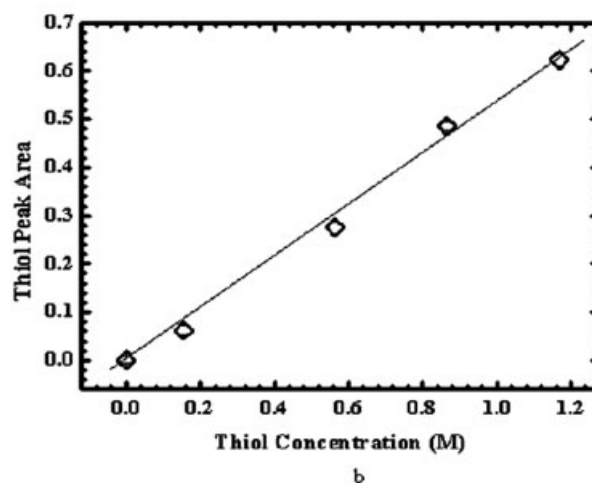
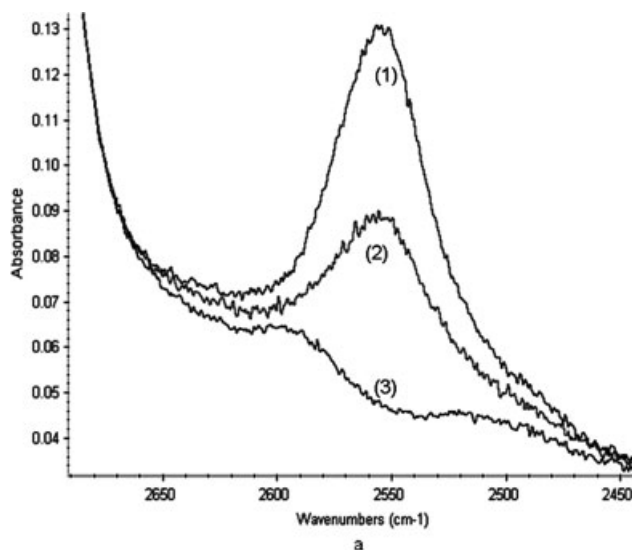
## RESULTS AND DISCUSSION

### Determining thiol concentrations in thiol-acrylate microdot arrays

Monomer solutions with known concentrations of thiol functional groups were printed onto glass slides that had been surface-modified with a methacrylated SAM to form arrays that varied in composition. The IR spectra of the various specimens were obtained using an IR microscope.

Figure 1(a) contains example FTIR spectra obtained from this technique to illustrate how thiol concentrations were quantified. In Figure 1(b), the thiol peak area is plotted as a function of the thiol functional group concentration for thiol-acrylate monomer solutions made from 0, 10, 30, 40, and 50 mol % thiol functional groups. A linear relationship between the thiol IR peak area and thiol functional group concentration was observed as expected. Since the monomer sample containing 50 mol % thiol functional groups represents the highest concentration of thiol functional groups in this study, and since minimal change in microdot volume was observed during any of the FTIR measurements, the slope of the line generated by these calibration experiments can be used to calculate thiol concentrations from the IR thiol peak areas.

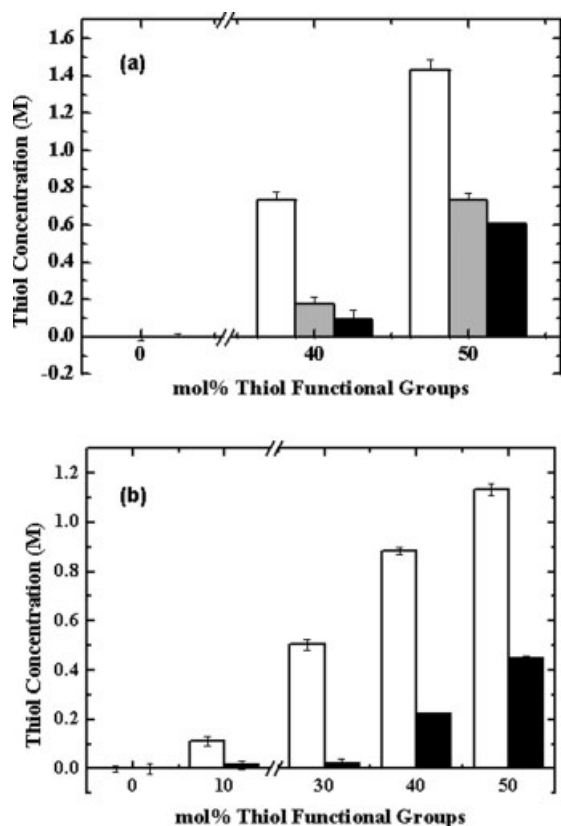
Next, the arrays of monomer microspots were photopolymerized into crosslinked thiol-acrylate networks and the samples were swollen in solvent to remove the sol fraction from each polymer spot. In Figure 2(a), the thiol concentrations are shown for the initial monomer spots, the corresponding polymer dots, and the same polymer dots after the sol fraction was extracted for networks made from 0, 40, and 50 mol % thiol functional groups. These results demonstrate how thiol concentration decreases as the networks are polymerized, and then further decreases (slightly) as the sol fraction is removed. Note that most of the unreacted thiol functional groups remain after the sol fraction extraction because they are tethered into the network as pendant groups. Because the thiol monomers are multifunctional, reaction of one or more thiol functional groups on a particular thiol monomer tethers any



**Figure 1.** (a) Example thiol FTIR spectra of a 50–50 mol % thiol-acrylate monomer spot (1), the polymerized spot (2), and the polymerized spot after modification with tetraethylene glycol divinyl ether (3). (b) The relationship between thiol functional group concentration and thiol peak area in the FTIR spectra of an array of thiol-acrylate monomer microspots. Monomer solutions were prepared with 0, 10, 30, 40, and 50 mol % thiol functional groups (corresponding to thiol concentrations of 0, 0.15, 0.56, 0.87, and 1.17 M).

remaining unreacted thiol groups on that monomer to the network. While a small portion of the original thiol monomers remain completely unreacted, once all of the acrylates are consumed and thus unextractable, most of the residual thiol peak area is from tethered thiol functional groups as demonstrated in Figure 2.

In Figure 2(b), the thiol concentrations of the initial monomer spots and the final polymer dots (with the sol fraction removed) are compared for all monomer formulations examined in this study. Again, a decrease in thiol functional group concentration is observed as the monomer solutions are converted into



**Figure 2.** (a) Thiol functional group concentrations determined from the FTIR thiol peak at  $2560\text{ cm}^{-1}$  for various thiol-acrylate monomer mixtures (white bars), polymer and any unreacted thiol species (gray), and polymer that was extracted in a 50:50 v:v acetone and methylene chloride mixture to remove all soluble monomer then dried before IR spectra were obtained (black). (b) Examination of a thiol-acrylate microarray as monomer (white), and the corresponding polymer spots (black bars, with the sol fraction extracted) for all thiol-acrylate formulations investigated in this study.

polymer networks. This data also reveal that when the polymer dots were formed from monomer solutions containing lower thiol concentrations (e.g. 10 and 30 mol % thiol functional groups), the majority of the thiol functional groups were consumed during network polymerization. In contrast, examination of the microdots formed from the monomer solutions containing 40 and 50 mol % thiol groups reveals at least 25% of thiol groups remain unreacted, even when the acrylate groups are fully consumed.

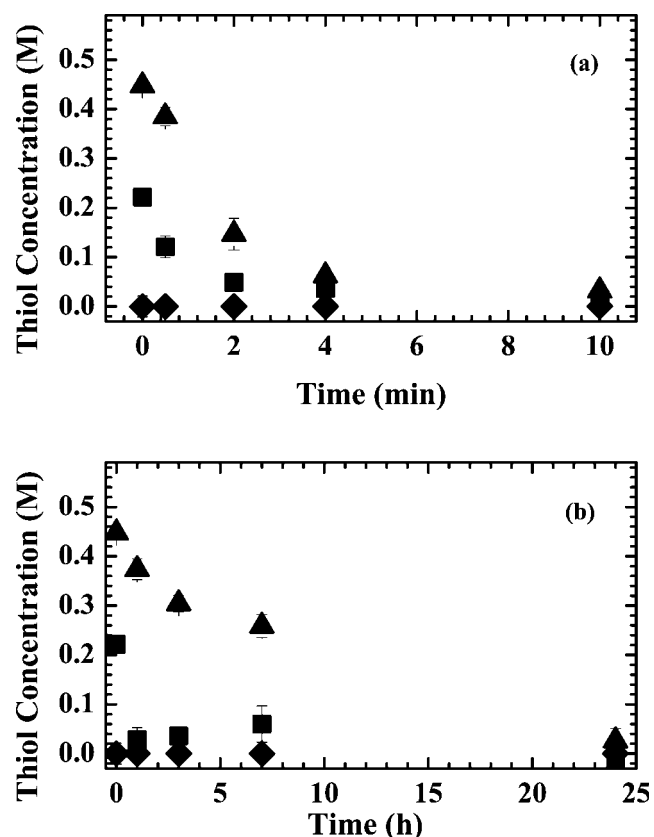
### Chemical modification of the pendant thiol groups

The presence of pendant thiol groups is a significant advantage of thiol-acrylate materials, permitting facile modification of the material's chemistry, which may influence aspects of biocompatibility (e.g., cell-material interactions). This work investigates two different methods for controlling material chemistry

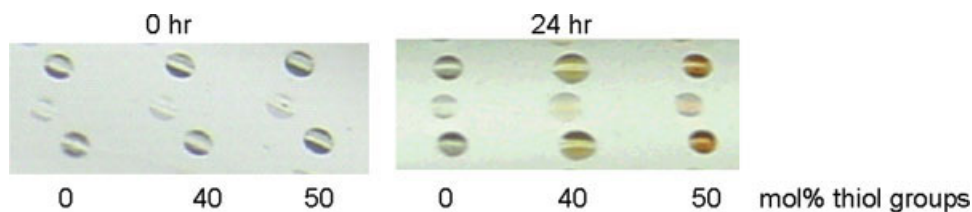
through thiol functional group modification: (1) photoinduced and (2) Michael-type addition coupling.

Figure 3 plots thiol functional group concentrations as a function of modification time for networks synthesized from monomer solutions containing 40 and 50 mol % thiol groups. These results indicated that as modification time increases, the thiol functional group concentrations decreased. The data shown in Figure 3(a) depict this decrease for networks where the pendant thiol groups are modified through photocoupling reactions with a vinyl ether-terminated monomer. These results indicate that photocoupling of the pendant thiols with vinyl ethers is fairly rapid (the thiol groups are fully consumed after 10 min under these conditions), but these observations depend upon the type of vinyl-reactive group, the chemistry of the modifier, and the amount of light energy delivered during the modification reaction. The observed decrease in thiol functional group concentrations is related to the extent of modification that occurs.

Figure 3(b) depicts how pendant thiol concentration changed as the Michael-type addition coupling



**Figure 3.** Disappearance of thiol functional groups as a function of time during (a) photo-coupling with tetraethylene glycol divinyl ether and (b) Michael-type addition coupling with  $5 \times 10^{-5}\text{ M}$  fluorescein-PEG-acrylate in 0.1 M PBS for polymer dots made from monomer solutions containing 0 (◆), 40 (■), and 50 (▲) mol % thiol functional groups.



**Figure 4.** Digital pictures of thiol-acrylate networks that have been exposed to  $5 \times 10^{-5}$  M fluorescein-PEG-acrylate in 0.1M PBS for (a) 0 and (b) 24 h. In both pictures, polymer dots in the left column are made from a monomer mixture containing 0 mol % thiol, the middle column from a 40 mol % thiol mixture, and the right column from a 50 mol % thiol mixture. Note: in both images the spots and their reflection off of the bottom of the glass slide are visible. The dimmer spots are the reflections of the actual polymer dots. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

proceeded. Even though the Michael-type addition coupling was much slower than photocoupling with the vinyl ether monomer, almost complete modification was observed for the samples after 24 h. In addition to these FTIR results, the samples modified with the acrylated fluorescein also exhibited an increase in color with increasing thiol monomer concentration and with increasing modification time. These results are shown in Figure 4, which depicts a portion of a microdot array both initially and after 24 h of Michael-type addition coupling with the acrylated fluorescein. Initially, all three types of polymer networks (synthesized from monomer formulations containing 0, 40, and 50 mol % thiol functional groups) show no difference in color. When these same samples were exposed to the acrylated fluorescein solution for 24 h, color was observed to increase as the pendant thiol concentration increased. Thus, samples that were modified through Michael-type addition coupling provided visual confirmation that the fluorescein was covalently attached to the thiol-acrylate material.

The results from these studies point to three main conclusions: (1) pendant thiol groups remain in thiol-acrylate photopolymer networks, (2) vinyl-group coupling of chemical molecules, including PEG and fluorescein, to the thiol-acrylate networks is achievable, and (3) the extent of chemical modification that occurs is easily controlled through variations in the pendant thiol group concentration and the duration of the modification procedure.

### Advantages of post-gelation modification

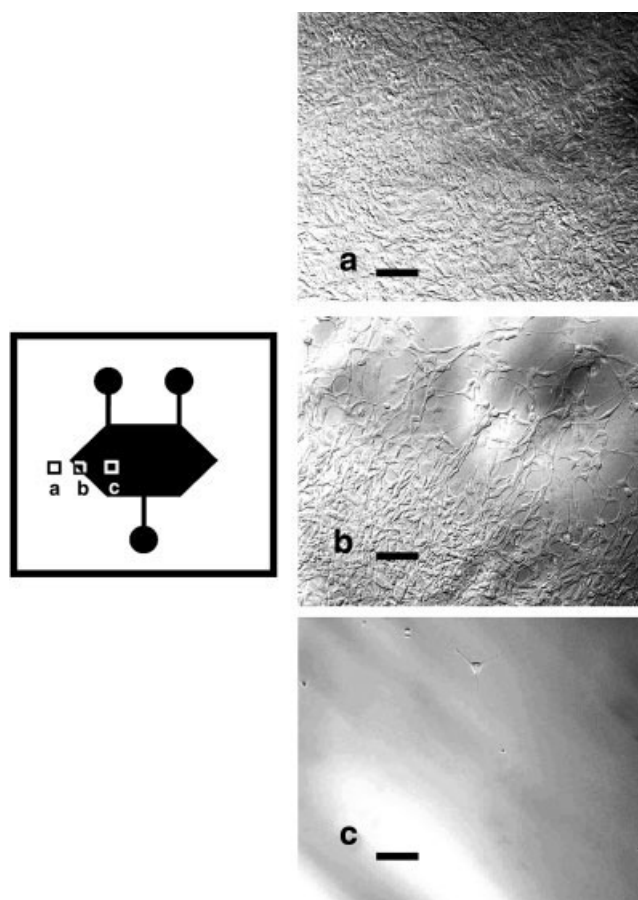
Chemical modification of crosslinked polymer networks is usually accomplished by incorporating the chemical modifier molecules into the solution of network precursors, whereby these molecules are randomly incorporated throughout the bulk of the network through copolymerization.<sup>1,6,7,11,16,36</sup> Chemical modification of the thiol-acrylate networks presented in this research is different in that pendant thiol

groups randomly form during network formation. The fact that these pendant groups are easily modified postpolymerization affords a number of advantages. The one that will be highlighted here is the creation of patterned devices using photoinduced modification in combination with standard photolithographic techniques.

In this experiment photocoupling was used to pattern a thin film of thiol-acrylate with a solution of acrylated-PEG-RGD to create regions within the same device which permit very different types of cell-material interactions. RGD is an attachment peptide sequence commonly found in native proteins such as fibronectin, vitronectin, laminin, and collagen.<sup>37</sup> Incorporating RGD into noncell adhesive biomaterials enables cell attachment and spreading,<sup>5</sup> which are necessary for the survival of anchorage-dependent cell types (e.g., the fibroblasts used in this study).

Here, a thin film of a polymerized thiol-acrylate network with a significant amount of residual thiol groups was photopatterned with an acrylated PEG-RGD monomer. When anchorage-dependent NIH 3T3 fibroblasts were seeded onto the surface of this patterned device, cell attachment was observed to vary significantly between regions with RGD modification compared with regions without RGD. Sample images of this behavior are shown in Figure 5. In this figure, the top image is of a region of the network that was modified through photocoupling of the RGD to the pendant thiols. The middle image is of a region at the boundary between where RGD coupling occurred and where it was blocked. The bottom image is of a region that was covered by the photomask and not exposed to light during the photocoupling reaction. Since the initial polymer substrate is the same for all images, the observed differences in cell attachment and spreading were attributed to the presence or absence of tethered RGD segments on the surface of the thiol-acrylate film.

While the modification conditions for the acrylate-PEG-RGD are not yet optimized, this initial result demonstrates that bioactive peptides, such as RGD, can be selectively patterned onto thiol-acrylate net-



**Figure 5.** 2D attachment of NIH 3T3 fibroblasts to a 50 mol % thiol-acrylate film that was photopatterned using  $1.5 \times 10^{-4}$  M acrylated RGD solution. The first image is a copy of the photomask that was used to pattern the photopolymer. The small boxes that are labeled "a," "b," and "c" correspond to the subsequent microscope images. These images demonstrate differences in cell attachment between regions of the same polymer sample that were and were not modified. The top image is of a region that was fully modified with RGD, the middle image is of a region where the bottom left of the image was modified and the top right was not, and the bottom image is of a region where no photocoupling occurred. Scale bar = 100  $\mu$ m.

works to control cell attachment. It further confirms the work of many other researchers that has identified the importance of providing the proper integrin receptors and binding domains to neighboring or encapsulated cells to maintain their viability and control their attachment. More importantly, it identifies these thiol-acrylate polymers as materials for both the development of improved biomedical devices, as well as for use as a research tool that is ideally suited for high-throughput cell-material interaction studies. The results presented in this paper demonstrate how pendant-thiol modification is a facile means to control signal (e.g., RGD) density through variations in the initial monomer formulation and the duration of the modification procedure.

Additionally, photopatterning could be used to generate devices rapidly that probe how the size and shapes of patterned regions impact cell-material interactions. Further, cell attachment to modified arrays of microspots may provide a high-throughput tool for rapidly screening cell-material interactions.

## CONCLUSIONS

Thiol-acrylate photopolymers provide a diverse platform for probing the impact of network chemistry on cell-material interactions. Networks containing pendant, reactive thiol groups are easily created, even at complete acrylate conversion. Additionally, variations in the stoichiometry of the thiol and acrylate functional groups in the initial monomer mixture control the concentration of pendant thiols within the photopolymer. This fact, combined with the length of time the pendant thiols are exposed to the modification conditions, allows intimate control over the concentration of modifier that is covalently attached to the thiol-acrylate network. Because these thiol-acrylate materials are capable of being chemically modified after network formation is complete, the material's chemistry and mechanical properties are decoupled, providing a material that can be precisely tuned for a variety of biomaterial applications.

Additionally, when the network chemistry is modified using photocoupling reactions, the extent of modification can be further controlled to generate polymers that are photopatterned with different modification moieties. When combined with high throughput analyses, these networks provide opportunities to advance the biomaterial field's understanding of many cell-material interactions by facilitating rapid screening and optimization of complex combinations of numerous ligands.

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