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# Design and Synthesis of Water-Soluble Multifunctionalizable Thiol-Reactive Polymeric Supports for Cellular Targeting

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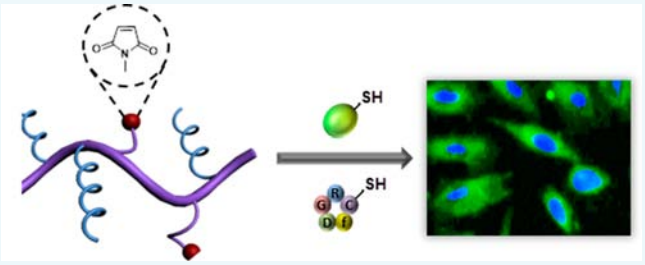
Supporting Information

**ABSTRACT:** Design and synthesis of novel water-soluble polymers bearing reactive side chains are actively pursued due to their increasing demand in areas such as bioconjugation and drug delivery. This study reports the fabrication of poly(ethylene glycol) methacrylate based thiol-reactive water-soluble polymeric supports that can serve as targeted drug delivery vehicles. Thiol-reactive maleimide units were incorporated into polymers as side chains by use of a furan-protected maleimide containing monomer. Atom transfer radical polymerization (ATRP) was employed to obtain a family of well-defined copolymers with narrow molecular weight distributions. After the polymerization, the maleimide groups were activated to their reactive form, ready for conjugation with thiol-containing molecules. Efficient functionalization of the maleimide moieties was demonstrated by conjugation of a tripeptide glutathione under mild and reagent-free aqueous conditions. Additionally, hydrophobic thiol-containing dye (Bodipy-SH) and a cyclic peptide-based targeting group (cRGDfC) were sequentially appended onto the maleimide bearing polymers to demonstrate their efficient multifunctionalization. The conjugates were utilized for in vitro experiments over both cancerous and healthy breast cell lines. Obtained results demonstrate that the conjugates were nontoxic, and displayed efficient cellular uptake. The presence of the peptide based targeting group had a clear effect on increasing the uptake of the dye-conjugated polymers into cells when compared to the construct devoid of the peptide. Overall, the facile synthesis and highly efficient multifunctionalization of maleimide-containing thiol-reactive copolymers offer a novel and attractive class of polyethylene glycol-based water-soluble supports for drug delivery.




## INTRODUCTION

The driving force for constant development of novel biocompatible polymeric materials is due to their ever-increasing applications in areas such as tissue engineering, gene therapy, drug delivery, and biosensing.[1−3] Synthetic polymeric supports incorporating various functional groups are routinely employed for conjugation of chemotherapy agents, biomolecules, peptides, and imaging agents.[4−8] Recently, polymer−peptide and polymer−protein assemblies have witnessed increasing interest due to the improved stability and pharmacological properties of peptides and proteins upon conjugation to polymers. Many of the pharmaceuticals based on peptides suffer from short circulating half-life due to rapid kidney clearance, immunogenicity, proteolytic degradation, and low solubility. Attachment of these proteins and peptides to polymers generally leads to improved pharmacological behavior such as longer half-life, reduced proteolytic degradation, slower clearance rate, and diminished immunogenicity.[9,10] The increasing demand of such polymers in biomedical fields necessitates expanding the toolbox of reactive water-soluble polymers that can be easily decorated with (bio)molecules of interest for particular applications.[11]

One of the most investigated polymers for biomedical applications, poly(ethylene glycol) (PEG), provides a number of advantages to improve the pharmacological properties of conjugated peptides and proteins.[12,13] It is well accepted that PEG improves the biocompatibility and hydrophilicity, and offers increased circulation time of the resultant conjugate in the body.[14,15] It is desirable that the conjugation of the active agent to the polymer can be accomplished under mild, preferably reagent-free conditions in a highly efficient manner. A brief survey of the literature reveals several well-established methods for the attachment of PEG chains to drugs, ligands, peptides, and proteins.[16−21] The conjugation method largely depends on the reactive groups present in the (bio)molecule of interest. Commonly present chemical functionalities include amine, carboxylic acid, hydroxyl, and thiol groups. For example, proteins containing amine groups on their surface can be conjugated to PEG-based polymers containing activated esters or aldehyde-functional groups in an efficient manner.[22,23]

Amine-based conjugations are widely employed for bioconjugation but oftentimes present a challenge since many peptides and proteins possess multiple amine groups. This may lead to unwanted cross-linking as well as generation of structurally poorly defined conjugates. As an alternative, utilization of thiol groups from cystein amino acids of proteins may allow construction of conjugates with desired specificity.[24] Although

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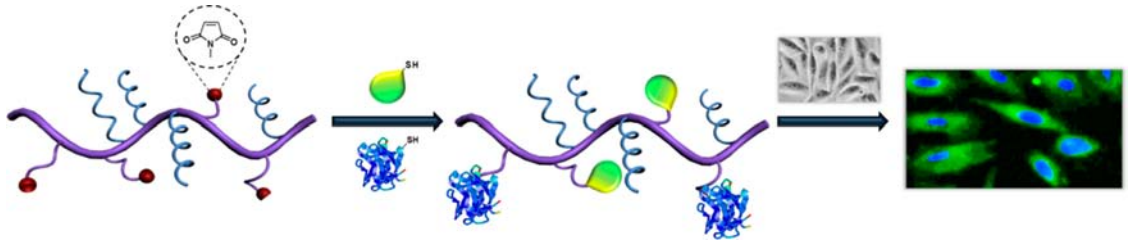
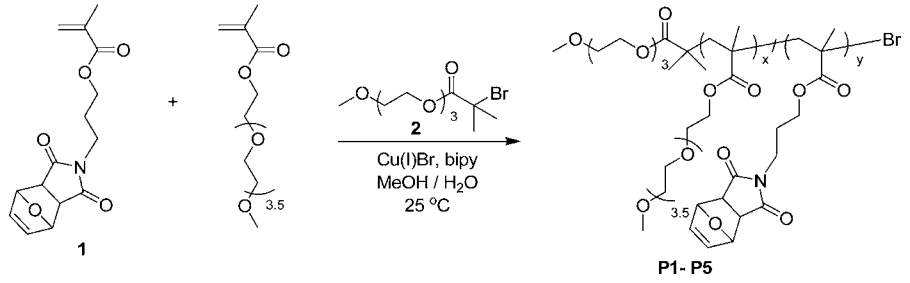


**Figure 1.** Schematic illustration of multifunctionalization and cellular targeting of maleimide-containing thiol-reactive copolymers.

**Scheme 1. Synthesis of Furan-Protected Maleimide Group Bearing Hydrophilic Copolymers**




thiol reactive polymers incorporating terminal maleimide, vinyl sulfone, and activated disulfides have been synthesized to achieve conjugation to proteins, there are very few reports of polymers with thiol reactive groups on the side chains. Commonly used polymers bearing pendant thiol-reactive functional groups contain pyridyl disulfide, allyl, or the epoxide group. The pyridyl disulfide allows attachment of thiol-containing molecules of interest through a disulfide linker which may be not desirable for some applications, as disulfides undergo cleavage in the presence or other thiols, while conjugations using the latter two groups, namely, allyl and epoxide, require the presence of a radical initiator or a strong base, respectively.[25−28] Among the available thiol-reactive functional groups, a maleimide group seems to be an attractive candidate since it is highly reactive toward thiols under mild conditions.[29−32] However, until recently, it was difficult to design polymeric materials containing reactive maleimide groups that are available for postpolymerization modification, because the maleimide group is prone to participate in polymerization due to the presence of an activated unsaturated double bond. Recently, advances have been made in this area by utilization of protected-maleimide based initiators and monomers to obtain polymers containing maleimide groups. In a pioneering study, Haddleton and co-workers disclosed an approach to obtain maleimide end-functionalized polymers for conjugation to cysteine residues on proteins.[33] They introduced the maleimide moiety by utilizing a furan-protected maleimide-based initiator for the polymerization followed by a deprotection step.

One can envision that water-soluble polymers containing multiple maleimide groups will be beneficial for many applications due to the multivalency offered. As a simple advantage, the limited loading capacity can be increased by linear PEG-based polymers. Additionally, in many cases multivalent display of functional groups will allow enhanced biomolecular recognition and targeting. In recent years, we have demonstrated that by using the protection/deprotection strategy of maleimide functional group using furan via the Diels−Alder/retro Diels−Alder (DA/rDA) cycloaddition reactions, maleimide functionalities can be incorporated as side chains of polymers.[34−39] Using this concept, maleimide-containing surface tethered polymer brushes were synthesized and demonstrated as an efficient method for surface modification with thiol-containing molecules.[40]

Herein, we report the design and synthesis of a family of well-defined poly(ethylene glycol) based water-soluble thiol-reactive copolymers using atom transfer radical polymerization (ATRP) and demonstrate their potential application toward fabrication of a targeted drug delivery platform via postpolymerization modifications with a thiol-containg dye and a cyclic peptide (Figure 1). To obtain such copolymers, a furan-protected masked maleimide monomer was copolymerized with water-soluble PEG-methacrylate monomer using ATRP. Activation of maleimide groups was achieved in a quantitaive fashion through retro Diels−Alder cycloreversion reaction. The copolymers obtained were characterized for their purity and maleimide content via $^1$H NMR spectroscopy. These PEG based thiol-reactive copolymers were evaluated for conjugation of free thiol group containing (bio)molecules such as reduced glutathione (a water-soluble tripeptide), a Bodipy-SH dye (a model hydrophobic drug/imaging agent), and cRGDfC peptide (a cyclic peptide based targeting group). To prove the potential of these reactive copolymers as delivery platforms for drugs and biomolecules, in vitro cytotoxicity experiments, cellular internalization, and targeting studies were performed.

## RESULTS AND DISCUSSION

**Synthesis of Copolymers Containing Maleimide Side-Chains.** Reactive hydrophilic polymeric scaffolds were obtained by the copolymerization of a PEG-based methacrylate monomer and a furan-masked maleimide-containing monomer. For this purpose, a methacrylate monomer possessing furan protected maleimide group was synthesized according to the literature.[37] Next, this reactive group containing monomer was copolymerized with a commercially available poly(ethylene glycol) monomethyl ether methacrylate (PEGMEMA, $M_n$ = 300 g mol$^{-1}$) monomer at room temperature to avoid unmasking of the maleimide moiety (Scheme 1).

Herein, PEGMEMA was chosen as a monomer to obtain hydrophilic copolymers that are also inherently anti-biofouling. ATRP was chosen as a method of polymerization since it proceeds at low temperatures, that are required to keep the


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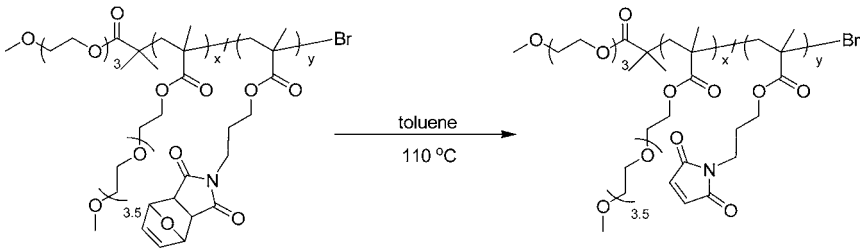
**Table 1. Polymerization Conditions and Characterization Results for the Synthesis of Latent-Reactive Monomer Containing Random Copolymers**

| | | | | | | | GPC[c] | | |
|---|---|---|---|---|---|---|---|---|---|
| no | polymer | feed ratio [M1]:[M2][a] | obtained ratio [M1]:[M2][a,b] | dilution MeOH: $H_2O$ (v/v) | time (min) | conv. (%) | $M_n$ (g/mol) | $M_w/M_n$ | $M_n$, theo (g/mol) |
| 1 | P1 | 1:1 | 0.86:1 | 5:1 | 30 | 52 | 10800 | 1.40 | 15540 |
| 2 | P2 | 2:1 | 1.62:1 | 5:1 | 30 | 55 | 15620 | 1.50 | 16510 |
| 3 | P3 | 4:1 | 4.2:1 | 1:1 | 15 | 82 | 20410 | 1.40 | 24710 |
| 4 | P4 | 8:1 | 8.3:1 | 1:1 | 10 | 61 | 30260 | 1.50 | 18430 |
| 5 | P5 | 16:1 | 15.8:1 | 1:1 | 10 | 73 | 35330 | 1.50 | 22090 |

[a]M1:PEGMEMA, M2:latent reactive monomer (1), $[I]_0:[M_{total}]_0:[CuBr]:[Bipy] = 1:100:1:2$. [b]Calculated using $^1$H NMR spectra. [c]Estimated by SEC eluted with THF, relative to linear PS standards.

**Scheme 2. Activation of Maleimide Groups via the Retro Diels–Alder Cycloreversion Reaction**




maleimide group in its masked form. In this study, we aimed to polymerize monomers in aqueous media. Since the masked maleimide monomer is insoluble in water, a mixture of water and methanol was used as solvent system. In particular, a triethylene glycol based water-soluble initiator was used as the alkyl halide initiator and polymerization was initiated by a complex Cu(I)Br and 2,2′-bipyridine.

A summary of different polymerization conditions investigated and the obtained results is outlined in Table 1. In the polymerizations listed, monomer:initiator:bpy:Cu(I)Br ratio was kept as 100:1:2:1. It was observed that the polymerization was very fast, as expected in highly polar solvents, and prolonged reaction times led to broadening in molecular weight distribution. Reaction time and solvent ratio were investigated for obtaining polymers with monomodal distribution and narrow polydispersities. A 6:1 (solvent:monomer) dilution ratio was observed to be most suitable since higher concentrations led to bimodal distributions and lower concentrations resulted in poor conversions. Methanol and water were used in equal amounts for polymers containing less maleimide (P3–5). Since the maleimide monomer is not soluble in water, the amount of methanol in the solvent was increased with increasing maleimide monomer in the feed to solubilize the hydrophobic monomer (Table 1). As expected, polymers with moderate molecular weight distributions were obtained (Supporting Information, Figures 14 and 15), since catalyst instability under aqueous conditions leads to deviations from a high level of control. It can be expected that utilization of Cu(0)-mediated radical polymerizations may provide better control, and efforts in this direction are currently underway.

After the polymerization, copolymers P3 and P4 were heated in toluene at 110 °C to remove the furan groups from the polymer via the retro Diels–Alder reaction to reveal the reactive maleimide units (Scheme 2). Complete cycloreversion of the furan-maleimide cycloadducts in a clean fashion was verified by $^1$H NMR spectroscopy.

Thus, synthesized copolymers were analyzed by $^1$H NMR spectroscopy to determine the degree of incorporation of maleimide monomer into the system and ratio of monomers after the polymerization. For example, characteristic peaks of furan-maleimide cycloadduct for P4 copolymer are easily seen at 6.52, 5.23, and 2.86 ppm (peaks a, b, and c respectively; Figure 2a). The ratio of integration values for these peaks with that of a singlet belonging to methoxy group of PEGMEMA monomer at 3.35 ppm (peak d, Figure 2a) reveals the monomer ratios incorporated into the polymer structure. In general, the obtained monomer ratio in the copolymers was found close to the feed ratio.

Comparison of $^1$H NMR spectrum of copolymer P4 with that of its retro product P7 (Figure 2a,b) clearly shows the disappearance of proton resonances belonging to the bicyclic moiety at 6.52, 5.23, and 2.86 ppm, and the appearance of a new proton resonance at 6.76 ppm belonging to the vinylic protons on the maleimide group indicates complete activation of all maleimide groups appended to polymer chains.

**Functionalization of Maleimide-Containing Copolymers.** *Conjugation of Polymer P6 with Glutathione.* Copolymer P6 (derived from P3 polymer) was functionalized with reduced glutathione, a free thiol containing tripeptide, to prove the reactivity of the polymers toward thiol groups. The Michael addition reaction between thiols and activated alkenes takes place efficiently under mild conditions in aqueous media (Scheme 3).

To demonstrate the efficiency of the conjugation reaction, copolymer P6 was reacted with 0.3, 0.6, and 1.0 equiv of glutathione and $^1$H NMR and CHNS data of modified copolymers indicated that quantitative functionalization was achieved in each case. In proton NMR spectra, it is clear that as the glutathione amount added into the reaction medium increases, the peak at 6.75 ppm (peak c, Figure 3a) corresponding to the double bond of the maleimide functionality decreases accordingly and expected peaks of glutathione at 2.77, 2.64, and 2.26 ppm (peaks e, f and g respectively, Figure 3b) become more apparent, which demonstrates the successful addition of glutathione.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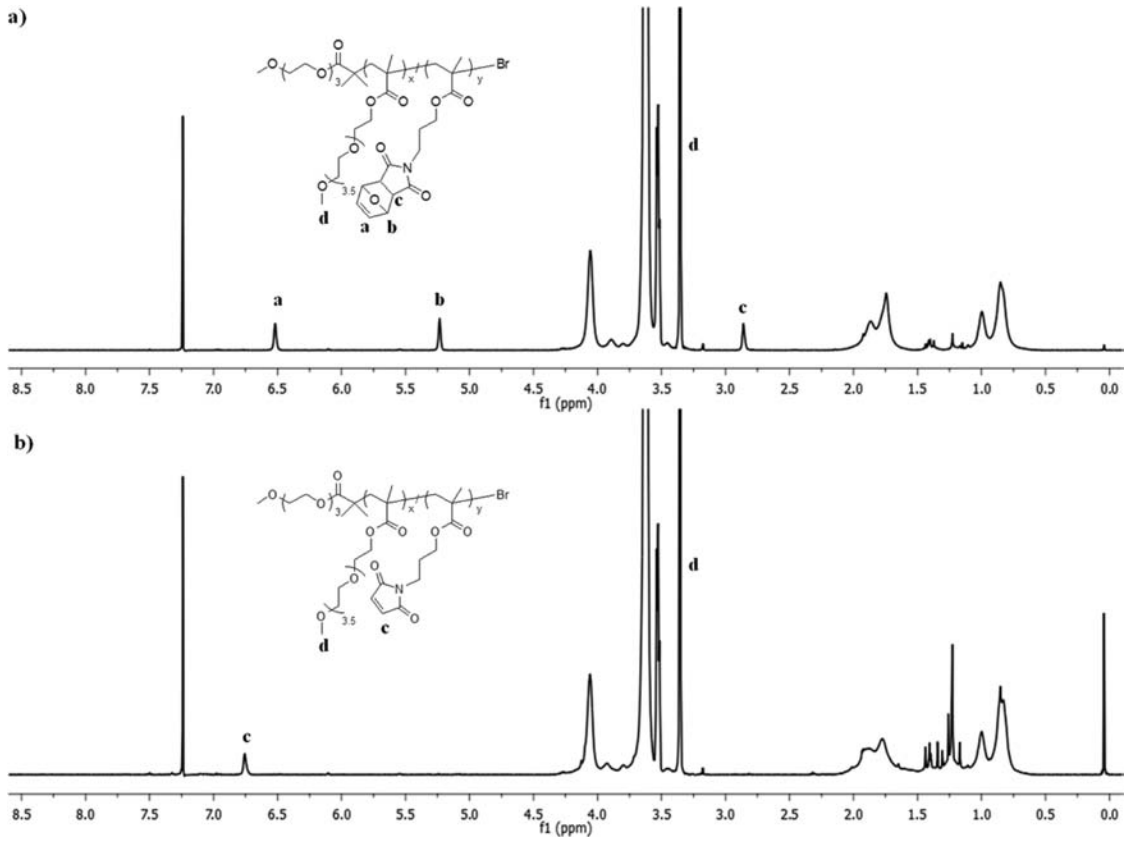
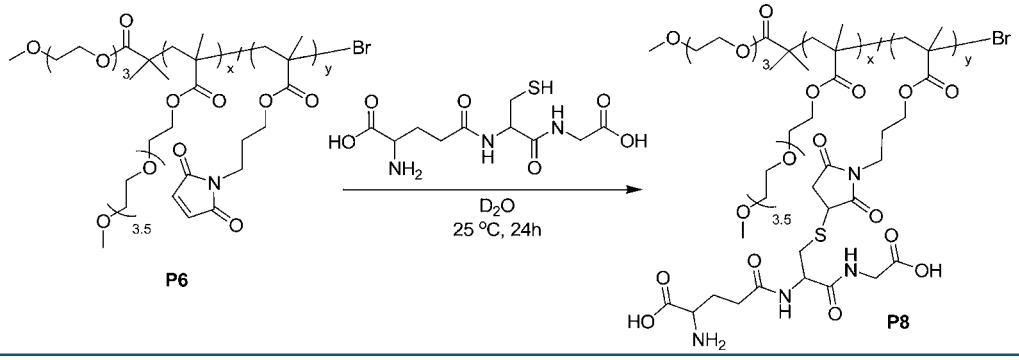



**Figure 2.** $^{1}$H NMR spectra of copolymers (a) P4 and (b) P7 in $CDCl_3$.

**Scheme 3. Functionalization of the Copolymer P6 with Glutathione**




Additionally, elemental analysis was used to probe the efficiency of conjugation to these copolymers via the thiol-maleimide conjugation. An increase in the amount of sulfur and nitrogen into the polymer structure upon conjugation of increasing amount of glutathione is evident (Table 2, Figure 4). Obtained results indicate that side-chain maleimide units can be utilized for multifunctionalization of these copolymers by sequential addition of nearly stoichiometric amount of different thiol bearing molecules.

*Conjugation of Copolymer P7 with BODIPY-SH and cRGDfC.* As an extension, postpolymerization functionalization of **P7** with free thiol-bearing biomolecules was accomplished in a stepwise manner. At first, Bodipy-SH, a hydrophobic dye, was conjugated to maleimide units at side chains of the copolymer. It has a core which consists of dipyrromethene complexed with a disubstituted boron atom. The fluorescence originating from this structure allows this class of dye to serve as an imaging agent. Additionally, BODIPY derivates are also known for their photodynamic properties and represents a model drug conjugation.[41]

Conjugation of the thiol-containing dye to copolymers was carried out in the presence of triethylamine (Scheme 4). The amount of dye per polymer chain can be adjusted by playing with the stoichiometric ratio of Bodipy-SH and maleimide units on the copolymer. A slighthly lower molar equivalent of the dye was used (maleimide:dye = 1.0:0.77) in order to obtain a polymer with residual maleimide units for further conjugation of a targeting unit. This reaction was carried out in THF due to the hydrophobic nature of the dye. However, conjugation of the dye to the PEG-based copolymers solubilized it in water, thus demonstrating potential advantage of the copolymer to improve solubility of any hydrophobic drug.

Efficiency of conjugation can be evaluated by comparing the integration of characteristic proton resonances belonging to the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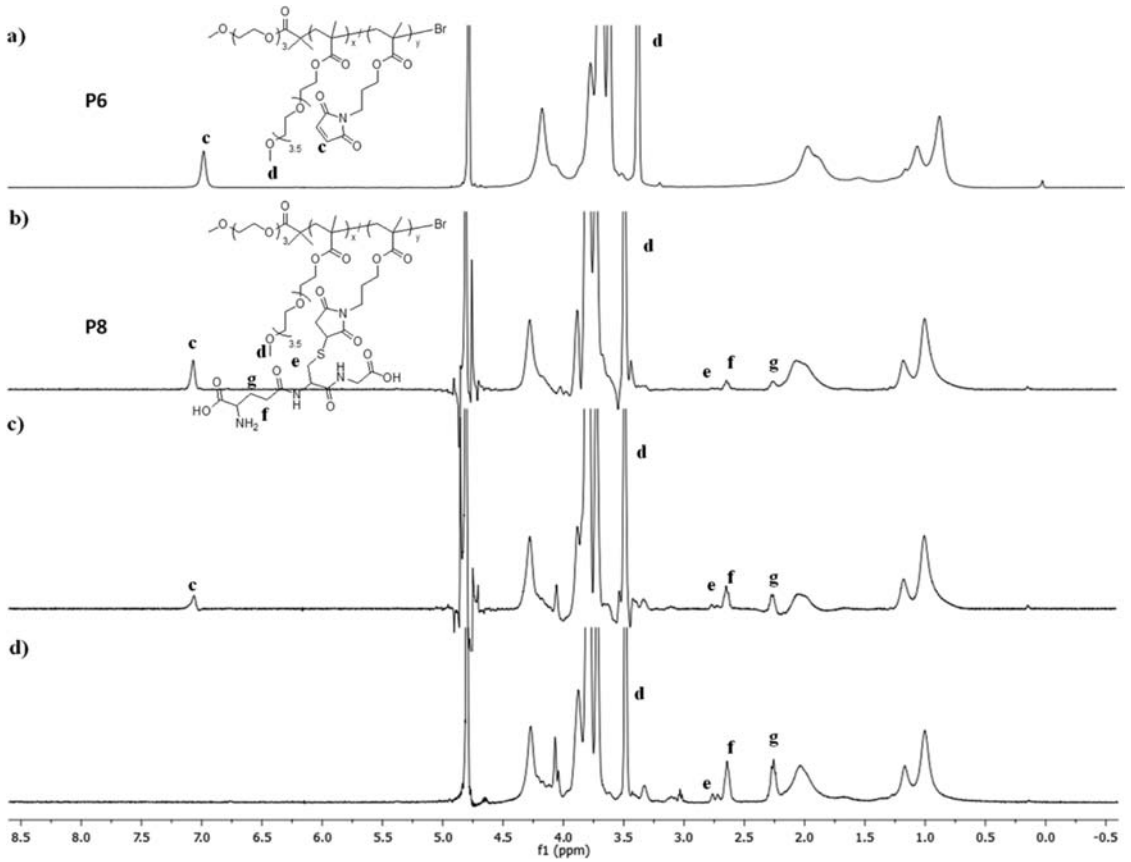


**Figure 3.** $^1$H NMR spectra of copolymer **P6** (a) conjugated with 0.3 (b), 0.6 (c), and 1.0 (d) equiv of glutathione, respectively, in $D_2O$.

**Table 2. Elemental Analysis Data for the Conjugation of Glutathione to Copolymer P6**

| no | glutathione equiv | N | C | H | S |
|---|---|---|---|---|---|
| 1 | 0 | 1.35 | 55.49 | 8.29 | 0 |
| 2 | 0.3 | 1.94 | 54.07 | 7.95 | 0.26 |
| 3 | 0.6 | 2.43 | 53.26 | 7.77 | 0.51 |
| 4 | 1.0 | 3.44 | 51.72 | 7.58 | 1.16 |

dye molecule and the polymer in $^1$H NMR spectra of modified copolymers. For example, in the case of polymer **P7**, the appearance of new peaks at 6.03, 2.49, and 2.40 ppm (peaks e, f, and g, respectively, Figure 5b) distinctly indicates the conjugation of dye where the first peak stands for the pyrrole moeity of the bodipy dye and the latter two peaks belong to methyl groups on the dye. Moreover, the integration value of the newly emerged bodipy peak at 6.03 ppm and the decrease in integration of proton resonance of the maleimide unit at 6.76 ppm (peak c, Figure 5b) correlate with each other, suggesting about 80% conjugation of total maleimide content in the polymer.

The remaining maleimide units of copolymer **P9** were utilized for the attachment of another thiol group containing cyclic peptide, cRGDfC. This peptide composed of five amino acids is a commonly used ligand for targeting the integrin receptors located on the outer surface of cell membrane, which are overexpressed in many types of cancerous cells. Due to the hydrophilic nature of the cyclic peptide cRGDfC, conjugation reaction was performed in aqueous medium (Scheme 5).

After removal of excess peptide from the reaction mixture by dialysis in distilled water, the resultant copolymer was characterized by $^1$H NMR spectroscopy. High efficiency for this reaction can be easily achieved from the complete disappearance of proton resonance of the maleimide group and the emergence of new peaks of peptide in the downfield region between 7 and 8 ppm (Figure 5c). Conjugation of peptide to the polymer backbone was further confirmed by proton NMR analysis performed in $D_2O$ and DMSO. In Figure 5d, proton resonances between 7.0 and 7.5 ppm point to the presence of the peptide cRGDfC along the polymer chains. Considering the hydrophobic nature of bodipy dye, it was not surprising to see the absence of any characteristic peaks belonging to dye in this spectrum acquired in $D_2O$ (referring to peaks e, f, and g). However, all proton resonances specifying the presence of dye, peptide, and polymer itself could be detected by performing $^1$H NMR spectroscopy in DMSO. The presence of resonances at 6.22 ppm originating from the proton on the pyrrole ring of bodipy dye, and labile hydrogens and aromatic protons of phenylalanine unit of peptide around 7−8 ppm, prove successful conjugation to copolymer (Figure 5e).

**In Vitro Experiments.** *In Vitro Cytotoxicity Assays.* Cytotoxic activity of the thiol reactive copolymer P4 and its conjugates with imaging agent, Bodipy-SH, and targeting group, cRGDfC, was investigated over MDA-MB-231 and MCF-10A human breast adenocarcinoma and healthy cell lines, respectively, and compared with each other. Cells adhered to 96-well plates were treated with drug and polymer−drug samples at 37 °C for 72 h. Cell viability was evaluated by a colorimetric assay; CCK-8 and obtained absorbance values at 450 nm were processed by GraphPad prism software using nonlinear regression curve with a sigmodial dose response equation. In this way, $EC_{50}$ values, the concentration at which the molecule displaces half of its toxic activity over cells, for all polymers were


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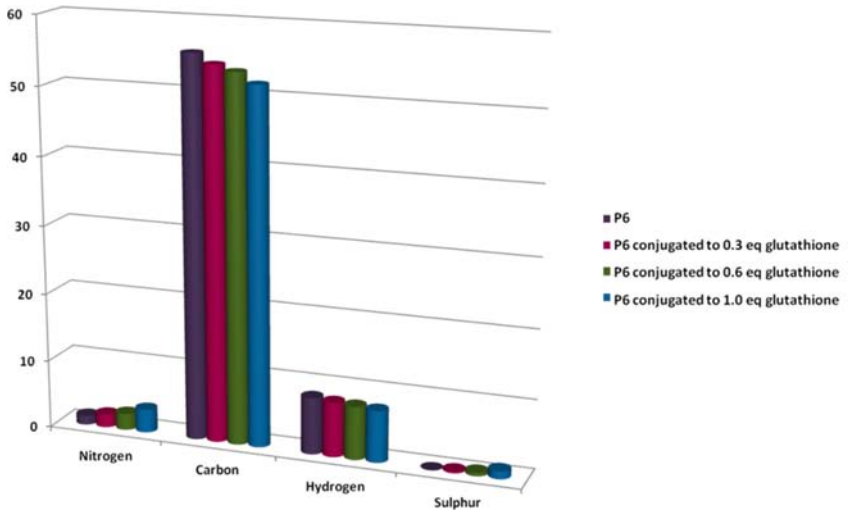
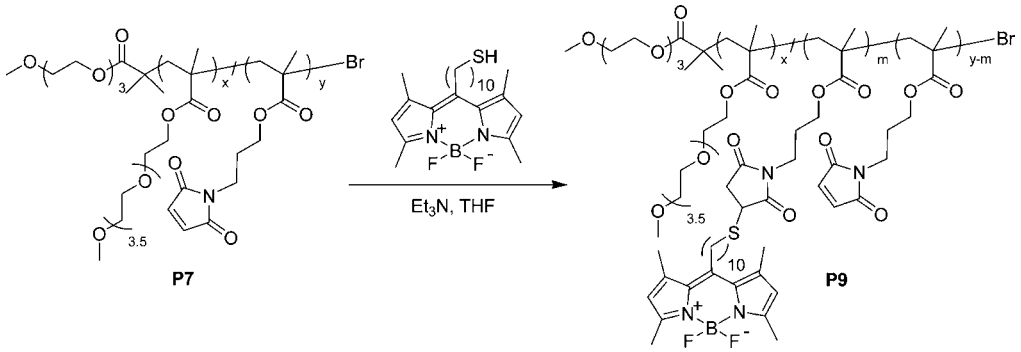



**Figure 4.** Changes in elemental compositions upon conjugation of **P6** with increasing amount of glutathione.

**Scheme 4. Thiol−ene Modification of Copolymer P7 with Bodipy-SH**




calculated from the cytoxicity graphs. The $EC_{50}$ values for all polymers were determined to be more than 100 $\mu$M, which reveals almost no toxicity for all. In each case, cell viability did not decrease remarkably even at high concentrations of polymers (Figure 6). Growth inhibition results showed that all conjugates are almost nontoxic to both cancerous and healthy breast cells, which supports their potential as delivery vehicles.

*In Vitro Internalization Assay.* The uptake of bodipy conjugated copolymers was examined over adenocarcinoma MDA-MB-231 human breast cell line. The bodipy molecule is fluorescent and can be visualized due to its characteristic green fluorescence. However, cell nuclei were detected by staining with 4′,6-diamidino-2-phenylindole (DAPI), which can be visualized by its blue fluorescence. Cultured cells were treated with polymer samples ($10^{-4}$ M) and incubated at 37 °C. A control experiment was performed by preincubating cells with integrin receptor antagonist c(RGDfV), which is a selective inhibitor for the integrin receptors with a higher affinity than the RGD peptide itself, and then treating them with polymer samples to probe if the uptake was indeed due to the targeting group.[42,43] At certain time points (3, 6, and 24 h) cells were examined to visualize the internalization. Figure 7 displays the fluorescence images of polymer treated cells, where copolymer P9 bears just bodipy dye and copolymer P10 consists of both bodipy and cRGDfC. As expected, cellular internalization increased as cells were incubated with polymer solutions for a longer time. However, green fluorescence intensity is remarkably higher for cells treated with cRGDfC containing copolymer P10, compared to cells treated with just the dye bearing polymer P9, as well as cells treated with cRGDfV and then P10 (Figure 7). The obtained result clearly demonstrates that the cellular internalization is enhanced due to the presence of the targeting group, cRGD, which strongly binds to integrin receptors overexpressed on the outer surface of MDA-MB-231 cells.[44,45]

## CONCLUSIONS

In summary, a novel class of multifunctional thiol reactive polymers was developed as potential platforms for targeted drug delivery. Maleimide groups were incorporated into the PEG-methacrylate based copolymers using a furan-protected maleimide containing monomer to enable the attachment of thiol group containing dyes (imaging agent) and peptides (targeting group). These water-soluble PEG-based copolymers were utilized to increase the hydrophilicity of the carrier and decrease nonspecific interactions with proteins. Copolymerizations were carried out using ATRP method since it provides polymers with good control over molecular weight and narrow polydispersity. Importantly, the polymerization can be conducted at ambient temperatures to avoid any unmasking of the reactive maleimide group. Maleimide groups in the copolymers were obtained quantitatively in their thiol-reactive form via the retro Diels−Alder reaction. Efficiency of postpolymerization functionalization was evaluated by conjugation of a tripeptide glutathione, a hydrophobic dye Bodipy-SH and a targeting cyclic peptide cRGDfC using Michael type nucleophilic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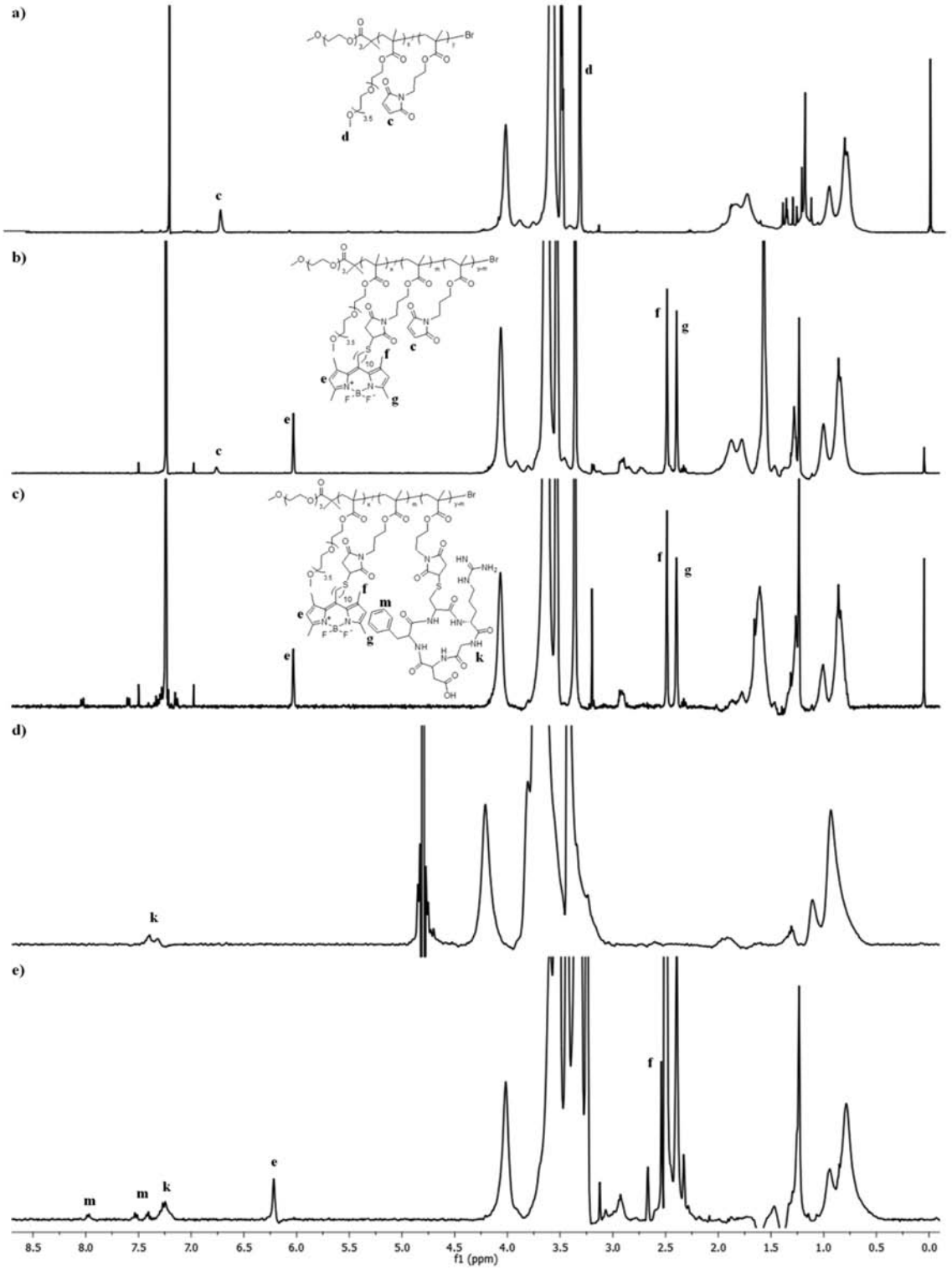


**Figure 5.** $^1$H NMR spectra of (a) **P7** in $CDCl_3$, (b) **P9** in $CDCl_3$, (c) **P10** in $CDCl_3$, (d) **P10** in $D_2O$, and (e) **P10** in DMSO.

thiol−ene chemistry. Cytotoxicity experiments conducted over both cancerous and healthy human breast cell lines revealed the nontoxic nature of obtained conjugates, and the cell internalization experiments demonstrated enhanced uptake of conjugates in the presence of a targeting group. Overall, facile synthesis and efficient multifunctonalization of these water-soluble thiol-reactive copolymers provides an attractive candidate for fabrication of a viable drug delivery platform.

## EXPERIMENTAL SECTION

**Materials.** *Chemicals.* All reagents were obtained from commercial sources and were used as received unless otherwise stated. Poly(ethylene glycol) methyl ether methacrylate ($M_n$ = 300 g mol$^{-1}$) (PEGMEMA, 99%, Aldrich) was passed through basic alumina column to remove inhibitor. Copper(I) bromide (CuBr, 99.9%, Aldrich), 2-(pyridin-2-yl)pyridine (2,2′-bipyridine, 99%, Aldrich), L-glutathione reduced (98%, Aldrich), and cRGDfV



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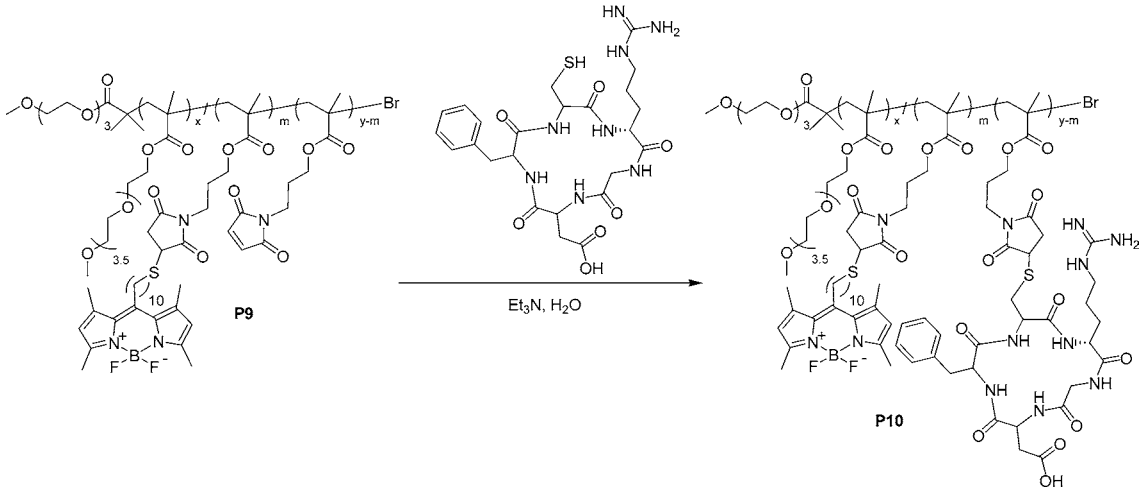
**Scheme 5. Thiol−ene modification of Bodipy-SH conjugated copolymer P9 with cRGDfC**



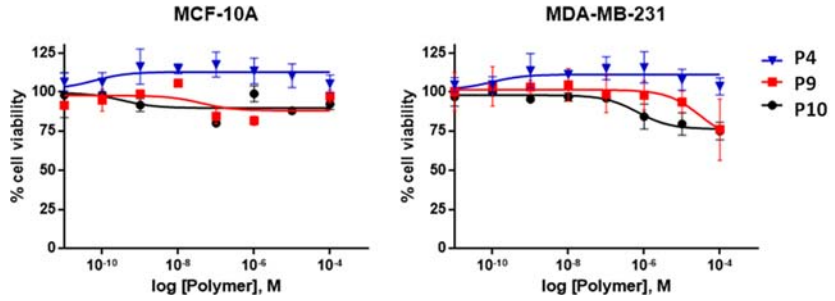




**Figure 6.** Inhibition of MDA-MB-231 and MCF-10A cell proliferation by P4 (thiol reactive copolymer), P9 (bodipy conjugated copolymer), and P10 (both bodipy and cRGDfC conjugated copolymer). Cell viability was determined by CCK-8 assay. Results were expressed as mean ± SD ($n$ = 4).

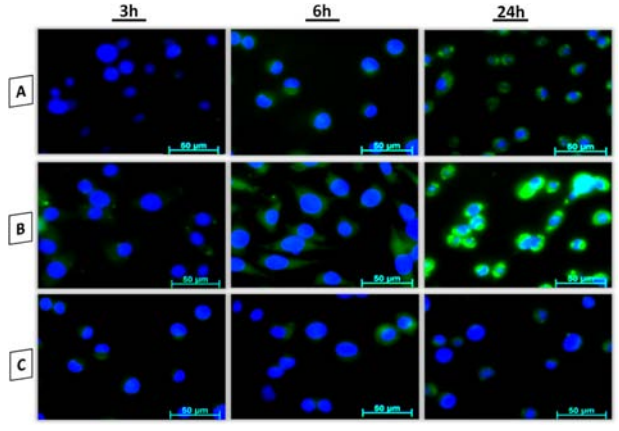



**Figure 7.** Merged fluorescence images of MDA-MB-231 cells following treatments with (A) P9, (B) P10, and (C) first with cRGDfV and then P10. Cells were incubated at 37 °C for different time points (3, 6, and 24 h). The scale bar is 50 $\mu$m.

(97%, Sigma) were used as received. BodipySH and cRGDfC were prepared according to the literature procedures.[46,47] Furan-protected maleimide-containing monomer (**1**) and 2-(2-(2-methoxyethoxy)ethoxy)ethyl 2-bromo-2-methylpropanoate (**2**) were synthesized by following previously reported protocol.[37,48] Anhydrous solvents ($CH_2Cl_2$, THF, toluene) were obtained from ScimatCo Purification System; other solvents were dried over molecular sieves. The dialysis bags (Spectra/Por Biotech Regenerated Cellulose Dialysis Membranes, MWCO 1000 Da) were purchased from Spectrum Laboratories. All other chemicals were used as received from the manufacturer (Merck, Aldrich, Alfa Aesar). Column chromatography was performed using Silicagel-60 (43−60 nm). Thin layer chromatography was performed using silica gel plates (Kiesel gel 60 F254, 0.2 mm, Merck).

*Cells.* Human breast adenocarcinoma MDA-MB-231 and healthy MCF-10A cell lines were obtained from ATCC (Wessel, Germany). Cells were kept in the logarithmic phase of cell growth for the duration of experiments. MDA-MB-231 cells were maintained in RPMI-1640 culture medium (Roswell Park Memorial Institute) [Gibco, Invitrogen] supplemented with 10% fetal bovine serum [FBS] [Lonza], 100 U/mL penicillin, 100 g/mL streptomycin, and 0.25 g/mL amphotericin B at 37 °C, 5% $CO_2$, and 95% relative humidity. MCF-10A cells were maintained in Endothelial Cell Growth Medium-2 (EMB-2) [Lonza, Invitrogen] supplemented with EGM-2 Bulletkit containing hEGF, hydrocortisone, GA-1000 (Gentamicin, Amphotericin-B), FBS (fetal bovine serum) 10 mL, VEGF, hFGF-B, R3-IGF-1, ascorbic acid, heparin, and 0.01% insulin [Lonza], at 37 °C, 5% $CO_2$, and 95% relative humidity. Cell counting kit-8 (CCK-8) and DAPI were obtained from Sigma-Aldrich.

**Instrumentation.** NMR spectra were recorded using a 400 MHz Varian spectrometer at 25 °C. $^1$H NMR measurements were made at frequency of 400 MHz, and calibrated with respect to the solvent signal. The measurements were performed in deuterated chloroform ($CDCl_3$), water ($D_2O$), and dimethyl sulfoxide (DMSO). The molecular weights of the copolymers were determined by size exclusion chromatography using a Shimadzu PSS-SDV (length/ID 8 × 300 mm, 10 mm particle size) linear Mixed C column calibrated with polystyrene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standards (1−400 kDa) using a refractive-index detector. Tetrahydrofuran (THF) was used as eluent at a flow rate of 1 mL/min at 30 °C. Elemental analysis data were obtained from Thermo Electron S.p.A. FlashEA 1112 Elemental Analyzer (CHNS separation column, PTFE; 2 m; 6 × 5 mm). Cell viability values for the cytotoxicity experiments were determined by measuring the absorbance of 96-well plates in use at 450 nm by Multiscan FC Microplate Photometer from Thermo Scientific equipped with a quartz halogen light source of a precision CV ≤ 0.2% (0.3−3.0 Abs). It has an excitation wavelength range 340−850 nm with excitation filters installed at 405, 450, and 620 nm. Cell internalization studies were performed with Zeiss Observer Z1 fluorescence microscope connected to Axiocam MRc5 using a Zeiss Filter set 38 (Excitation BP 470/40, Emission BP 525/50) for imaging the functionalized polymers and a Zeiss Filter set 49 (Excitation G365, Emission BP 445/50) was used for imaging DAPI stained nuclei. Images were obtained to visualize cell nuclei and morphology using Zeiss AxioVision software.

**General Procedure for the Copolymerization of Latent Reactive Monomer.** In a typical experiment (for P4 copolymer), the furan protected maleimide monomer (1) (0.64 g, 0.22 mmol) dissolved in minimum amount of degassed MeOH was introduced into a flask containing 2,2′ bipyridine (6.15 mg, 0.040 mmol), CuBr (2.80 mg, 0.020 mmol), and PEGMEMA (0.50 mL, 1.75 mmol) dissolved in degassed MeOH (1.65 mL) and $H_2O$ (1.65 mL) under stirring. To the stirring mixture was added TEGME-Br initiator (2) (6.16 mg, 0.020 mmol) and the reaction was stirred at room temperature for 10 min. At the end of the reaction, flask was opened to atmosphere and MeOH was evaporated under vacuum and water was removed using a lyophilizer. The polymer was dissolved in minimum amount of dichloromethane and precipitated in cold diethyl ether and then passed through aluminum oxide to remove the residual copper complex. Copolymer P4 was obtained as a viscous solid.

For **P1**: ($[M]_0/[I]_0$ = 100, [1]:[PEGMEMA] = 1:0.86, $[I]_0$:[CuBr]:[PMDETA] = 1:2:4, conversion = 52%, $M_{n,theo}$ = 15540, $M_{n,GPC}$ = 10800, $M_w/M_n$ = 1.40, relative to PS). $^1$H NMR ($CDCl_3$, $\delta$, ppm) 6.50 (s, 2H, C*H*═C*H*), 5.21 (s, 2H, C*H* bridgehead protons), 4.05 (s, 2H, OC$H_2$ ester protons of PEGMEMA), 3.89 (br s, 2H, OC$H_2$), 3.62−3.50 (m, 4H, OC$H_2$ of PEGMEMA and NC$H_2$), 3.34 (s, 3H, OC$H_3$ of PEGMEMA), 2.84 (s, 2H, C*H*−C*H*, bridge protons), 1.92−0.82 (m, 7H, $NCH_2CH_2CH_2O$, C$H_2$ and C$H_3$ along polymer backbone).

For **P2**: ($[M]_0/[I]_0$ = 100, [1]:[PEGMEMA] = 1:1.62, $[I]_0$:[CuBr]:[PMDETA] = 1:2:4, conversion = 55%, $M_{n,theo}$ = 16510, $M_{n,GPC}$ = 15620, $M_w/M_n$ = 1.50, relative to PS). $^1$H NMR ($CDCl_3$, $\delta$, ppm) 6.51 (s, 2H, C*H*═C*H*), 5.23 (s, 2H, C*H* bridgehead protons), 4.06 (s, 2H, OC$H_2$ ester protons of PEGMEMA), 3.89 (br s, 2H, OC$H_2$), 3.63−3.52 (m, 4H, OC$H_2$ of PEGMEMA and NC$H_2$), 3.35 (s, 3H, OC$H_3$ of PEGMEMA), 2.85 (s, 2H, C*H*−C*H*, bridge protons), 1.92−0.83 (m, 7H, $NCH_2CH_2CH_2O$, C$H_2$ and C$H_3$ along polymer backbone).

For **P3**: ($[M]_0/[I]_0$ = 100, [1]:[PEGMEMA] = 1:4.2, $[I]_0$:[CuBr]:[PMDETA] = 1:2:4, conversion = 82%, $M_{n,theo}$ = 24710, $M_{n,GPC}$ = 20410, $M_w/M_n$ = 1.40, relative to PS). $^1$H NMR ($CDCl_3$, $\delta$, ppm) 6.51 (s, 2H, C*H*═C*H*), 5.23 (s, 2H, C*H* bridgehead protons), 4.05 (s, 2H, OC$H_2$ ester protons of PEGMEMA), 3.89 (br s, 2H, OC$H_2$), 3.63−3.52 (m, 4H, OC$H_2$ of PEGMEMA and NC$H_2$), 3.35 (s, 3H, OC$H_3$ of PEGMEMA), 2.85 (s, 2H, C*H*−C*H*, bridge protons), 1.87−0.83 (m, 7H, $NCH_2CH_2CH_2O$, C$H_2$ and C$H_3$ along polymer backbone).

For **P4**: ($[M]_0/[I]_0$ = 100, [1]:[PEGMEMA] = 1:8.3, $[I]_0$:[CuBr]:[PMDETA] = 1:2:4, conversion = 61%, $M_{n,theo}$ = 18430, $M_{n,GPC}$ = 30260, $M_w/M_n$ = 1.50, relative to PS). $^1$H NMR ($CDCl_3$, $\delta$, ppm) 6.52 (s, 2H, C*H*═C*H*), 5.23 (s, 2H, C*H* bridgehead protons), 4.06 (s, 2H, OC$H_2$ ester protons of PEGMEMA), 3.89 (br s, 2H, OC$H_2$), 3.63−3.52 (m, 4H, OC$H_2$ of PEGMEMA and NC$H_2$), 3.35 (s, 3H, OC$H_3$ of PEGMEMA), 2.86 (s, 2H, C*H*−C*H*, bridge protons), 1.92−0.85 (m, 7H, $NCH_2CH_2CH_2O$, C$H_2$ and C$H_3$ along polymer backbone).

For **P5**: ($[M]_0/[I]_0$ = 100, [1]:[PEGMEMA] = 1:15.8, $[I]_0$:[CuBr]:[PMDETA] = 1:2:4, conversion = 73%, $M_{n,theo}$ = 22090, $M_{n,GPC}$ = 35330, $M_w/M_n$ = 1.50, relative to PS). $^1$H NMR ($CDCl_3$, $\delta$, ppm) 6.53 (s, 2H, C*H*═C*H*), 5.24 (s, 2H, C*H* bridgehead protons), 4.06 (s, 2H, OC$H_2$ ester protons of PEGMEMA), 3.89 (br s, 2H, OC$H_2$), 3.64−3.52 (m, 4H, OC$H_2$ of PEGMEMA and NC$H_2$), 3.36 (s, 3H, OC$H_3$ of PEGMEMA), 2.87 (s, 2H, C*H*−C*H*, bridge protons), 1.87−0.85 (m, 7H, $NCH_2CH_2CH_2O$, C$H_2$ and C$H_3$ along polymer backbone).

**General Procedure for the Activation of Copolymers by Retro Diels/Alder Reaction.** Polymer P4 (40.0 mg, 1.32 $\mu$mol) was dissolved in anhydrous toluene and heated at 110 °C for 24 h. $^1$H NMR analysis indicated a quantitative conversion of the oxabicyclic moiety to the maleimide functional group to yield copolymer **P7** (36.0 mg, 92% yield).

For polymer **P6**: ([1]:[PEGMEMA] = 1:4.2) $^1$H NMR ($CDCl_3$, $\delta$, ppm) 6.76 (s, 2H, C*H*═C*H*), 4.06 (s, 2H, OC$H_2$ ester protons of PEGMEMA), 3.92 (br s, 2H, OC$H_2$), 3.63−3.61 (m, 2H, OC$H_2$ of PEGMEMA), 3.54−3.52 (m, 5H, OC$H_3$ and NC$H_2$), 3.35 (s, 3H, OC$H_3$ of PEGMEMA), 1.92−0.82 (m, 7H, $NCH_2CH_2CH_2O$, C$H_2$ and C$H_3$ along polymer backbone); $^1$H NMR ($D_2O$, $\delta$, ppm) 6.99 (s, 2H, C*H*═C*H*), 4.19 (s, 2H, OC$H_2$ of PEGMEMA), 4.08 (br s, 2H, OC$H_2$), 3.79−3.54 (m, 4H, OC$H_2$ of PEGMEMA and NC$H_2$), 3.40 (s, 3H, OC$H_3$ of PEGMEMA), 2.00−1.92 (m, 7H, $NCH_2CH_2CH_2O$, C$H_2$ and C$H_3$ along polymer backbone). ($M_{n,GPC}$ = 18380, $M_w/M_n$ = 1.40, relative to PS).

For polymer **P7**: ([1]:[PEGMEMA] = 1:8.3) $^1$H NMR ($CDCl_3$, $\delta$, ppm) 6.76 (s, 2H, C*H*═C*H*), 4.06 (s, 2H, OC$H_2$ ester protons of PEGMEMA), 3.92 (br s, 2H, OC$H_2$), 3.64−3.52 (m, 5H, OC$H_2$ of PEGMEMA and NC$H_2$), 3.35 (s, 3H, OC$H_3$ of PEGMEMA), 1.92−0.83 (m, 7H, $NCH_2CH_2CH_2O$, C$H_2$ and C$H_3$ along polymer backbone). ($M_{n,GPC}$ = 31300, $M_w/M_n$ = 1.50, relative to PS).

**Conjugation of P6 with Glutathione via Thiol−ene Reaction.** Copolymer **P6** (10.0 mg, 0.49 $\mu$mol) was dissolved in $D_2O$ (0.50 mL) and degassed for 30 min. Glutathione was added 0.3, 0.6, and 1.0 equiv from the stock solution of 10 mg/mL in $D_2O$ and reacted with the maleimide groups of the copolymer at room temperature for 24 h. $^1$H NMR analysis proved quantitative addition of the glutathione molecules to the maleimide functional group.

Modified copolymer **P8**: $^1$H NMR ($D_2O$, $\delta$, ppm) 4.17 (s, 2H, OC$H_2$ of PEGMEMA), 4.12 (br s, 2H, OC$H_2$), 3.87−3.72 (m, 4H, OC$H_2$ of PEGMEMA and NC$H_2$), 3.48 (s, 3H, OC$H_3$ of PEGMEMA), 2.62 (br s, 2H, SC$H_2$ of glutathione), 2.26 (br s, 4H, COC$H_2$C$H_2$ of glutathione), 2.10−1.00 (m, 7H, $NCH_2CH_2CH_2O$, C$H_2$ and C$H_3$ along polymer backbone). ($M_{n,GPC}$ = 23430, $M_w/M_n$ = 1.40, relative to PS).

**Conjugation of P7 with Bodipy-SH via Thiol−ene Reaction.** Polymer **P7** (15.0 mg, 0.40 $\mu$mol) and Bodipy-SH (1.60 mg, 3.70 $\mu$mol) were dissolved in THF (0.15 mL) and triethylamine (0.07 $\mu$L, 0.46 $\mu$mol) was added into the reaction mixture. The reaction was allowed to proceed at room temperature for 24 h. Unreacted dye was removed by washing the conjugate with cold diethyl ether several times. $^1$H NMR analysis revealed that 80% of maleimides appended to the


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polymer chains were attached to Bodipy-SH. Dye conjugated polymer was obtained as a fluorescent viscous solid P9 (12 mg, 71% yield).

For polymer **P9**: $^1$H NMR ($CDCl_3$, $\delta$, ppm) 6.76 (s, 2H, C*H*═C*H*), 6.03 (s, 2H, *H*C═CCH$_3$ of Bodipy), 4.06 (s, 2H, OC*H*$_2$ ester protons of PEGMEMA), 3.92 (br s, 2*H*, OC*H*$_2$), 3.64−3.53 (m, 5H, OC*H*$_2$ of PEGMEMA and NC*H*$_2$), 3.36 (s, 3H, OC*H*$_3$ of PEGMEMA), 3.20−3.12 (m, 1H, C*H*SCH$_2$), 2.93−2.83 (m, 3H, C*H*$_2$S of Bodipy and C*H*$_2$CHS), 2.76−2.69 (m, 1H, C*H*$_2$CHS), 2.49 (s, 6H, NCC*H*$_3$ of Bodipy), 2.40 (s, 6H, C*H*$_3$C═C of Bodipy), 2.33 (t, 2H, $J$ = 7.6 Hz, CC*H*$_2$ of Bodipy), 1.88−0.84 (m, 23H, NCH$_2$C*H*$_2$CH$_2$O, C*H*$_2$ and C*H*$_3$ of Bodipy, C*H*$_2$ and C*H*$_3$ along the polymer backbone).

**Conjugation of P9 with cRGDfC via Thiol−ene Reaction.** Fluorescent dye conjugated polymer **P9** (5.0 mg, 0.15 $\mu$mol) and cRGDfC (0.49 mg, 0.86 $\mu$mol) were dissolved in distilled $H_2O$ (0.10 $\mu$L) and the mixture was degassed for 3 min. Then, triethylamine (0.02 $\mu$L, 0.14 $\mu$mol) was added into the reaction mixture. The reaction was allowed to proceed at room temperature for 24 h. Unreacted peptide was removed by dialysis over distilled water using 1000 Da cutoff cellulose generated membrane. $^1$H NMR analysis revealed that rest of maleimide groups appended to the polymer chains were conjugated to cRGDfC. Both dye and peptide conjugated polymer was obtained as a fluorescent viscous solid P10 (4.3 mg, 83% yield) ([Bodipy-SH]:[cRGDfC] = 4:1).

Copolymer **P10**: $^1$H NMR ($CDCl_3$, $\delta$, ppm) 6.03 (s, 2H, *H*C═CCH$_3$ of Bodipy), 4.07 (s, 4H, OC*H*$_2$ ester protons of PEGMEMA and OC*H*$_2$), 3.64−3.53 (m, 5H, OC*H*$_2$ of PEGMEMA and NC*H*$_2$), 3.36 (s, 3H, OC*H*$_3$ of PEGMEMA), 3.23−3.17 (m, 1H, C*H*SCH$_2$), 2.93−2.83 (m, 2H, C*H*$_2$S of Bodipy), 2.49 (s, 6H, NCC*H*$_3$ of Bodipy), 2.40 (s, 6H, C*H*$_3$C═C of Bodipy), 2.33 (t, 2H, $J$ = 7.7 Hz, CC*H*$_2$ of Bodipy), 1.66−0.84 (m, 25H, NCH$_2$C*H*$_2$CH$_2$O, C*H*$_2$ of Bodipy, C*H*$_2$ of cRGDfC, C*H*$_2$ and C*H*$_3$ along polymer backbone). $^1$H NMR ($D_2O$, $\delta$, ppm) 7.46−7.27 (m, 1H, N*H* of cRGDfC), 4.21 (br s, 4H, OC*H*$_2$ ester protons of PEGMEMA and OC*H*$_2$), 3.81−3.66 (m, 5H, OC*H*$_2$ of PEGMEMA and NC*H*$_2$), 3.42 (br s, 3H, OC*H*$_3$ of PEGMEMA), 2.06−0.63 (m, 25H, NCH$_2$C*H*$_2$CH$_2$O, C*H*$_2$ of Bodipy, C*H*$_2$ of cRGDfC, C*H*$_2$ and C*H*$_3$ along polymer backbone). $^1$H NMR (DMSO, $\delta$, ppm) 8.00−7.93 (m, 2H, Ar-*H* of cRGDfC), 7.56−7.36 (m, 3H, Ar-*H* of cRGDfC), 7.34−7.14 (m, 1H, N*H* of cRGDfC), 6.22 (s, 2H, *H*C═CCH$_3$ of Bodipy), 4.02 (br s, 4H, OC*H*$_2$ ester protons of PEGMEMA and OC*H*$_2$), 3.60−3.43 (m, 5H, OC*H*$_2$ of PEGMEMA and NC*H*$_2$), 3.25 (br s, 3H, OC*H*$_3$ of PEGMEMA), 3.12 (br s, 1H, C*H*SCH2), 2.98−2.75 (m, 4H, C*H*$_2$S of Bodipy and C*H*$_2$CHS), 2.67 (br s, 2H, C*H*$_2$S of cRGDfC), 2.54 (s, 6H, NCC*H*$_3$ of Bodipy), 2.39 (br s, 2H, C*H*$_2$ of cRGDfC), 2.33 (br s, 3H, N*H* and N*H*$_2$ of cRGDfC), 1.73−0.79 (m, 25H, NCH$_2$C*H*$_2$CH$_2$O, C*H*$_2$ of Bodipy, C*H*$_2$ of cRGDfC, C*H*$_2$ and C*H*$_3$ along polymer backbone).

**In Vitro Experiments.** *Cytotoxicity Experiments.* Cytotoxicity of thiol reactive copolymer **P4** and its imaging agent and targeting group conjugated versions were assessed by CCK-8 viability assay against adenocarcinoma MDA-MB-231 and healthy MCF-10A human breast cell lines. Cells (5000 cells/well) were seeded in a 96-well plate as quadruplet in 100 $\mu$L appropriate culture medium and incubated at 37 °C for 24 h for cells to adhere completely. Naked and functionalized copolymers were dissolved in DMSO at $1 \times 10^{-2}$ M stock concentration and seven different concentrations (0.1 nM to 0.1 mM) of samples were prepared via serial dilutions. Cells were treated with these samples and incubated at 37 °C for 72 h. After removal of sample solutions, wells were washed with 100 $\mu$L PBS twice. The amount of viable cells was determined by CCK-8 cell viability assay by adding 10 $\mu$L CCK-8 reagent in 100 $\mu$L fresh medium onto wells and at the end of 1 h incubation absorbance values at 450 nm were measured via a microplate reader. $EC_{50}$ values were calculated by GraphPad prism software using nonlinear regression mode.

*Internalization Experiment.* Adenocarcinoma MDA-MB-231 human breast cells (10 000 cells/well) were seeded in a 24-well plate as triplicate in 500 $\mu$L appropriate culture medium. A separate plate of cells was added with 20 $\mu$M cRGDfV, an integrin receptor antagonist, and all cells were incubated at 37 °C for 24 h for cells to adhere completely. Then the culture medium was aspirated and replaced with fresh medium containing imaging agent functionalized copolymers at a concentration of $1 \times 10^{-4}$ M. The experiments were completed in triplicate. After incubation at 37 °C for several time points (3, 6, and 24 h), sample solutions were removed. Wells were washed with 500 $\mu$L PBS twice and cells were mounted with PBS containing DAPI (5 mg/mL) at 37 °C for 30 min. Then washed with PBS three times, cells were fixed with 4% formaline solution at 37 °C for 5 min. Images were collected using Zeiss Observer Z1 fluorescence microscope at room temperature.

## ■ ASSOCIATED CONTENT

### Supporting Information

$^1$H NMR analysis results and GPC traces of the synthesized copolymers and conjugates. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00182.

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### Notes

The authors declare no competing financial interest.

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