Light-Activated Immobilization of Biomolecules to Agarose Hydrogels for Controlled Cellular Response

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We describe a new method of synthesizing photolabile hydrogel materials for convenient photoimmobilization of biomolecules on surfaces or in 3-D matrixes. Dissolved agarose was modified with photolabile *S*-(2-nitrobenzyl)cysteine (*S*-NBC) via 1,1′-carbonyldiimidazole (CDI) activation of primary hydroxyl groups. *S*-NBC-modified agarose remained soluble and gelable with up to 5% *S*-NBC substitution, yet gelation was slower and the elastic modulus of the resulting gel was lower than those of unmodified agarose. Irradiating *S*-NBC-grafted agarose resulted in the loss of the protecting 2-nitrobenzyl groups, thereby exposing free sulfhydryl groups for biomolecular coupling. When appropriately activated with sulfhydryl-reactive groups, either peptides or proteins were effectively immobilized to the photoirradiated hydrogel matrixes, with the irradiation energy dose (i.e., irradiation time) used to control the amount of biomolecule immobilization. When the GRGDS peptide was immobilized on agarose, it was shown to be cell-adhesive and to promote neurite outgrowth from primary, embryonic chick dorsal root ganglion neurons. The immobilized GRGDS surface ligand concentration affected the cellular response: neurite length and density increased with GRGDS surface concentration at low adhesion ligand concentration and then plateaued at higher GRGDS concentration. Grafting 2-nitrobenzyl-protected compounds to hydrogel materials is useful for creating new photolabile hydrogel substrates for light-activated functional group generation and biomolecular immobilization.

1. Introduction

The immobilization of biomolecules to hydrogels is an important tool to control both the chemical and physical properties of the gel while at the same time providing bioactive materials for a variety of biotechnology applications, including biomimetic materials for cell adhesion, affinity chromatography sorbents, and solid-phase biocatalysts.[1−3] The ease and convenience of using light as the switch to control the temporal and spatial features of a chemical process make it attractive for use in biomolecular immobilization. The most commonly used compounds for biomolecular photoimmobilization include aryl azide, diazirine, and benzophenones.[4−7] The active intermediates derived from these photosensitive moieties can react with the surrounding chemicals by nonspecific insertion into chemical bonds; however, in most situations, the efficiency of these photoreactions is limited by water, requiring dehydrated materials or anhydrous organic solvents to optimize photoreaction yields.[8,9] Consequently, these common photoimmobilization biomolecules are of limited utility in aqueous environments, including those of 3-D hydrogels or for in vitro and in vivo chemistry.

2-Nitrobenzyl and its derivatives are important photolabile protecting groups used for a wide range of nucleophilic functional groups, such as amine, hydroxyl, carboxylic acid, sulfhydryl, among others.[10,11] The photogeneration of functional groups by cleaving 2-nitrobenzyl has a satisfactory yield in aqueous environments of 20−70% at pH 7.5.[12,13] By attaching these protecting groups to active amino acids, peptides, and proteins through the functional groups in amino acids, "caged" compounds can be created—that is, compounds with their bioactivity blocked temporarily and recovered upon photoirradiation. The soluble caged amino acids, peptides, and proteins are significant tools in many applications, from physiological studies,[10,14,15] to drug discovery,[16] to imaging.[17] Immobilizing a 2-nitrobenzyl-protected biomolecule to a hydrogel presents opportunities to modify the local chemistry through laser technologies[18] or masks,[19] for example, to deliver specific signals to cells in 3-D matrixes.[20]

Agarose is a thermosensitive natural polysaccharide polymer, consisting of disaccharide ((1→4)-3,6-anhydro-α-L-galactopyranosyl-(1→3)-β-D-galactopyranose) repeating units. Agarose hydrogels have been used as substrates for model 3-D cell culture studies, cell encapsulation, and tissue engineering.[21−23] Like other polysaccharide polymers, agarose can be conveniently modified through the activation of hydroxyl groups as has been demonstrated with several reagents including cyanogen bromide, glycidol, 4-nitrophenyl chlorocarbonate, 1,1-carbonyldiimidazole, among others.[2,24−26]

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The hydroxyl groups on agarose can be modified to elicit a specific response, and because agarose gels are transparent, they can be modified with light. Because agarose itself is nonadhesive to cells yet allows cell infiltration (and cell visualization), it is attractive for studies investigating specific cellular interactions.[22]

In this study, we report the synthesis of a new type of agarose hydrogel material bearing immobilized 2-nitrobenzyl-protected biomolecules that are amenable to further peptide and protein immobilization. *S*-(2-Nitrobenzyl)cysteine was grafted to hydroxyl groups of dissolved agarose polymer, and free functional sulfhydryl groups were generated upon photoirradiation. When the biomolecular compounds (peptides and proteins) were activated with sulfhydryl-reactive groups, e.g., maleimide, they were immobilized to agarose hydrogel matrixes, demonstrating the breadth of molecules possible to immobilize. The extent of immobilization was controlled by the energy dose (i.e., irradiation time) delivered locally, as determined by fluorescence measurements of fluorescently labeled peptides and proteins. A fibronectin-derived oligopeptide, GRGDS, was immobilized and used in cell studies where the bioavailability of immobilized GRGDS was tested by measuring neurite outgrowth of cultured primary embryonic chick dorsal root ganglion cells as a function of ligand concentration. Ultimately, these new materials may be useful tools to study cell behavior.

2. Experimental Section

All chemicals were purchased from Sigma and used as received unless otherwise specified. Water was distilled and deionized using Millipore Milli-RO 10 Plus and Milli-Q UF Plus (Bedford, MA) at 18 MΩ resistance. Water for cell culture was sterile-filtered through a 0.22 μm filter (Millipore, MA).

2.1. Synthesis of *S*-(2-Nitrobenzyl)cysteine (*S*-NBC). *S*-NBC was synthesized by modifying a published procedure.[27] L-Cysteine (1.27 g or 10 mmol), dissolved in 30 mL of distilled water, was neutralized by triethylamine (1.412 mL or 9.2 mmol). The solution was cooled to 0 °C, and then added dropwise to a 2-nitrobenzyl bromide solution (2 g or 9.2 mmol in 30 mL of methanol). The reaction was maintained at 0 °C for 30 min and then at room temperature for another 30 min. The crude product of *S*-NBC was obtained after the pale yellow precipitate was collected and washed with ethyl acetate and water. By recrystallizing the crude product in hot water, purified *S*-NBC was obtained as pale yellow long needles. The UV peak absorption (in phosphate buffer, pH 7.4) was 266 nm, $\epsilon = 4470$ L·mol^{-1}·cm^{-1}. The ^{1}H NMR spectrum of *S*-NBC in acidic water (D_2O:CF_3COOD = 90:10, v/v) showed the relevant peaks at δ 2.95 (2H, m, ArCH$_2$SC*H*$_2$CHR), δ 4.0−4.2 (3H, m, ArC*H*$_2$SCH$_2$C*H*R), and δ 7.3−8 (4H, Ar). Only one spot (R_f 0.75) was observed on silica gel plates eluted with 2-propanol/2-butanol/acetic acid/water (5:5:3:3), further confirming the purity of the product.

2.2. Synthesis of *S*-NBC-Grafted Agarose Polymer and (2-Aminoethoxy)ethylamine (AEA)-Grafted Agarose Polymer. Under a N_2 gas environment, 0.2 g of ultralow gelling temperature (ULGT) agarose was added to 5 mL of DMSO and heated to 80 °C until totally dissolved. The agarose solution was cooled to room temperature, and then 1,1′-carbonyldiimidazole (CDI; 0.01−0.09 g dissolved in 2 mL of DMSO) was added dropwise. After 1 h of activation, 0.12 g of *S*-NBC in 3 mL of DMSO (dissolved by heating) was added. The reaction was maintained at room temperature for 12 h and then dialyzed against water for 3 d. The dry, white *S*-NBC-grafted ULGT agarose polymer was obtained after the aqueous solution was lyophilized. The absorption profile of *S*-NBC-grafted agarose polymer was characterized by UV−vis spectrophotometry (Ultraspec 4000, Biopharmacia). The substitution level of *S*-NBC was calculated by comparing the absorptions of modified agarose polymers to that of the control, unmodified agarose polymer at 266 nm.

To better understand how the ionic properties of *S*-NBC affected its grafting to agarose, a nonionic-amine-containing compound, AEA, was grafted to agarose using CDI activation and then purified following the same procedure described above for *S*-NBC. The substitution level of AEA in the resulting agarose was determined by analyzing the amine concentration in the polymer solution by 2,4,6-trinitrobenzenesulfonic acid (TNBSA) using a standard protocol.[28] Briefly, TNBSA was dissolved in 0.1 M sodium bicarbonate at a concentration of 0.01% (w/v) and was reacted with the modified agarose. By comparing the absorbance (335 nm) of the chromogenic TNBSA derivative formed after reaction relative to a calibration curve, the amine content in agarose was determined.

2.3. Characterization of *S*-NBC-Grafted Agarose Polymer. All characterization and biomolecular photoimmobilization studies were performed on agarose synthesized with 0.07 g of CDI/0.2 g of agarose in DMSO. Agarose solution and hydrogel matrixes were prepared using phosphate-buffered saline (PBS) solution at pH 7.4.

2.3.1. Gelation of *S*-NBC-Grafted Agarose Polymer. The gelability of *S*-NBC-grafted agarose polymers with different *S*-NBC concentrations was tested at 0.5 wt % agarose concentration in water. *S*-NBC-grafted agarose (200 μL) was dissolved by heating and then cooled to room temperature followed by refrigeration at 4 °C to create a gel. The time required for gelation was estimated qualitatively by monitoring whether the gel flowed when turned upside down. If *S*-NBC−agarose gels did not visibly flow after being turned upside down for 30 min, they were considered to have gelled. Other spectroscopic techniques for gelation were not appropriate because agarose forms a transparent gel.

2.3.2. Reological Characterization of *S*-NBC-Grafted Agarose Polymer. The rheological properties of *S*-NBC-grafted agarose and unmodified ULGT agarose were compared by dynamic shear tests. Agarose solution (0.5 mL, 1% or 0.5%) was loaded between the cone (diameter 6 cm and angle 0.5°) and plate geometry of a dynamic rheometer (AR 2000, TA Instruments) and cooled at 4 °C for 90 min to solidify. The temperature was raised to 37 °C (to simulate the cell culture/physiologic temperature) and equilibrated for 5 min. The frequency sweep was performed from 0.1 to 100

rad/s with 1% shear strain applied to the sample. Dynamic shear moduli (storage and loss moduli) were measured.

2.3.3. Photolysis of *S*-NBC-Grafted Agarose Polymer Results in Free Sulfhydryl Groups. Generation of free sulfhydryl groups in *S*-NBC-grafted agarose polymer solution upon photoirradiation was tested as a function of photoirradiation time and analyzed using Ellman's reagent. *S*-NBC-modified agarose solution (200 μL, 1 wt %, in a 48-well plate) was cooled on ice and irradiated for a prescribed time (from 0 to 120 s) using a mercury UV lamp (X-cite, EFOS) with a light output at 0.4 W. Ellman's reagent was added to the photoirradiated solution and reacted for 15 min. The concentrations of photoreleased sulfhydryl groups were determined by the solution absorption at 412 nm.

2.4. Synthesis of Biomolecules with Sulfhydryl-Reactive Groups. **2.4.1. Activation of Peptides with Maleimidyl (MI) and Acrylyl (A) Groups That React with Sulfhydryl**. Maleimidyl- and acrylyl-activated oligopeptides were custom-synthesized on a solid-state peptide synthesizer (Pioneer, Applied Biosystems, California) on a 0.1 mmol scale. The desired oligopeptide sequence, i.e., GRGDS or GDPGYIGSR, was first synthesized (0.1 mmol) without cleaving the side chain protecting groups on a peptide synthesizer. One of either 3-maleimidopropionic acid or acrylic acid (1 mmol) was activated using dicyclohexylcarbodiimide (1 mmol) in dichloromethane (10 mL) for 30 min under nitrogen protection, and a white precipitate was filtered. The filtrate was collected and reacted with the amine terminal of the peptide on the resin for 2 h. The resin was washed sequentially with dichloromethane, 2-propanol, and methanol before being dried under vacuum. The MI- or A-modified peptide *N*-α-(3-maleimidopropionyl)-GRGDS (MI-GRGDS), *N*-α-(3-maleimidopropionyl)-GDPGYIGSR (MI-GDPGYIGSR), acrylyl-GRGDS (A-GRGDS), or acrylyl-GDPGYIGSR (A-GDPGYIGSR) was deprotected and cleaved from the resin using 95% aqueous trifluoroacetic acid (TFA; 2 h) and then lyophilized. The molecular weight and qualitative purity of the oligopeptides were analyzed by MALDI mass spectrometry (Voyager-DE STR, PerSpective Biosystems, California).

2.4.2. Activation of Proteins with Maleimidyl and Dithiopyridyl Groups That React with Sulfhydryl. Proteins were modified with either maleimidyl or dithiopyridyl according to the published procedure of Hermanson et al.[28] Bovine serum albumin (BSA) was the protein used to study protein immobilization. A 1 mg sample of a bifunctional cross-linker, either *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) or sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC), was dissolved in 100 μL of DMSO and added to 1 mL of a 10 mg/mL BSA solution in PBS at pH 7.4 for 2 h. The modified BSA was then passed through a Sephadex G-75 column to remove the excessive, unreacted SPDP or sulfo-SMCC. The fractions containing the modified protein product were evident by absorption at 280 nm (Ultraspec 4000, Biopharmacia) and collected. The activated BSA was lyophilized and stored at −20 °C in a desiccator until use.

2.4.3. Modifying Activated Peptides and Proteins with Fluorescent Groups. Fluorescein-labeled peptides were synthesized in the same way as described in section 2.4.1, except that a fluorescein-labeled lysine, *N*-α-Fmoc-*N*-ϵ-(5/6-carboxyfluorescein)-L-lysine (Anaspec, California) was added to the amine terminal of the oligopeptide sequence before the terminal functional compound 3-maleimidopropionic acid was added. The full structure of the fluorescein-labeled maleimidyl-activated GRGDS was *N*-α-(3-maleimidopropionyl)-*N*-ϵ-(fluorescein)lysine-GRGDS (MI-fluo-K-GRGDS). Fluorescein-labeled BSA was synthesized as described in section 2.4.2, except that a labeling compound, NHS-fluorescein, was mixed with sulfo-SMCC or SPDP (7:10, w/w) and reacted with BSA in the activation step.

2.5. Conjugating Activated Oligopeptides to Agarose Hydrogel Matrixes. **2.5.1. Fluorescence Imaging of Photoimmobilization**. The immobilized peptides and proteins were detected and imaged by fluorescence of fluorescein-labeled peptides and proteins. Hydrogel samples (1%) were mixed with activated peptide or proteins (10 wt % fluorescein-labeled reagent) for 30 min and irradiated for 1 s with a focused 3.5 mW laser beam (Omnichrome, model 3074R-S-A03). The irradiated hydrogel matrixes continued to react with activated peptide for 4 h before the hydrogel samples were thoroughly washed with PBS to remove excessive unreacted peptides or proteins. After irradiation and photoimmobilization, the chemical domains of peptides or proteins were imaged by a confocal microscope using a 10× objective (FV300, Olympus).

2.5.2. Fluorescence Intensity Detection for Photoimmobilization. To study the photoimmobilization yield, agarose hydrogel matrixes were irradiated for different lengths of time and analyzed for fluorescence intensity using a spectrofluorimeter (Gemini, Molecular Devices). *S*-NBC-modified agarose gel samples (75 μL of a 0.5% solution in a 96-well plate) were cooled on ice and irradiated for a prescribed time (from 0 to 120 s) by an EFOS UV lamp (0.4 W). Irradiated samples were reacted with maleimidyl-activated GRGDS oligopeptide (MI-fluo-GRGDS:MI-GRGDS = 1:9, w/w) as described above. The immobilized GRGDS peptide was detected by the spectrofluorimeter.

2.6. Cellular Response to Peptides of Different Concentrations Immobilized on Agarose Hydrogel Surfaces. *S*-NBC−agarose solution was sterile-filtered through a 0.22 μm filter (Millipore, MA), dispensed in 75 μL quantities in each well of a 96-well plate, and refrigerated at 4 °C for 90 min to solidify the gel. Hydrogel samples were irradiated for a prescribed time varying from 1 to 60 s and then reacted with MI-GRGDS oligopeptide as described above. Chick dorsal root ganglia (DRG) cells were harvested from E9-11 chick embryos that were removed from fertilized eggs according to published procedures.[29] Briefly, connective tissue capsules were removed from DRGs and axons severed. Explanted DRGs were incubated with trypsin solution (0.0375% in HANKS buffer) for 20 min to degrade extracellular matrixes. DRGs were collected by centrifuge (1500 rpm, 5 min), immersed in cell culture medium, and dissociated by gentle trituration. After being diluted and plated on substrates (2×10^4 cells/cm^2), the dissociated DRG cells were cultured in MEM α medium containing 10% horse serum, 1% glutamine, 1% penicillin/streptomycin, and 50

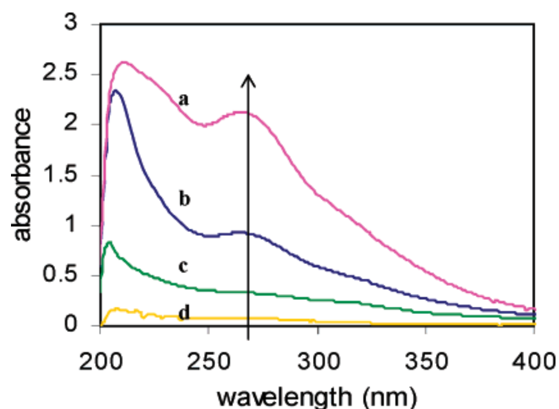

Figure 1. UV spectra of *S*-NBC-grafted agarose with varying degrees of CDI activation. Agarose polymers were processed with (a) 0.04 g of CDI/0.2 g of agarose, (b) 0.01 g of CDI/0.2 g of agarose, and (c) 0 g of CDI/0.2 g of agarose. (d) is the spectrum for unprocessed plain ULGT agarose.

Table 1. Yields of Immobilization of *S*-NBC to Agarose Polymer by CDI Activation

sample code	CDI/agarose (g/g)	*S*-NBC concn in 1% agarose gel (mM)	deg of substitution (mol %)	*S*-NBC/CDI (mol %)
A	0.0/0.2	0.173	0.56	
B	0.04/0.2	0.578	1.9	4.7
C	0.07/0.2	0.801	2.5	3.8
D	0.09/0.2	1.395	4.5	5.0

ng/mL NGF (all supplied by Gibco) in a standard cell culture incubator (37 °C, 5% CO_2, and 100% humidity). After 24 h, neurite growth on the surfaces was recorded using the phase contrast microscope (LM410, Zeiss) to calculate average neurite density and neurite length. The neurite length reported is the average and standard deviation of 70 neurites except in those cases where the neurite number was less than 70, in which case the total countable neurites were averaged and noted.

3. Results

3.1. Synthesis and Characterization of 2-Nitrobenzyl-Grafted Agarose Hydrogel. *S*-NBC was covalently immobilized to the ULGT agarose gel as evidenced by the absorption profile of the modified agarose polymer solution vs unmodified controls, as shown in Figure 1. The UV absorption peak at 266 nm corresponds to the 2-nitrobenzyl moiety covalently bound to agarose. The extent of *S*-NBC modification is a function of CDI activation, where greater CDI activation results in greater *S*-NBC modification of agarose (part a vs b of Figure 1). In controls where *S*-NBC was mixed with unactivated (i.e., no CDI) agarose, the peak at 266 nm was largely absent (Figure 1c). The peak was also absent in plain agarose (Figure 1d). The resulting degree of substitution and concentration of *S*-NBC in 1% agarose gels are directly correlated with the amount of *S*-NBC-modified agarose and the amount of CDI activation, as summarized in Table 1. These data demonstrate that CDI is an efficient way to activate agarose hydroxyl groups and covalently bind amine-containing compounds, such as peptides and proteins. However, the yields of grafting *S*-NBC to agarose were limited.

Table 2. Yields of Modification of Diamine Compound to ULGT Agarose

CDI/agarose (g/g)	diamine concn in 1% agarose gel (mM)	immobilized AEA/CDI (mol %)
0.04/0.2	5.9	50
0.02/0.2	2.8	48

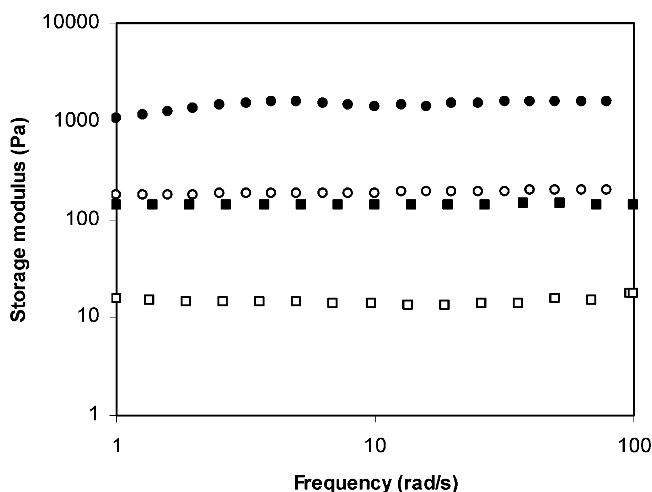

Figure 2. Comparison of modified (hollow symbols) and unmodified (solid symbols) agarose hydrogel in mechanical strength. The storage moduli for *S*-NBC-modified agarose at (○) 1% and (□) 0.5% were significantly lower than those of unmodified ULGT agarose at (●) 1% and (■) 0.5%.

To gain greater insight into the coupling of *S*-NBC to agarose using CDI, a nonionic amine, AEA, was grafted to agarose using the same method. The diamine was added in excess, and each grafted moiety contained a free amine. The final modification level was characterized by determining the free amine content in the modified agarose. As summarized in Table 2, the yield of grafting AEA to ULGT agarose was almost 50%, which is an order of magnitude higher than that of *S*-NBC, likely reflecting the difference in reactivity of *S*-NBC and AEA in DMSO.

Since 2-nitrobenzyl is hydrophobic, only moderately modified agarose (i.e., up to 5%) remained both soluble in water at high temperature and optically transparent upon cooling to the hydrogel. A higher substitution level of *S*-NBC resulted in a solid agarose that would not dissolve in boiling water. Dissolving *S*-NBC-modified agarose requires heating the polymer in water at a much higher temperature (usually 100 °C in boiling water) in comparison with the unmodified ULGT agarose, which can be easily dissolved at approximately 50 °C. Although the polymers listed in Table 1 remained gelable, the agarose solution showed a prolonged gelling time compared to unmodified agarose, which gels within 15 min. For 0.5% *S*-NBC−agarose, the gelation time increases with modification to 30, 50, and 90 min for *S*-NBC concentrations of 0.578, 0.801, and 1.395 mM, respectively.

One consequence of chemical modification of agarose with *S*-NBC is a reduction in mechanical properties relative to those of unmodified agarose. Dynamic mechanical tests were performed to quantify the stiffness of the modified vs unmodified hydrogels. As can been seen from Figure 2, the storage moduli of *S*-NBC-modified agarose hydrogel ma-

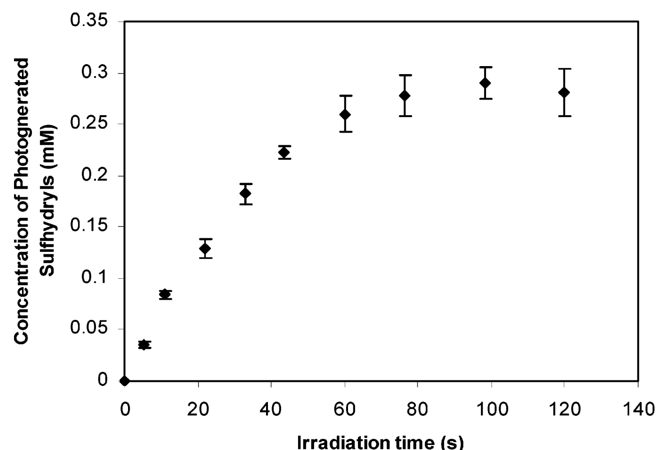

Figure 3. Concentration of photogenerated free sulfhydryl groups (mean ± standard deviation) in an agarose hydrogel increases with irradiation time until a plateau is reached at 60 s.

trixes (with a 2.5% degree of *S*-NBC substitution, i.e., entry C of Table 1) were significantly lower than those of unmodified agarose matrixes by approximately an order of magnitude. This decrease is likely due to a disruption of the hydrogen-bonding mechanism required for gelation of agarose.

S-NBC remained photosensitive when immobilized to the agarose polymer. The release of sulfhydryl groups in agarose hydrogel upon photoirradiation was studied using Ellman's reagent. As shown in Figure 3, the amount of free sulfhydryl groups increased with increased irradiation time and likely reached a plateau when either all of the 2-nitrobenzyl groups were cleaved or, more likely, the highest yield was achieved. Therefore, controlling the dose of light energy through irradiation time can be used to obtain the desired biomolecule concentration. When the concentration of *S*-NBC in modified agarose (by UV) was compared with the concentration of free sulfhydryl groups in agarose (by Ellman's reaction), the yield of photocleavage was estimated at 35%, which is significantly higher than those other photochemical reactions in water of 1%[30] to 9%.[8]

3.2. Photoimmobilization of Biomolecules to Agarose Hydrogel. **3.2.1. Activation of Peptides and Proteins with Sulfhydryl-Reactive Groups**. To immobilize biomolecules to *S*-(2-nitrobenzyl)cysteine by photoirradiation, peptides and proteins were modified with sulfhydryl-reactive groups. Many chemical groups react with sulfhydryl groups in aqueous environments, including maleimide and unsaturated carbonyl; acrylyl groups react with sulfhydryl, forming stable thioether linkages via Michael addition.[28,31] Pyridyl disulfide[28,32] also reacts with sulfhydryl groups and results in disulfide bonds. In this study, we modified peptides with maleimide and acrylyl during solid peptide synthesis and activated proteins with maleimide and pyridyl disulfide.

Peptides were modified during solid-state synthesis with the crude protected peptide on the resin. After reaction with activated 3-maleimidopropionic acid or acrylic acid (1 mmol) on the resin, the peptide products coupled with the sulfhydryl-reactive groups and were successfully precipitated from the cleavage cocktail. As maleimide is susceptible to hydrolysis, contact with aqueous solution was minimized. The mass spectrometry data of acrylyl- and maleimide-activated GRGDS and GDPGYIGSR are summarized in Table 3. While the molar mass of the acrylyl-activated peptides did not correspond to the theoretical value, the molar mass of maleimide-activated peptides did correspond to the theoretical value, and thus these were used further, in reactions with *S*-NBC-modified agarose.

Table 3. Mass Spectroscopic Analysis of Oligopeptide Products

oligopeptide	theoretical MW	MS analysis
A-GRGDS	544.5	single peak, 617.0
MI-GRGDS	641.6	single peak, 642.2
A-GDPGYIGSR	975.0	single peak, 1048.0
MI-GDPGYIGSR	1072.07	single peak, 1071.2

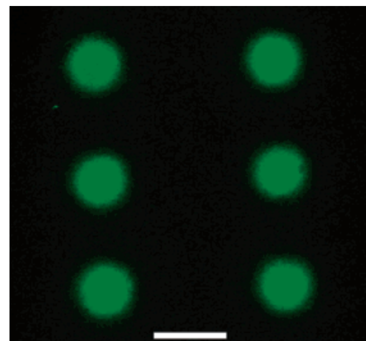

Figure 4. Photoimmolibized BSA domains in an agarose hydrogel are visualized by confocal microscopy of fluorescein-labeled BSA (scale bar 200 μm).

Proteins were modified by reacting the amine groups with succinimide, followed by dithiopyridyl, maleimide, and fluorescein. BSA that was modified with either SPDP or sulfo-SMCC showed weak absorption at 340 nm, while fluorescein-modified BSA showed strong absorption at 495 nm. The modified protein (activated by either SPDP or sulfo-SMCC) was photoimmobilized to agarose using a laser beam.

3.2.2. Imaging Photoimmobilized Biomolecules. Photoimmobilization of MI-peptides was achieved using a He−Cd focused laser that was used to irradiate the hydrogel matrixes in selected regions. Fluorescein-labeled compounds were used to visualize the modified regions. Both maleimide-activated peptides and maleimide/pyridyl disulfide-modified BSA were effectively immobilized to sulfhydryl-functionalized agarose hydrogel matrixes, indicating that the functional groups attached to the biomolecules were reactive. A representative image of the BSA protein domains is shown in Figure 4, where fluorescent regions indicate the presence of the agarose-immobilized BSA. A similar pattern was obtained in peptide immobilization to agarose. Controls that had either *S*-NBC−agarose (not irradiated) or unmodified agarose mixed with activated protein/peptide had no visible fluorescence and were black (not shown).

3.2.3. Fluorescence Intensity Detection of Photoimmobilized Biomolecules. Using the fluorescence-labeled oligopeptide MI-fGRGDS, the relative amount of peptide immobilized to the hydrogel matrix surfaces was investigated in terms of fluorescent intensity using a spectrofluorimeter. As the fluorescence intensity is related to peptide concentration, the GRGDS immobilization yield increased with the irradiation time (Figure 5), in a fashion similar to that

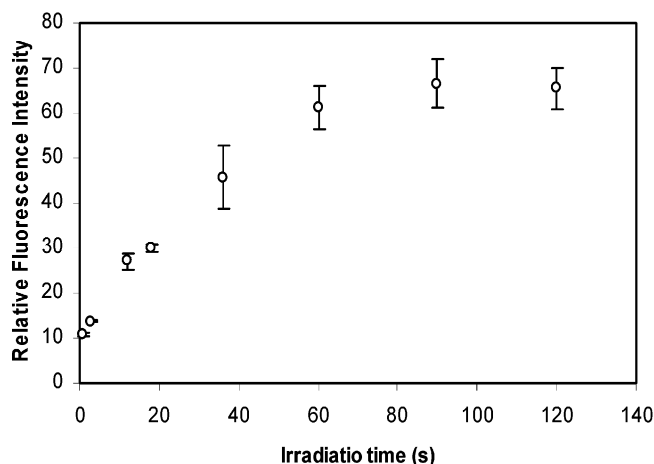

Figure 5. Relative fluorescence intensity (mean ± standard deviation) of immobilized GRGDS peptide. The immobilization exhibited a pattern similar to that of the photogeneration of sulfhydryl groups: it increased with irradiation time until a plateau was reached at 60 s.

observed for the photoreleased free sulfhydryl groups. The relative fluorescent intensity, or concentration of peptide immobilized, reached a plateau after ~60 s using our irradiation conditions, demonstrating a 6-fold increase in the concentration of immobilized peptide relative to that achieved after 1 s. It is important to note that the plateau was not due to the onset of fluorescence quenching because the fluorescence signal at the plateau was within the linear fluorescence signal range (data not shown).

3.3. DRG Cellular Response to Photoimmobilized GRGDS on Agarose Surfaces. Because neurons are adhesion-dependent for neurite extension, both an adhesive substrate and a nerve-growth-factor-supplemented medium are required. While agarose itself is nonadhesive to cells, we expected that GRGDS-modified agarose would be cell-adhesive. We studied the cellular response of chick DRG neurons to agarose surfaces modified with different concentrations of photoimmobilized GRGDS, where the irradiation time controlled the GRGDS concentration. Cells were observed 24 h after plating. On nonirradiated agarose controls, cells formed aggregates and did not extend neurites (Figure 6a). With 1 s of irradiation, GRGDS was immobilized to agarose and stimulated cell adhesion and extension of short neurites (Figure 6b), indicating that the immobilized GRGDS was bioactive. By increasing the irradiation time to 3 s (Figure 6c) and then to 12 s (Figure 6d), both the neurite density and length increased. As shown in Figure 7, the neurite density (Figure 7a) and neurite length (Figure 7b) leveled off after 12 s. Despite the more than 2-fold increase in the immobilized GRGDS concentration from 12 to 60 s (cf. Figure 5), cells behaved very similarly on these substrates.

4. Discussion

4.1. Synthesis of a Photolabile Hydrogel Material Containing a 2-Nitrobenzyl-Protected Moiety. In this study, CDI was used to activate the hydroxyl groups and immobilize a photosensitive amine-containing compound to agarose. As hydroxyl groups are present in many hydrogels composed of either natural polysaccharides or synthetic polymers, this synthetic route can be used to introduce photolabile *S*-NBC to a diversity of polymers to generate functional groups in hydrogel matrixes for bulk photoreaction.

Using a protected cysteine was advantageous because the carboxylic acid group on cysteine enhanced the hydrophilicity of the 2-nitrobenzyl moiety, thereby promoting conjugation to the hydrophilic agarose polymer. Nonetheless, the yield of *S*-NBC immobilized over CDI activation was low and <5% (cf. Table 1), possibly because the reactivity of *S*-NBC was limited in the aprotic solvent (DMSO) used. In contrast, the yield of grafting the diamine (AEA) to the ULGT agarose is much higher than that of *S*-NBC. This suggests that the modification yield could be improved if a hydrophilic, nonionic photolabile compound were used.

The photochemistry of 2-nitrobenzyl derivatives is highly dependent on pH.[33] Chang et al.[34] found that a higher conversion yield was obtained when the caged cysteine peptide was irradiated at pH 6.0 (50%) compared to pH 8.5 (30%), the latter of which is similar to the results obtained in this study. As free sulfhydryls are not stable and susceptible to oxidation in air, a reducing reagent, DTT, can be added to improve the photoreaction yield. However, because DTT contains free sulfhydryls which compete for the reaction with protein or peptide biomolecules, no DTT was added to reduce the oxidation of sulfhydryl groups.

Due to the hydrophobic nature of the 2-nitrobenzyl groups of *S*-NBC, the extent of modification was limited to preserve the gelation behavior of agarose. As can been seen from studying the physical properties of the modified agarose hydrogel, even at moderate modification levels, both prolonged gelation times (Table 1) and decreased mechanical strength (Figure 2) were observed. The change in physical properties of agarose reflects a decreased association of polymer chains due to the partial loss of intermolecular interactions associated with agarose hydroxyl groups. This decreased number of intermolecular interactions likely results from the presence of both *S*-NBC-modified groups and *S*-NBC side products. To increase the extent of photolabile moiety modification, the chemical structure of the 2-nitrobenzyl group may have to be modified to be more compatible with hydrophilic hydrogels. For example, α-carboxy-2-nitrobenzyl is more hydrophilic than 2-nitrobenzyl, and may be more compatible with the hydrophilic agarose as a protecting sulfhydryl group for biomolecular immobilization.[34]

4.2. Photoimmoblization of Peptides and Proteins to Agarose Hydrogels. Immobilizing peptides to the bulk or the surface of a material is an important step in the creation of biomimetic substrates for controlling cell attachment and function. Many conjugation chemistries of peptides have been described, including the reaction of carboxylic acid-modified materials with the amine termini of oligopeptides, such as GRGDS, to form stable amide bonds.[35−37] More chemoselective approaches, such as taking advantage of the free sulfhydryl group of cysteine to react with acryloyl-, maleimide-, or gold-functionalized materials, allows peptide coupling and presentation to be better controlled.[38,39]

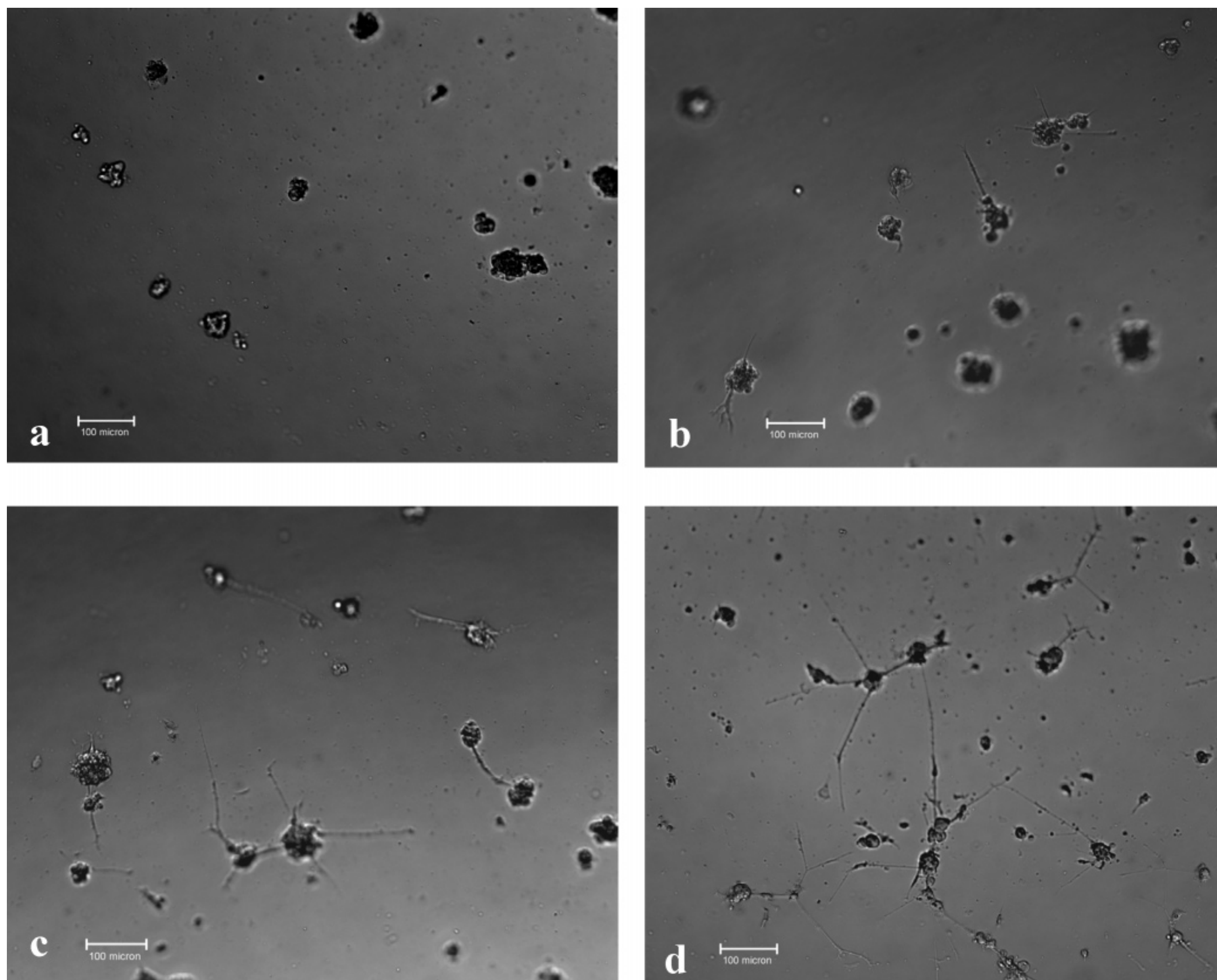

Figure 6. DRG neurons were plated on 1% agarose gels that had been irradiated to photoimmobilize GRGDS peptides for a series of times: (a) 0 s, (b) 1 s, (c) 3 s, and (d) 12 s. Cells were imaged under a light microscope 24 h after plating. Cells aggregated with each other on unmodified agarose (a) and extended neurites on modified agarose (b−d), with greater lengths and numbers of neurites observed with greater irradiation times (scale bar 100 μm).

In this study, we introduced sulfhydryl reactive groups (i.e., maleimide) to the amine terminus of the oligopeptide during solid-state peptide synthesis, which allowed efficient conjugation to sulfhydryl-functionalized agarose hydrogels. Maleimide is stable during both the deprotection of the peptide side groups and the cleavage of the peptide from the resin using the TFA cleavage cocktail. This method, however, has some limitations and cannot be used readily with amino acid sequences containing either cysteine or lysine, both of which will react with maleimide.

The immobilization of proteins, especially enzymes to substrates, finds wide applications in biotechnology.[2,4,40] Studies have shown that by introducing thiol-reactive functional groups, enzymes can be effectively conjugated to thiolated agarose beads.[41] In this study, we demonstrated the feasibility of immobilizing a model protein, BSA, using bifunctional cross-linkers. Proteins covalently bonded to the hydrogel through photoreleased sulfhydryl groups and formed disulfide and thioether bonds after activation by SPDP and sulfo-SMCC, respectively. As SPDP and sulfo-SMCC reacted with the lysine residues in the protein, immobilization may have affected the protein conformation and bioactivity. Given that immobilization chemistry affects bioactivity,[4,42,43] additional studies will be necessary to understand the bioactivity of the proteins photoimmobilized herein.

4.3. Ligand Density and DRG Cellular Response to Photoimmobilized GRGDS on Agarose Surfaces. Controlling the adhesive ligand density on substrates is often of great interest in biomaterial design, as it has been shown to affect cellular response to substrates in terms of adhesion strength, receptor expression, and function.[44−46] We demonstrated the bioactivity of coupled adhesive peptides by observing neuritogenesis on modified agarose gel surfaces. Although excessive 2-nitrobenzyl and thiol residue may be toxic to cells, no adverse cellular response was observed in our study. An advantage of our photoimmobilization method is the ability to control the adhesive oligopeptide concentration, by varying the irradiation time, and thereby direct cellular response on hydrogel substrates.

In our cell culture system, the neuritogenesis was likely induced by the interaction of the signaling pathways activated by integrin and trk A, NGF receptors on the cell membrane. Upon binding to the extracellular adhesion ligand, GRGDS, integrin receptors activate a series of intracellular protein

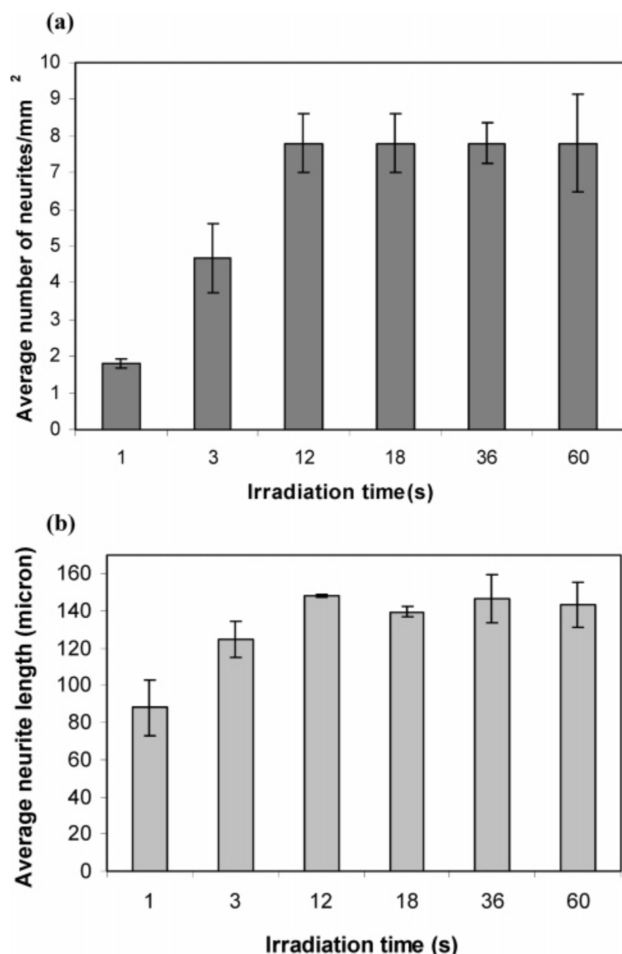

Figure 7. Adherent DRG neurons were quantified in terms of neurite number and neurite length 24 h after plating. The mean ± standard deviation for (a) neurite number and (b) neurite length were calculated for cells on 1% agarose hydrogel surfaces photoimmobilized with GRGDS, for a series of irradiation times. Both the number and length of neurites increase with irradiation time (or GRGDS concentration) up to 12 s of irradiation, after which a plateau in the cellular response was observed ($n = 70$ cells).

tyrosine kinases, including the focal adhesion kinases.[47] In addition to initiating cytoskeleton synthesis and organization, some of the kinase activations coordinate with the growth factor receptor signal transduction to regulate neuritogenesis.[47−49]

In this study, a biphasic cellular response was observed. Below a ligand concentration threshold (achieved with 12 s of irradiation here), neurite outgrowth increased with increased GRGDS concentration. When the surface ligand concentration reached a given amount, the neurite length and number on agarose hydrogels reached a plateau. This suggests that the integrin receptor needs a certain adhesion ligand concentration to exhibit normal function. Below this level, the integrin-associated activity, such as promoting neuritogenesis, is enhanced by increased adhesion ligand concentration. Above a threshold ligand concentration, the integrin expression can adjust to the extracellular environment to regulate the cell behavior, as proposed by Letourneau et al.[50] When Letourneau et al. varied the surface concentration of laminin by 10-fold, almost equivalent neurite outgrowth activity was observed because the expression level of integrin receptors on nerve cells was regulated by the extracellular adhesive ligand concentration. Neurite growth was also reported to slow at high adhesion ligand concentraions. We did not observe this decrease in our study, likely because the total adhesion ligand concentration in dilute agarose gel was too low to cause an inhibitory effect on neurite growth.

In summary, the new method for photoimmobilization of biomolecules to hydrogel matrixes established in this study offers the possibility to tune the ligand density to control cell behavior. Activation by light also makes it feasible to immobilize multiple types of ligands with spatial control in two and three dimensions.

5. Conclusions

2-Nitrobenzyl-protected cysteine grafted to agarose provides a new mechanism for the photoimmobilization of biomolecules to substrates. Both peptides and proteins can be activated through convenient chemistries to become active with photogenerated sulfhydryl functional groups in hydrogel matrixes. Ongoing studies will address how to increase the compatibility of photolabile moieties with the skeletal polymer to facilitate control of the material properties. The new photolabile materials may be used to create novel substrates with controlled 3-D chemical structures for cell biology and tissue engineering studies.[20]

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