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ARTICLE

Fabrication of Maleimide Containing Thiol Reactive Hydrogels via Diels−Alder/Retro-Diels−Alder Strategy

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ABSTRACT: Poly(ethylene glycol) methacrylate-based hydrogels containing thiol reactive maleimide functional groups have been synthesized using a novel Diels−Alder cycloaddition/cycloreversion-based strategy. Masked maleimide groups are directly incorporated into the hydrogel matrix during the gelation process by utilization of a furan protected maleimide containing methacrylate monomer. During the polymerization, the thermal deprotection of the maleimide groups in some of the monomer results in the formation of an *in situ* cross-linker that results in gelation. After gelation, the protected maleimide groups can be activated to their reactive forms via a thermal cycloreversion step. The efficiency of the gel formation, maleimide incorporation, and functionalization of the hydrogel were investigated. These reactive maleimide group embedded hydrogels can be efficiently derivatized with thiol containing molecules such as a fluorescent dye, BodipyC10SH. Thiolated biotin derivatives were covalently attached to these hydrogels under mild, reagent-free conditions. It was found that the extent of immobilization of FITC-streptavidin onto these biotinylated gels can be tailored by varying the density of maleimide groups in the parent hydrogels.

Introduction

Hydrogels have become materials of interest in a wide variety of fields such as biomolecular immobilization, tissue engineering, sensors, implant materials and implant coatings, and drug delivery.[1−6] Such amplified interest also necessitates synthesis of novel hydrogel materials to widen the scope of intended applications of such materials. The functionalization of the hydrogels is generally accomplished either by physiabsorption or by covalent attachment of molecules of interest. For applications such as scaffolds for protein delivery in tissue engineering, guided cell growth and controlled drug delivery, control over attachment, and immobilization using covalent attachment of molecules of interest may be more desirable.[7−13] Hydrogels that contain molecules of interest integrated into the material through covalent attachment usually utilizes employment of a functional acrylate containing comonomer during the hydrogel synthesis. Heterobifunctional PEG such as acryloyl-PEG-NHS polymer has been widely utilized. This polymer contains an activated ester at one terminus that allows attachment of any molecule of interest using the amidation chemistry, while the acrylate group at the other end allows covalent integration into the hydrogel. In most cases, the macromonomer containing the *N*-hydroxysuccinimide-based activated ester is functionalized with the peptide fragment of interest prior to gelation. Hydrogels containing covalently attached biotin and the cell adhesive peptide RGDS have been fabricated using this approach.[14−16] Alternatively, addition of monomers containing reactive groups that would allow postgelation modification into the feed would afford functionalizable hydrogels. Incorporation of glycidyl methacrylate as a reactant during gelation renders the obtained hydrogel reactive toward functionalization using electrophilic oxirane ring-opening reactions.[17] The latter approach that allows postfunctionalization of hydrogels is attractive since it circumvents the requirement of converting molecules of interest such as peptides or oligonucleotides into reactive acrylates. Smart hydrogel design can allow the postfunctionalization step to allow spatially controlled activation within a 3D hydrogel. Shoichet and co-workers reported fabrication of hydrogel matrix containing photolabile protected sulfhydryl groups. Upon spatially controlled photodeprotection, the reactive thiol groups were utilized to immobilize maleimide-labeled biomolecules such as fluorescein-tagged maleimide-terminated GRGDS peptide unit.[18]

For immobilization of biomolecules, a much sought after functionalization is via thiols since many biomolecules either contain or can be incorporated with cysteine residues at specific sites. Sulfhydryl group of the cysteine residues in biomolecules undergo facile reactions with maleimides,[19,20] orthopyridyl disulfide units,[21−23] and vinyl sulfones.[24−28] The aforementioned functional groups have been incorporated in various polymers to provide a handle for conjugation of biomolecules to polymers to obtain polymer−biomolecule conjugates. Although the maleimide group has been extensively exploited in biomolecular immobilization using monolayers on various metallic and glass surfaces,[29−32] examples of polymeric materials with maleimide side chains have been very limited. The limitation stems from the tendency of the reactive double bond of maleimide to participate in radical polymerization. Recently, remarkable advances have been made in this area by utilization of protected maleimide-based initiators and monomers to obtain polymers with maleimide as their end groups and as side chains using a Diels−Alder reaction-based maleimide protection−deprotection strategy.[33−38] Our approach here outlines an extension of the concept to fabricate maleimide containing antibiofouling hydrogels amenable toward specific covalent functionalization.

Herein we describe the synthesis, characterization, and conjugation studies of a novel maleimide functional group containing thiol reactive hydrogel. The synthetic strategy utilizes a furan protected maleimide-based methacrylate monomer that undergoes an *in situ* activation to a cross-linker during free radical polymerization to induce gelation. Control over incorporation of reactive maleimide units within the gel via temperature and feed

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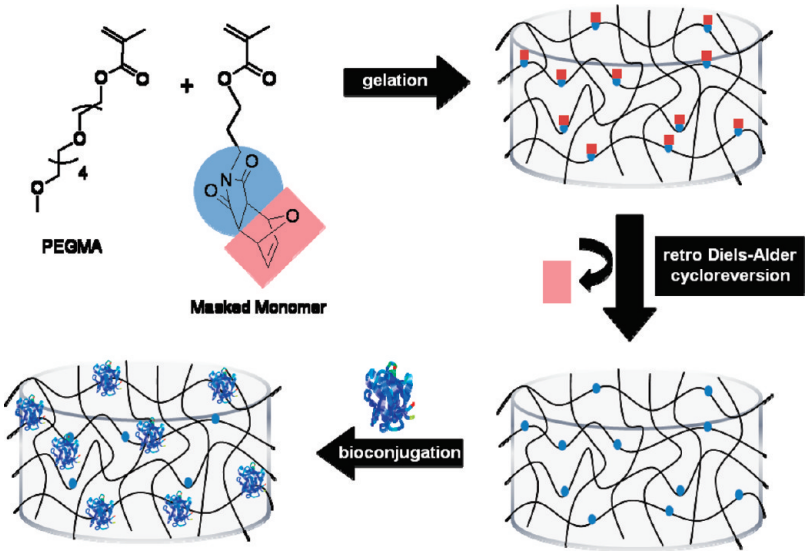

Figure 1. Overall scheme for the preparation and functionalization of maleimide containing hydrogels.

Table 1. Properties of Hydrogels with Variations in Feed Ratios, Temperature, and PEG M_w

item	hydrogels	temp (°C)	PEGMA M_w	monomer **I**:PEGMA[a]	furan (%) observed[b]	furan (%) theoretical[c]	gel content (%)[d]
1	**H1**	75	300	1:10	2.21	2.06	96.0
2	**H2**	75	300	2:10	3.54	3.79	93.1
3	**H3**	75	300	4:10	5.11	6.51	96.2
4	**H4**	90	300	1:10	1.59	2.06	90.4
5	**H5**	90	300	2:10	2.37	3.79	92.3
6	**H6**	90	300	4:10	3.22	6.51	94.6
7	**H7**	75	750	4:10	2.81	3.14	95.7
8	**H8**	75	1100	4:10	2.26	2.23	94.1

[a] Based on mole ratio. [b] Calculated from the amount of furan released as observed in TGA thermograms (wt %). [c] Calculated based upon feed ratio of comonomer prior to gelation (wt %). [d] Gel content = (dry gel weight/total weight of monomer used) × 100.

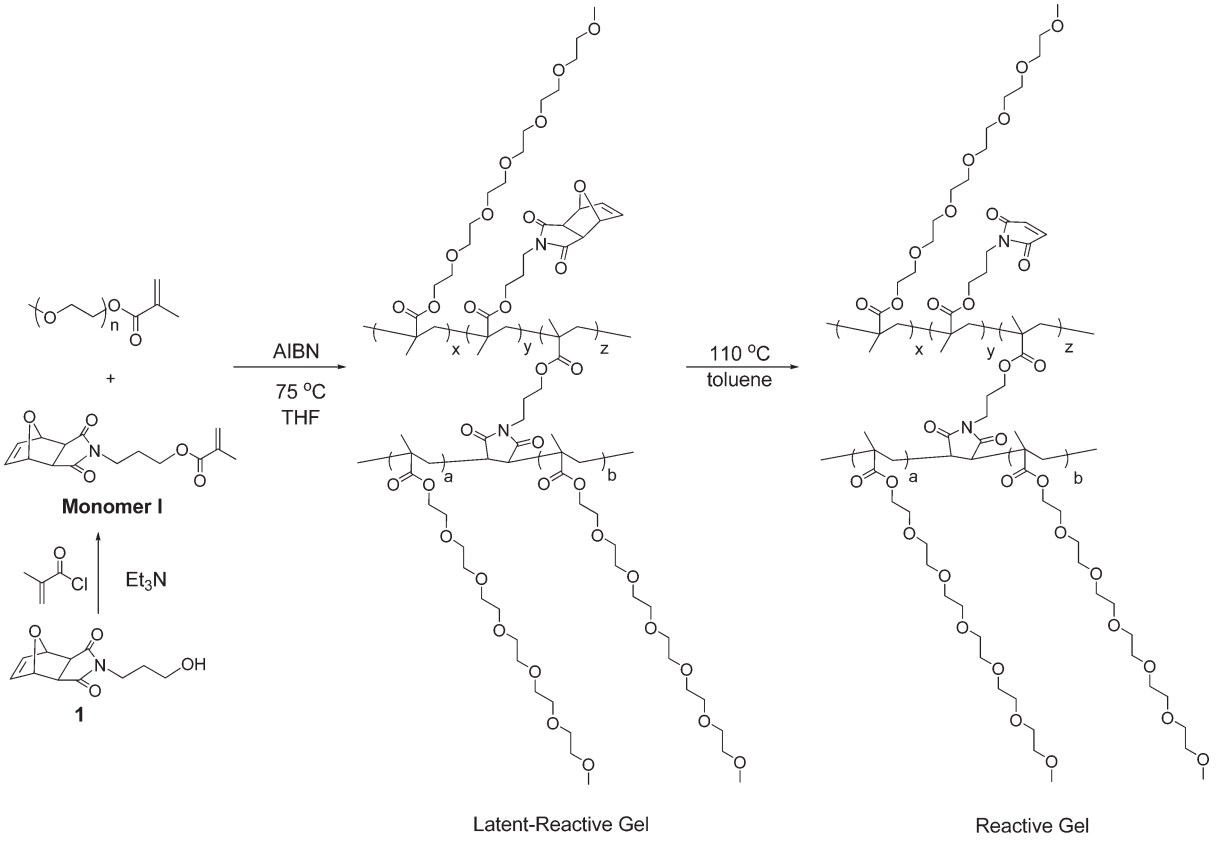

Figure 2. Overall reaction sequence for the synthesis of maleimide containing hydrogels.

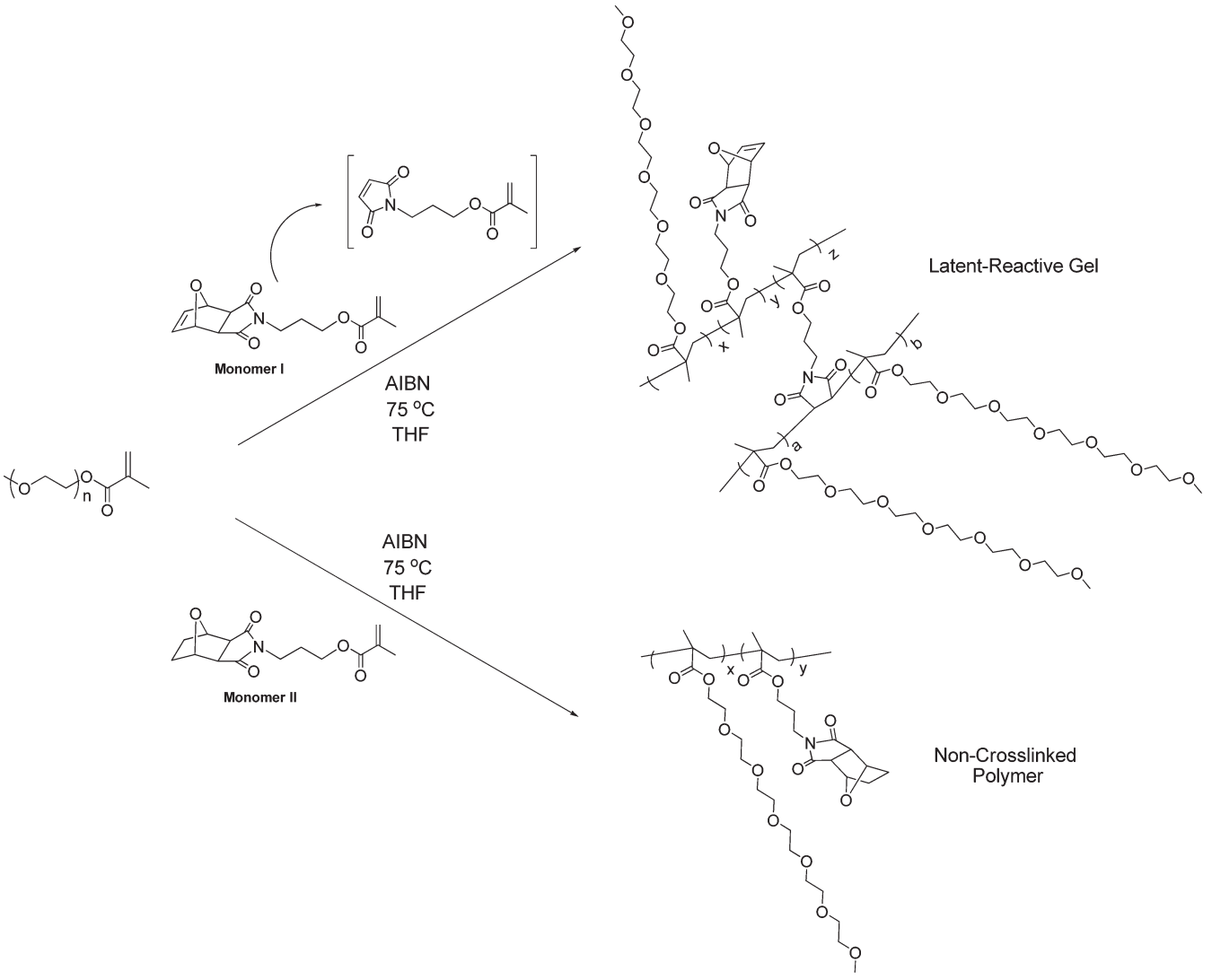

Figure 3. Investigation of the origin of cross-link formation in the hydrogels.

ratio is investigated. The control on maleimide reactive group density within the gel allows a handle on degree of covalent functionalization of the hydrogel matrix.

Experimental Section

Materials and Methods. Poly(ethylene glycol) monomethyl ether methacrylate (PEGMA, MW 300) and 2,2-azobis(isobutyronitrile) (AIBN) were purchased from Aldrich Chemical Co. 2,2-Azoisobutyronitrile (AIBN) was purified by recrystallization from ethanol. Poly(ethylene glycol) monomethyl ether methacrylate (PEGMA, MW 750) was purchased from Alfa Aesar. Methacryloyl chloride was obtained from Alfa Aesar and used as received. Biotiylated (triethylene glycol) undecanethiol was obtained from Nanoscience Instruments (Phoenix, AZ). Fluorescein (FITC) conjugated streptavidin is obtained from Pierce and used as received. BodipyC10SH was synthesized according to literature procedure.[39] Alcohol *exo*-3a,4,7,7a-tetrahydro-2-(3-hydroxypropyl)-4,7-epoxy-1*H*-isoindole-1,3(2*H*)-dione (**1**) was prepared according to the literature.[40] Thermogravimetric analysis (TGA) was carried out on a TA Instruments at a heating rate of 10 °C/min under a nitrogen atmosphere.

Synthesis of Monomer I. The furan protected maleimide monomer was prepared as reported before.[33] Briefly, to a solution of the alcohol, *exo*-3a,4,7,7a-tetrahydro-2-(3-hydroxypropyl)-4,7-epoxy-1*H*-isoindole-1,3(2*H*)-dione (**1**) (2.00 g, 8.86 mmol), and triethylamine (1.05 mL, 10.63 mmol) in dichloromethane (120 mL) at 0 °C was added methacryloyl chloride (0.91 mL, 9.39 mmol) in 0.1 mL portions over 30 min. The clear solution was stirred for 2 h at 0 °C. To the reaction mixture was added dichloromethane (40 mL), and the mixture was washed with saturated $NaHCO_3$ (2 × 40 mL) and H_2O (2 × 40 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated to give a yellow residue that was purified by flash chromatography on SiO_2 (EtOAc:CH_2Cl_2 1:1), affording 2.50 g (96% yield) monomer as a white waxy solid. ^{1}H NMR ($CDCl_3$, δ, ppm): 6.49 (s, 2H, C*H*=C*H*), 6.11 (s, 1H, CH_2=C), 5.55 (m, 1H, CH_2=C), 5.24 (s, 2H, C*H* bridgehead protons), 4.09 (t, 2H, J = 6.2 Hz, OCH_2) 3.59 (t, 2H, J = 7.0 Hz, NCH_2), 2.82 (s, 2H, C*H*−C*H*, bridge protons), 1.98−1.91 (m, 5H, $CH_2CH_2CH_2$ and CH_3). ^{13}C NMR ($CDCl_3$, δ, ppm): 176.0, 167.1, 136.4, 136.1, 125.4, 80.8, 61.4, 47.3, 35.7, 26.6, 18.2. FTIR (cm^{-1}) 1705.8.

Representative Hydrogel Synthesis. Three series of hydrogels were synthesized at both 75 and 90 °C containing different monomer ratios as represented in Table 1. Briefly, to a vial containing furan protected maleimide monomer (12.3 mg, 0.042 mmol) and 2,2-azobis(isobutyronitrile) (AIBN, 4.6 mg, 0.028 mmol) was added degassed PEGMA (0.12 mL, 0.42 mmol) in dry, degassed THF (0.14 mL). Then, the reaction vials were sealed and placed in an oil bath for 30 min. The hydrogels were purified by washing several times with methanol under sonication to remove any unreacted monomers. Conversions determined gravimetrically based on gel content were usually above 90%.

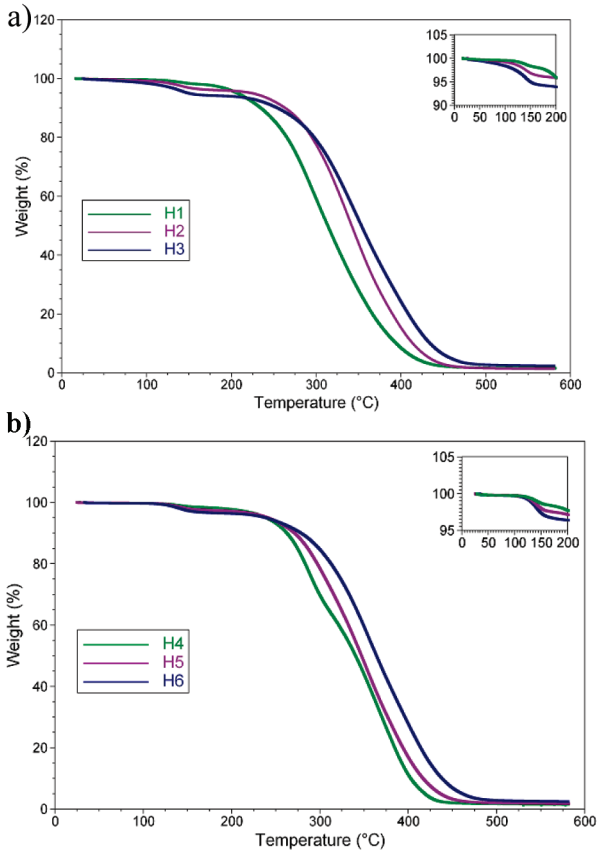

Figure 4. TGA thermograms of (a) hydrogels (**H1**−**H3**) prepared at 75 °C and hydrogels (**H4**−**H6**) prepared at 90 °C. The above thermograms illustrate the effect of the feed ratio on the amount of incorporation of masked maleimide monomer into the hydrogels.

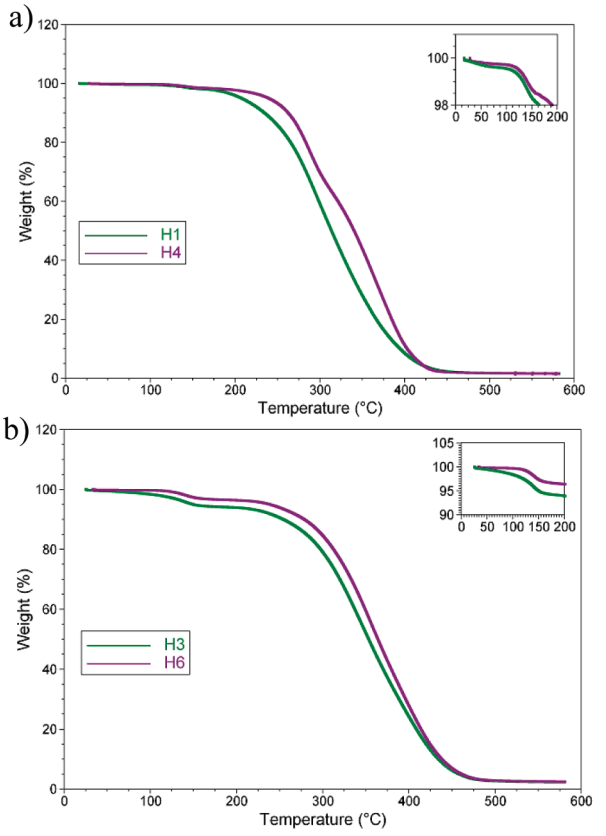

Figure 5. TGA thermograms of (a) hydrogels **H1** and **H4** prepared at 75 °C and (b) hydrogels **H3** and **H6** prepared at 90 °C. The above thermograms illustrate the effect of the temperature on the amount of incorporation of masked maleimide monomer into the hydrogels.

Activation of the Hydrogels. Dried hydrogels were heated at 110 °C in anhydrous toluene for 4 h. Thermogravimetric analysis (TGA) demonstrated that quantitative conversion of the oxabicyclic moiety to the maleimide functional group was achieved. The hydrogel samples obtained after this thermal activation step did not show any significant weight loss corresponding to the loss of furan. TGA of hydrogel samples prior to activation step showed expected weight loss due to release of furan (see Results and Discussion).

Swelling Studies. A circular piece of purified and dried hydrogel was transferred to a flask containing distilled/deionized water at room temperature. The mass of the hydrogel sample was recorded regularly after removing the hydrogel from solution and drying the surface with a filter paper.

Scanning Electron Microscopy. Swollen hydrogels were lyophilized and scanning electron microscopy was used to characterize the morphology of the hydrogels. The hydrogel was immersed in liquid nitrogen and broken, and images were taken using ESEM-FEG/EDAX Philips XL-30 (Philips, Eindhoven, The Netherlands) instrument using an accelerating voltage of 10 kV.

Functionalization with Fluorescent dye BodipyC10SH. Hydrogels were functionalized with a fluorescent dye, BodipyC10SH, after activation. To a degassed solution of BodipyC10SH (2.7 mg) in dry THF (0.5 mL) was added a dried sample of **H1** (Figure 2) (10.0 mg, 2.21% furan) and reacted for 12 h. Hydrogel was washed several times with THF, and fluorescent images were taken. As a control experiment to account for absence of entrapment of the dye within the gel matrix, the same hydrogel was incubated with a solution of BodipyC10Br and washed several times with THF.

Functionalization with Streptavidin. To a degassed solution of Biotin-SH (4.2 mg) in MeOH (0.5 mL) was added a dried sample of **H1** (Figure 2) (10.0 mg, 2.21% furan) and reacted for 12 h. Hydrogel was washed several times with MeOH and incubated with a solution of FITC conjugated streptavidin (0.1 mg/mL PBS) for 20 min. After incubation, the sample was washed with PBS and deionized water several times, and fluorescent images were taken. As a control, hydrogel without the biotinylation procedure was incubated with a solution of streptavidin and was washed with PBS and deionized water several times before fluorescence microscopy.

Results and Discussion

Synthesis and Characterization of Hydrogels. The latent-reactive monomer **I** (Figure 2) was synthesized by the reaction of the alcohol, *exo*-3a,4,7,7a-tetrahydro-2-(3-hydroxypropyl)-4,7-epoxy-1*H*-isoindole-1,3(2*H*)-dione (**1**), with methacryloyl chloride in the presence of triethylamine at 0 °C (Figure 2). The furan protected maleimide containing alcohol was obtained according to a reported literature procedure[40] in pure *exo* form. Copolymerization of the latent-reactive maleimide monomer **I** with PEGMA, initiated by AIBN in degassed THF, at high concentrations in a sealed glass vial led to gelation of the reaction mixture within 30 min. Obtained gels remain insoluble in various solvents such as water, methanol, and DMF at high temperatures.

It is proposed that the observed cross-linking during the formation of these covalently functionalizable gels occurs due to the *in situ* retro-Diels−Alder reaction of the furan protected monomer. The release of furan produces a monomer containing two reactive double bonds. This fragment acts as a cross-linker. The amount of this reactive fragment generated during the reaction is dependent on the reaction temperature. As a control experiment, gelation reaction was attempted using a nonlatent reactive monomer **II**, incapable

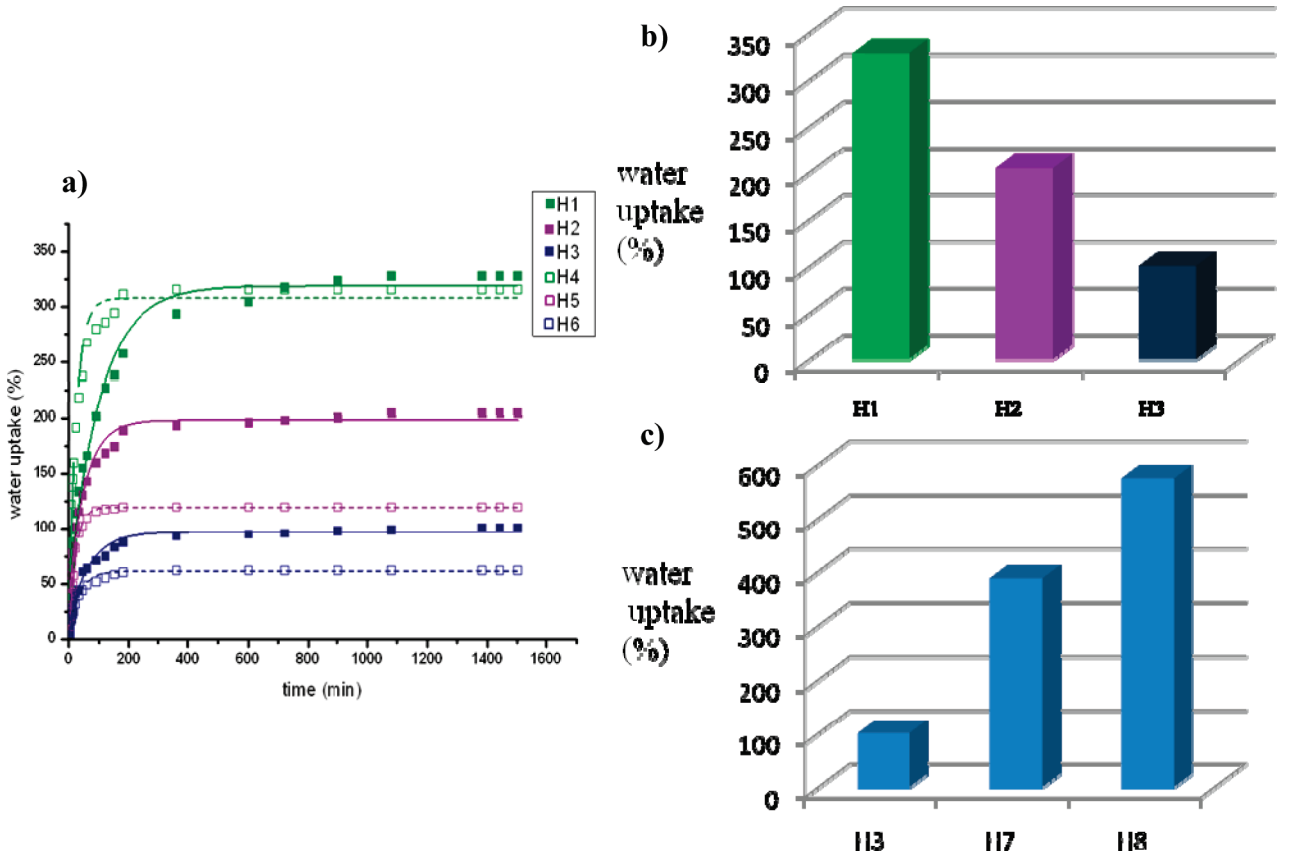

Figure 6. (a) Effect of temperature and feed ratio on the degree of swelling of the hydrogels. (b) Effect of increasing amount of maleimide monomer on relative swelling capacities of hydrogels. (c) Effect of increasing PEG chain lengths on swelling capacities of the hydrogels.

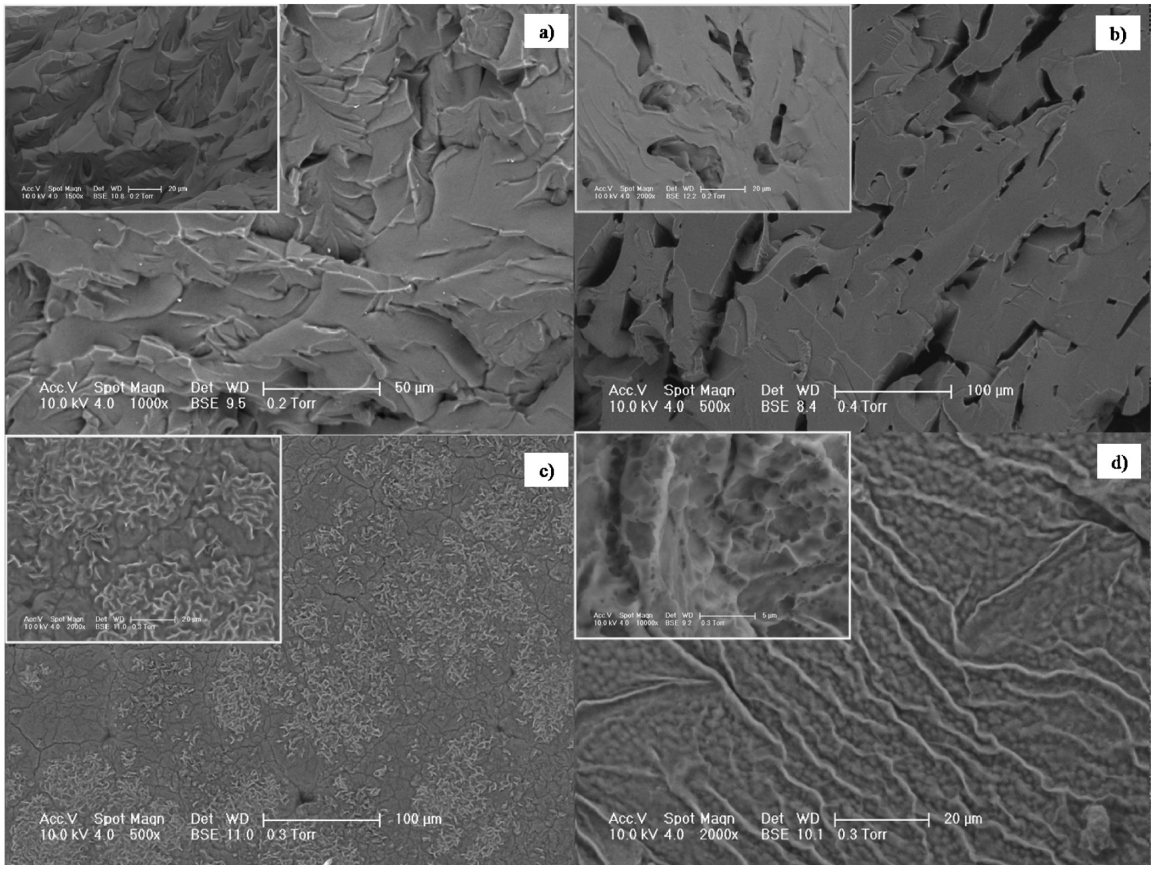

Figure 7. Representative SEM micrographs of hydrogels (a) **H4**, (b) **H3**, (c) **H7**, and (d) **H8**.

of undergoing a retro-Diels−Alder cycloreversion to generate the cross-linker. As expected, upon using various amounts of this nonlatent monomer in the reaction feed, no gels but only soluble polymers were obtained with monomer **II** incorporated as side chains.

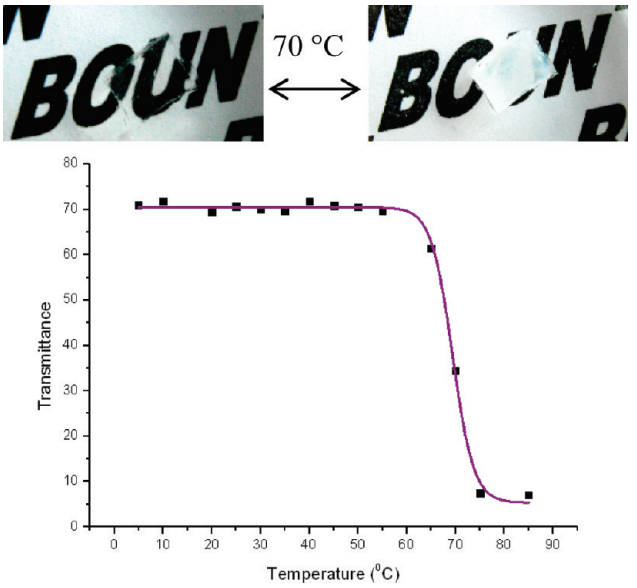

Figure 8. Thermoreversible nature of bulk hydrogel **H2** monitored by heating the gel. Transition temperature was measured by monitoring the loss of transmittance upon heating.

The physical and chemical characteristics of the hydrogels would be dependent on the experimental condition such as temperature of gelation, monomer feed ratio, hydrophilicity of monomers, and postgelation treatment. A variety of different hydrogels were synthesized in order to investigate the effect of gelation temperature, feed ratio, and chain length of poly(ethylene glycol) in PEGMA on their properties. These results are summarized in Table 1, and the observations are discussed thereafter.

Effect of Feed Ratio on the Amount of Maleimide Group Incorporation in the Hydrogel. One can expect that an increase in the amount of protected maleimide monomer in the feed ratio will result in formation of gels with higher content of protected maleimide units under the same experimental conditions. The amount of protected maleimide units within the gel can be probed using thermogravimetric analysis (TGA). The weight loss between 60 and 180 °C corresponds to the removal of the furan molecules generated during the retro-Diels−Alder reaction.[41] As expected, an increase in the loss of furan moiety is observed for gels with higher content of latent reactive monomer (Table 1 and Figure 4). This trend was observed for hydrogels prepared at 75 °C (**H1**−**H3**) and 90 °C (**H4**−**H6**). Thus, the amount of incorporated reactive maleimide group within the gels can be tailored by adjusting the feed ratio.

Effect of Temperature on the Amount of Maleimide Group Incorporation in the Hydrogel. Increase in the reaction temperature should lead to more *in situ* fragmentation of the latent reactive monomer to produce more of the cross-linking reactions. This would result in decrease in the amount of protected maleimide units within the gel. Hydrogels were prepared at

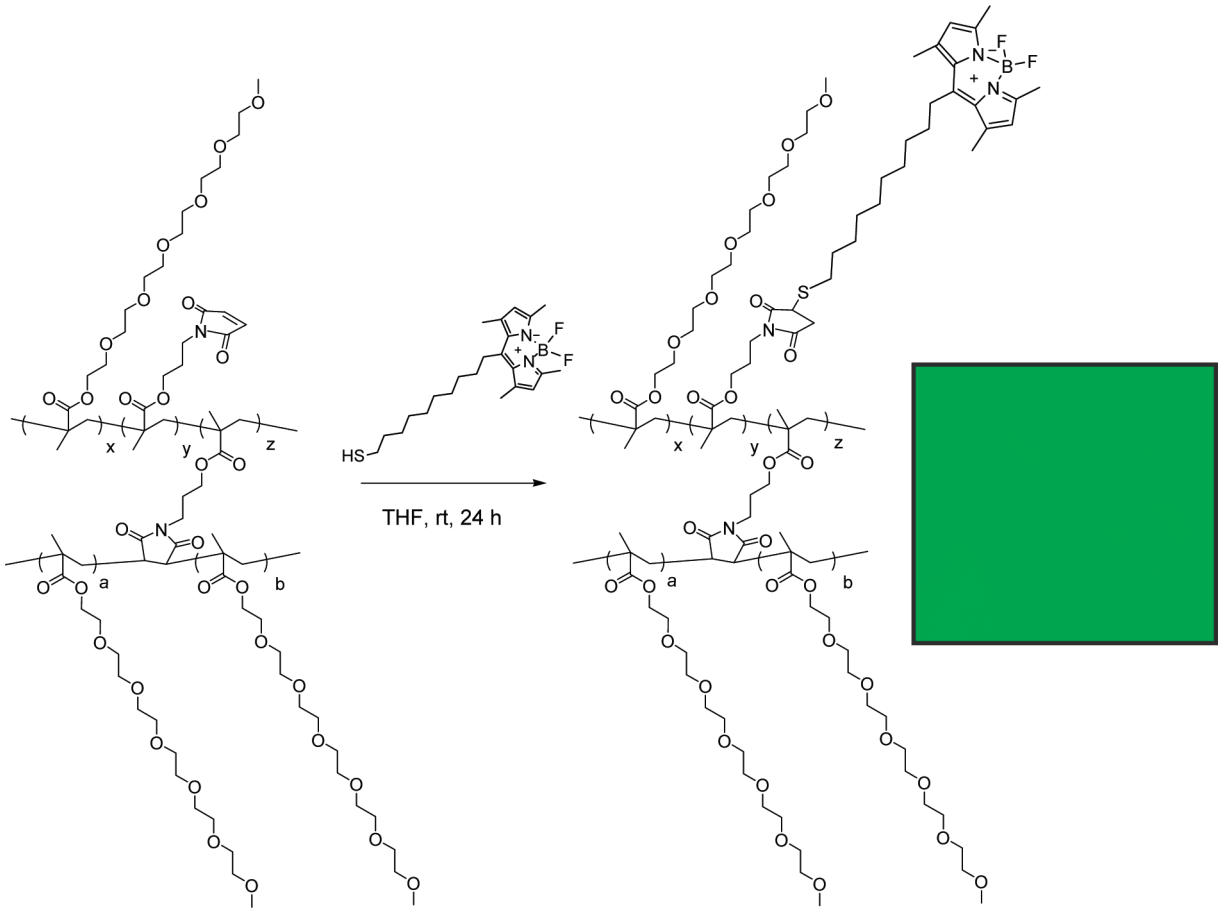

Figure 9. Functionalization of hydrogels with fluorescent dye BODIPYC10SH. Color inset shows a fluorescence microscope image of BODIPY functionalized hydrogel **H1**.

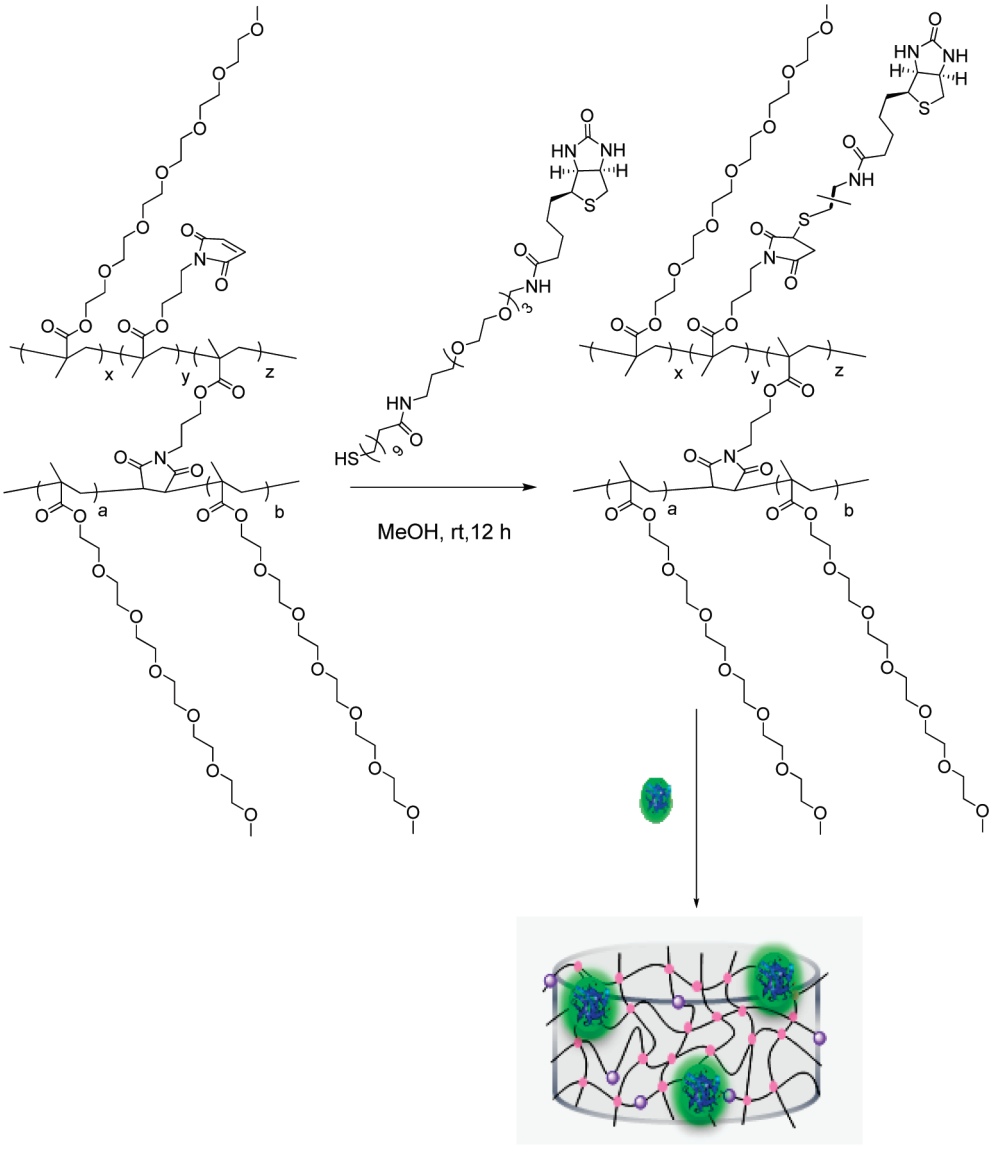

Figure 10. Immobilization of FITC-streptavidin on hydrogels after covalent biotinylation.

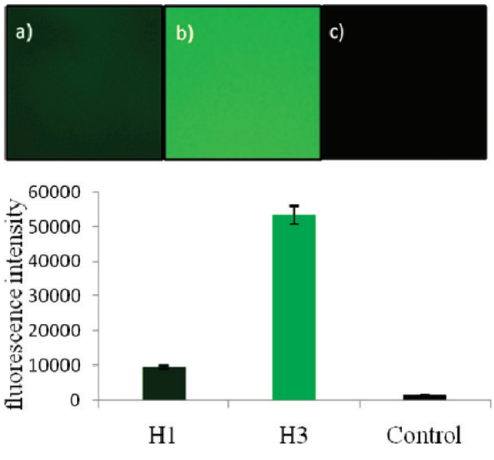

Figure 11. Fluorescence microscope images of FITC-streptavidin bound (a) **H1**, (b) **H3**, and (c) **H3** control hydrogels. Graph shows the relative fluorescence intensities of enzyme bound **H1**, **H3**, and **H3** control gel. Control gels did not contain any covalently bound biotin ligands.

different temperatures for the same feed ratio of the monomers. As expected, the TGA analysis of the gels revealed a decrease in the amount of furan released as the temperature of gelation was increased.

Swelling Studies of the Hydrogels. Swelling behavior of these hydrogels was probed gravimetrically by recording the water uptake by the hydrogel over time until they reach equilibrium. It was observed that an increase in the amount of monomer **I** in the feed ratio led to a decrease in the swelling capacity of the hydrogel (Figure 6). This behavior could be either due to the increase in the fraction of hydrophobic monomer within the hydrogel matrix or due to increased cross-linking obtained upon increasing the amount of furan protected maleimide monomer. Increase in cross-linking upon the increase of monomer **I** in feed ratio was also evident from the TGA results as discussed before. Swelling capacity can be notably increased by utilizing a higher molecular weight PEGMA monomer (Figure 6c).

Surface Morphology of the Hydrogels. Microstructures of the hydrogels were investigated with scanning electron microscopy (Figure 7). For low molecular weight PEGMA-based hydrogels, a continuous gel structure was observed. From the SEM micrographs, some evidence of increase in porous microstructures could be seen in the hydrogels obtained with higher molecular weight hydrophilic monomers. Less porous

microstructure of these gels are perhaps not very surprising, considering that these are cross-linked along the backbone of the polymers as opposed to microporous gels commonly obtained using polymers cross-linked at chain ends.

In recent years, poly(ethylene glycol) methacrylate has emerged as an alternative building block to the well-known *N*-isopropylacrylamide-based polymers for the fabrication of thermoresponsive materials. It has been well documented in recent years that polymers containing pendent oligoethylene chains exhibit temperature stimuli behavior.[42] The presence of PEGMA in the scaffold the maleimide containing hydrogels renders them temperature sensitive. While the parent hydrogels were clear and transparent, loss of transparency was observed upon warming the gel (Figure 8). For hydrogels fabricated with PEGMA ($M_w = 300$) a transition temperature near 70 °C was obtained upon monitoring the loss of transmittance with increase in temperature using a UV−vis spectrophotometer. This change was reversible as complete recovery was transparency was observed upon cooling of the gel.

Functionalization of Hydrogels via Michael Type Thiol−Ene Addition. The efficiency of functionalization of these gels was studied by conjugation of a thiol containing fluorescent dye BodipyC10SH. Hydrogel **H1** was selected for this purpose and was incubated with BodipyC10SH and washed with methanol to remove any unbound dye. As a control experiment, gels were incubated with a solution of BODIPY-Br and washed with methanol to remove any physiabsorbed dye. While the gel treated with BODIPY-SH was highly fluorescent, the hydrogel in the control experiment showed no fluorescence (Figure 9). This indicates that successful conjugation was achieved via the Michael type addition of the thiol to the maleimide groups within the hydrogel. Near-quantitative efficiency (>97%) of the BODIPY-SH conjugation to hydrogel **H3** was measured by quantification of the depletion of the dye in the supernatant solution using UV−vis spectroscopy, after immersion of maleimide containing hydrogels with the dye for 24 h (see Supporting Information).

As a next step, the potential of these hydrogels to act as templates for bioimmobilization of enzymes in a controlled manner was evaluated (Figure 10). Thiol containing biotin derivative, biotinylated (triethylene glycol) undecene thiol, was covalently attached to the gels, and their availability for immobilization of streptavidin was investigated. Biotin is a ligand known to bind onto streptavidin through strong specific noncovalent binding. Hydrogels **H1** and **H3** with different degree of maleimide groups were reacted with excess thiol containing biotin. After washing off excess biotin from the hydrogel to remove any unbound ligands, hydrogels were exposed to FITC label streptavidin. After washing off physiabsorbed streptavidin from the hydrogel, the extent of immobilization was investigated using fluorescence microscopy.

As expected, gels containing higher amounts of covalently bound biotin ligands were able to immobilize higher amounts of streptavidin. Maleimide containing gels which are not biotinylated are used as controls. These gels were exposed to FITC-streptavidin and similar washing protocols. Lack of any significant fluorescence relative to biotinylated gels shows that the enzymes are immobilized via covalently bound biotin ligand and that the PEG matrix acts as an effective antibiofouling scaffold that minimizes nonspecific adsorption of streptavidin (Figure 11).

Conclusions

Hydrogels containing maleimide functional groups that are reactive toward thiol containing molecules are synthesized using a novel Diels−Alder/retro-Diels−Alder strategy. The amount of reactive maleimide groups within the hydrogel matrix was tailored via amount of masked maleimide containing comonomer in the feed ratio and the gelation temperature. Efficient conversion of the masked maleimide groups into their reactive forms was monitored by thermogravimetric analysis. Swelling characteristics of these hydrogels were found to be dependent on the molecular weight of PEGMA monomer, feed ratio, and gelation temperature. The maleimide containing hydrogels were efficiently functionalized with small molecules such as fluorescent thiol containing dye, BODIPY-SH, and a thiol containing biotin ligand. Extent of immobilization of FITC-streptavidin onto these biotinylated gels could be tailored by varying the density of maleimide groups in the parent hydrogels.

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Supporting Information Available: H NMR spectra of *exo*-alcohol **1**, H NMR spectra of monomer **I**, H NMR spectra of monomer **II**, IR spectra of monomer **II**, H NMR spectra of PEG-*co*-poly(monomer **II**)-soluble copolymer, and IR spectra of PEG-*co*-poly(monomer **II**)-soluble copolymer. This material is available free of charge via the Internet at http://pubs.acs.org.

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