Articles

Three-dimensional Chemical Patterning of Transparent Hydrogels

Jordan H. Wosnick[†‡] and Molly S. Shoichet*[,†‡§||]

Department of Chemical Engineering and Applied Chemistry, Centre for Cellular and Biomolecular Research, Institute of Biomaterials and Biomedical Engineering, and Department of Chemistry, University of Toronto, 160 College Street, Toronto, Ontario M5S 3E1, Canada

Received April 29, 2007. Revised Manuscript Received August 2, 2007

Covalent modification of agarose with a 6-bromo-7-hydroxycoumarin (Bhc) sulfide derivative yields a hydrogel that generates bound thiol groups upon excitation with either UV light or a pulsed infrared laser. Using a multiphoton confocal microscope as the patterning platform, intricate internal chemically modified volumes of stable nucleophilic thiol groups are created within these hydrogel samples, which are in turn modified with biomolecules without causing hydrogel cross-linking or changes in its physical properties. The use of automated scripts and microscope stage control allows, for the first time, biologically relevant molecules of interest to be photochemically immobilized within the hydrogel in complex patterns with feature sizes comparable to those of mammalian cells. The resulting *chemically* patterned hydrogels have applications in tissue engineering, where they can be used to control cell behavior.

Introduction

Tissue engineering requires a biomimetic matrix to guide cell growth and differentiation within a three-dimensional system. While several groups (including our own) have created three-dimensional analogs of the extracellular matrix, most 3D scaffolds have been filled with physical void volumes that, on the cellular level, appear as flat surfaces.[1] Some synthetic extracellular matrices have been created with enzyme-labile cross-linked gels;[2] however, in these systems, cells must actively cleave the cross-links for penetration therein, which significantly retards cell mobility. To date, no matrix has fulfilled the requirement of providing cells with a true 3D environment in which they are surrounded by stimuli for guided regeneration, except for previous reports from our laboratory where cells were guided in cylindrical, chemically patterned volumes inside a hydrogel.[3,4] These demonstrations were achieved with both agarose and hyaluronan that had been covalently modified with *S*-2-nitrobenzylcysteine, a derivative of cysteine in which the thiol moiety is blocked with a photocleavable protecting group. When 2–3 mm thick samples of these gels are irradiated with a simple UV laser, the laser beam, which has a diameter of ca. 150 μm, causes removal of the protecting groups along its entire path through the gel. The laser irradiation thus provides cylindrically patterned regions (approximately 150 μm in diameter and 2–3 mm in length) that extend through the full thickness of the hydrogel samples. Treating these patterned gels with maleimide-modified oligopeptides resulted in the site-specific covalent immobilization of these biomolecules within the patterned cylinders via Michael-type addition, leading to bulk substrates with cell-adhesive channels defined in a purely chemical fashion (i.e., without altering the local mechanical properties of the gel).

Although the resulting cylindrical channels proved useful for control of cell growth in a spatially directed manner, in order to better mimic the complex cell-adhesive architecture of the central nervous system, we sought to create more sophisticated, truly 3D-modified volumes such as chemical channels, subsurface tunnels, and networks. Accordingly, we examined multiphoton excitation as a means for creating intricate, 3D, chemically defined patterns below the surface of gel samples, avoiding techniques such as conventional photoinitiated polymerization or gel cross-linking[5] that may change the mechanical properties of the materials being patterned. Multiphoton excitation is an important depth-sectioning tool in

* To whom correspondence should be addressed. E-mail: molly@chem-eng.utoronto.ca.
† Department of Chemical Engineering and Applied Chemistry.
‡ Centre for Cellular and Biomolecular Research.
§ Institute of Biomaterials and Biomedical Engineering.
|| Department of Chemistry.

(1) (a) Holy, C. E.; Davies, J. E.; Fialkov, J.; Shoichet, M. S. *J. Biomed. Mater. Res.* **2003**, *65A*, 44. (b) Ishaug, S. L.; Crane, G. M.; Miller, M. J.; Yasko, A. W.; Yaszemski, M. J.; Mikos, A. G. *J. Biomed. Mater. Res.* **1997**, *36*, 17. (c) Mikos, A. G.; Bao, Y.; Cima, L. G.; Ingber, D. E.; Vacanti, J. P.; Langer, R. *J. Biomed. Mater. Res.* **1993**, *27*, 183.

(2) (a) West, J. L.; Hubbell, J. A. *Macromolecules* **1999**, *32*, 241. (b) Levesque, S.; Shoichet, M. S. *Bioconjugate Chem.* **2007**, *18*, 874. (c) Schense, J.; Bloch, J.; Aebisher, P.; Hubbell, J. A. *Nat. Biotechnol.* **2001**, *18*, 415. (d) Kim, S.; Chung, E. H.; Gilbert, M.; Healy, K. E. *J. Biomed. Mater. Res. A* **2005**, *75*, 73.

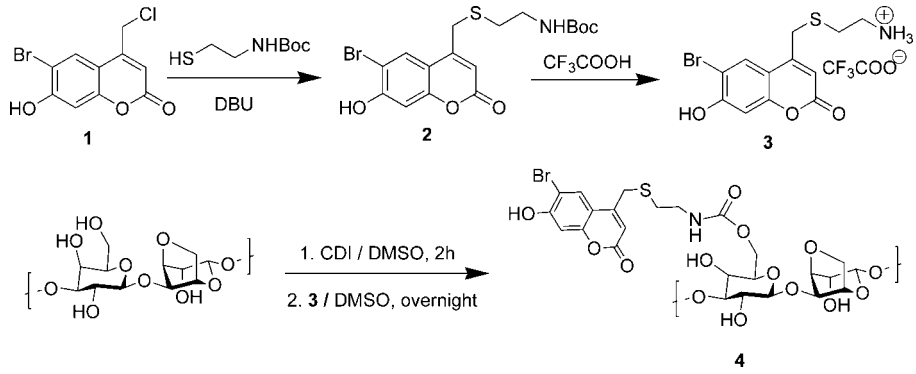
(3) (a) Luo, Y.; Shoichet, M. S. *Nat. Mater.* **2004**, *3*, 249. (b) Luo, Y.; Shoichet, M. S. *Biomacromolecules* **2004**, *5*, 2315.

(4) Musoke, P.; Shoichet, M. S. *Biomed. Mater.* **2006**, *1*, 162.

(5) (a) Hahn, M. S.; Miller, J. S.; West, J. L. *Adv. Mater.* **2006**, *18*, 2679. (b) Albrecht, D. R.; Underhill, G. H.; Wassermann, T. B.; Sah, R. L.; Bhatia, S. N. *Nat. Methods* **2006**, *3*, 369. (c) Flaim, C. J.; Chien, S.; Bhatia, S. N. *Nat. Methods* **2005**, *2*, 119.

10.1021/cm071158m CCC: $40.75
Published on Web 10/06/2007

Scheme 1. Synthesis of Coumarin-Derivatized Agarose 4[a]



[a] A 0.2% degree of substitution. Coumarin moieties are indicated on the polymer repeat structure for clarity.

fluorescence microscopy[6,7] and relies on the large power density present at the focal point of a pulsed laser to excite chromophores using two or more low-energy photons. The requirements of high laser power and tight beam focusing under these conditions ensure that excitation occurs only in a small volume around the focal point of the irradiating laser, while chromophores elsewhere in the laser path are exposed only to isolated low-energy photons. The 3D control of excitation provided by this technique has made it very useful in microlithography, in which objects (including photonic devices) with submicrometer feature sizes can be made by the photoinitiated cross-linking of oligomers in resin form.[8] Recently, formation of solid structures by multiphoton-induced cross-linking of soluble proteins in aqueous media has also been described.[9,10] Our challenge was to harness the multiphoton processing to create chemically distinct volumes in gels and not the physical shapes that had been used in the past.

We anticipated that the localization of multiphoton excitation in 3D hydrogels would provide chemically differentiated patterns that could be used as handles for site-specific covalent immobilization of bioactive molecules in the gels; these in turn could be used to simulate the complex pattern of biochemical cues that are found in natural tissue. In this paper, we describe the realization of these goals in the form of 3D chemical pattern writing in a coumarin-modified thiolated agarose derivative. This coumarin−agarose derivative can be efficiently cleaved using multiphoton excitation on a computer-controlled confocal microscope stage, making it useful for formation of repeated, complex biochemical patterns inside hydrogel samples.

Results and Discussion

Recognizing the relatively low efficiency of multiphoton laser deprotection of nitrobenzyl-based chromophores,[11] we were drawn to the 6-bromo-7-hydroxycoumarin (Bhc)-based protecting groups[12] as the basis for a photosensitive "pattern-writing medium"in hydrogels with chemically masked functionality. The Bhc unit, which is well-attested as an efficient, multiphoton-labile ($\delta_u \approx 1$ GM at 740 nm) protecting group for amines,[12] alcohols and phenols,[13] aldehydes,[14] and diols,[15] has also been used extensively as a phototrigger inside biological systems,[10,16] making it attractive from a tissue-engineering standpoint. However, the ubiquity of amines and low reactivity of alcohols in biological environments required us to consider adapting the Bhc chromophore to the protection of thiols, which, when photochemically unmasked, would provide handles for immobilization of easily prepared, maleimide-modified biomolecules. On the basis of the proposed reaction mechanism for photocleavage of the Bhc group,[15,17] we reasoned that Bhc−sulfide derivatives would similarly generate the corresponding thiols on irradiation. Accordingly, we prepared, for the first time, the sulfide-containing Bhc−amine **3** (λ_{max} = 373 nm, ϵ = 14500 M^{-1} cm^{-1} in 0.1 M HEPES, pH 7) in two steps from the previously reported chloride derivative **1** and commercially available Boc-protected mercaptoethylamine (Scheme 1—see Experimental Section). Conjugation of **3** to agarose was carried out in DMSO using 1,1-carbonyldiimidazole (CDI).[3] Following dialysis and freeze drying, derivatized agarose **4** was obtained in good yield with ca. 0.2% coumarin substitution (based on moles of **3** per agarose repeat unit).

(6) Denk, W.; Strickler, J. H.; Webb, W. W. *Science* **1990**, *248*, 73.
(7) (a) Recent reviews Helmchen, F.; Denk, W. *Nat. Methods* **2005**, *2*, 932. (b) Diaspro, A.; Chirico, G.; Collini, M. *Q. Rev. Biophys.* **2005**, *38*, 97. (c) Svoboda, K.; Yasuda, R. *Neuron* **2006**, *50*, 823. (d) Rubart, M. *Circ. Res.* **2004**, *95*, 1154.
(8) (a) For example, see Cumpston, B. H. *Nature* **1999**, *398*, 51. (b) Lu, Y.; Hasegawa, F.; Goto, T.; Ohkuma, S.; Fukuhara, S.; Kawazu, Y.; Totani, K.; Yamashita, T.; Watanabe, T. *J. Mater. Chem.* **2004**, *14*, 75. (c) Kuebler, S. M.; Braun, K. L.; Zhou, W.; Cammack, J. K.; Yu, T.; Ober, C. K.; Marder, S. R.; Perry, J. W. *J. Photochem. Photobiol., A* **2003**, *158*, 163.
(9) (a) Hill, R. T.; Lyon, J. L.; Allen, R.; Stevenson, K. J.; Shear, J. B. *J. Am. Chem. Soc.* **2005**, *127*, 10707. (b) Allen, R.; Nielson, R.; Wise, D. D.; Shear, J. B. *Anal. Chem.* **2005**, *77*, 5089. (c) Kaehr, B.; Allen, R.; Javier, D. J.; Currie, J.; Shear, J. B. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 16104.
(10) (a) Basu, S.; Cunningham, L. P.; Pins, G. D.; Bush, K. A.; Taboada, R.; Howell, A. R.; Wang, J.; Campagnola, P. J. *Biomacromolecules* **2005**, *6*, 1465. (b) Basu, S.; Wolgemuth, C. W.; Campagnola, P. J. *Biomacromolecules* **2004**, *5*, 2347.
(11) For example, see Brown, E.; Shear, J. B.; Adams, S. R.; Tsien, R. Y.; Webb, W. W. *Biophys. J.* **1999**, *76*, 489.
(12) Furuta, T.; Wang, S. S.-H.; Dantzker, J. L.; Dore, T. M.; Bybee, W. J.; Callaway, E. M.; Denk, W.; Tsien, R. Y. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 1193.
(13) Suzuki, A. Z.; Watanabe, T.; Kawamoto, M.; Nishiyama, K.; Yamashita, H.; Ishii, M.; Iwamura, M.; Furuta, T. *Org. Lett.* **2003**, *5*, 4867.
(14) Lu, M.; Fedoryak, O. D.; Moister, B. R.; Dore, T. M. *Org. Lett.* **2003**, *5*, 2119.
(15) Lin, W.; Lawrence, D. S. *J. Org. Chem.* **2002**, *67*, 2723.

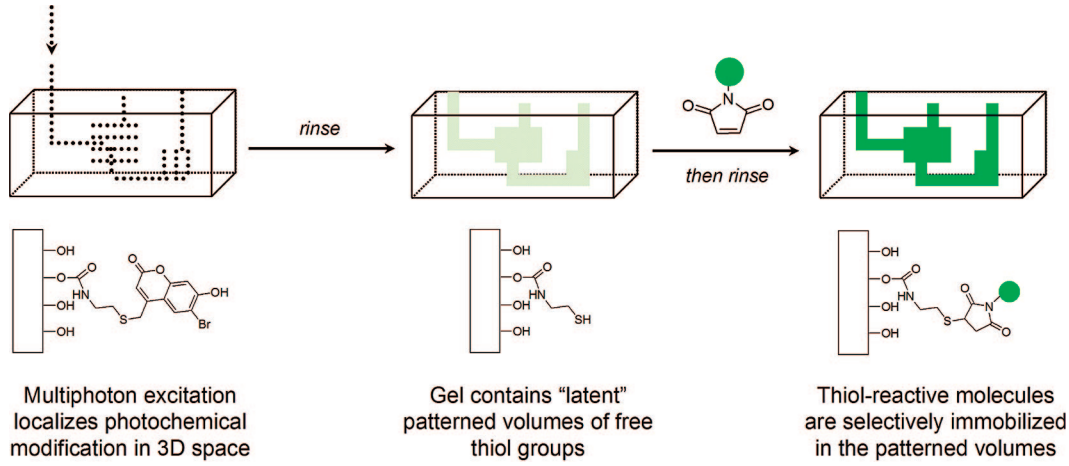


Figure 1. Schematic representation of multiphoton chemical patterning in hydrogels.

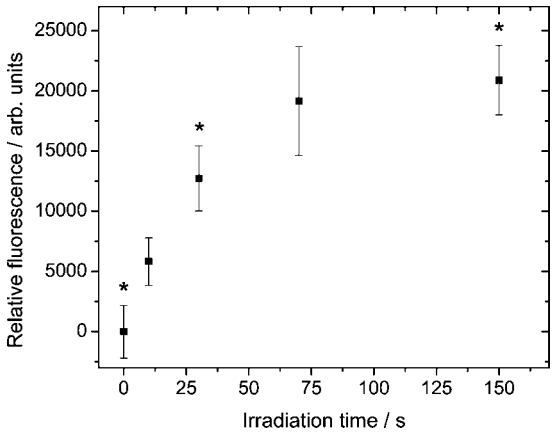


Figure 2. Extent of fluorescein-5-maleimide immobilization in hydrogels of **4** as a function of irradiation time. (Data is shown as mean ± standard deviation, $n = 4$. Asterisks indicate data points that differ significantly from one another at 95% confidence with $p < 0.05$—see Experimental Section for details of ANOVA.)

To verify the ability of hydrogels of **4** to serve as "pattern-writing media"by forming agarose-bound thiols on irradiation, solutions of **4** (1.25% in HEPES buffer, pH 7.0) were irradiated with UV light (350 nm) for various time intervals. Aliquots of the irradiated solution were removed, mixed with excess fluorescein-5-maleimide (a thiol-reactive fluorescent dye), cooled at 4 °C for 2 h to induce gelation, and washed extensively with buffer. Emission from the immobilized fluorescein groups in the resulting gels was found to increase with irradiation time (Figure 2), clearly demonstrating a light-dependent immobilization process.

We tested the ability to create distinct chemical patterns below the surface of hydrogels of **4** by irradiating gelled samples (0.5 mm thick gels from 1% **4** in 0.1 M PBS, pH 7.4) containing a thiol-reactive fluorescent dye (0.5 mg/mL

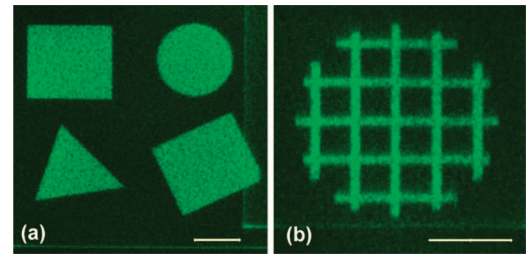


Figure 3. Confocal micrographs of fluorescent (a) geometric shapes and (b) a grid created by photochemical immobilization of AF488-Mal in a hydrogel sample of **4** using multiphoton irradiation guided by software-delineated regions of interest. Scale bars are 50 μm.

Alexa Fluor 488 Maleimide[AF488-Mal], Invitrogen) with a commercial Ti−Sapphire femtosecond laser (Spectra Physics Mai Tai). Gels were placed on the sample stage of a Leica TPS SP2 confocal microscope with a HC PL Fluotar 20×/0.5 lens, and the microscope control software was used to delineate geometric "regions of interest"(ROIs) for raster scanning of the laser focal point within a plane set to arbitrarily chosen depths below the surface of the gel. After irradiation, the gel samples were washed exhaustively to remove nonimmobilized AF488-Mal dye, revealing sharp-edged green fluorescent areas (some as small as 2 μm in width) corresponding to the patterned ROIs (Figure 3). We found that a single 1-s scan at the maximum available laser power (100 mW average at 740 nm) was sufficient for creation of clear patterns following washing and that these patterned areas maintained their shape, size, and bright fluorescence after 2 months storage in aqueous buffer under ordinary room light.

After confirming the suitability of gelled samples of **4** as a "writeable"medium using multiphoton excitation, we studied the specificity and mechanism of the immobilization reaction by attempting multiphoton laser patterning under a variety of conditions. Pattern formation was found to be efficient only at laser wavelengths in the 710–800 nm range, consistent with the two-photon action profile expected for **3**, which has an estimated maximum two-photon absorption around 740–750 nm ($\approx 2\lambda_{max}$). Additionally, we found that pattern writing could also be successfully carried out by irradiating the gels in the *absence* of a thiol-reactive dye, waiting several hours, and then immersing the gel in a

(16) (a) Goard, M.; Aakalu, A.; Fedoryak, O. D.; Quinonez, C.St.; Julien, J.; Poteet, S. J.; Schuman, E. M.; Dore, T. M. *Chem. Biol.* **2005**, *12*, 685. (b) Perdicakis, B.; Montgomery, H. J.; Abbott, G. L.; Fishlock, D.; Lajoie, G. A.; Guillemette, J. G.; Jervis, E. *Bioorg. Med. Chem.* **2005**, *13*, 47. (c) Ando, H.; Furuta, T.; Tsien, R. Y.; Okamoto, H. *Nat. Genet.* **2001**, *28*, 317.

(17) (a) Schade, B.; Hagen, V.; Schmidt, R.; Herbrich, R.; Krause, E.; Eckardt, T.; Bendig, J. *J. Org. Chem.* **1999**, *64*, 9109. (b) Eckardt, T.; Hagen, V.; Schade, B.; Schmidt, R.; Schweitzer, C.; Bendig, J. *J. Org. Chem.* **2002**, *67*, 703.

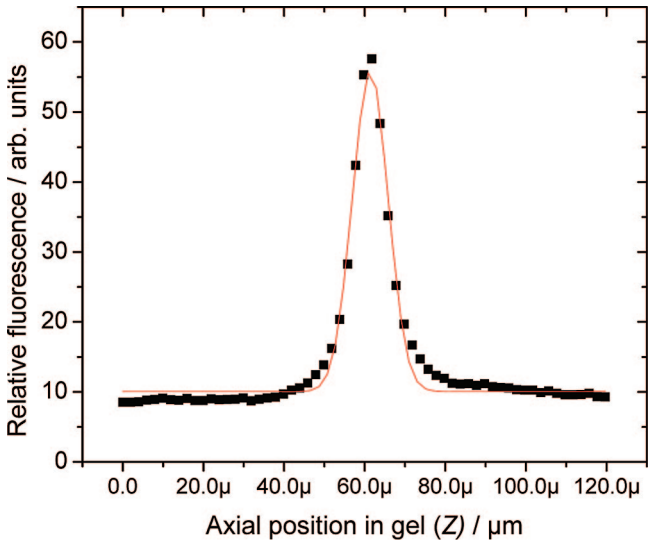


Figure 4. Axial distribution of immobilized AF488-Mal (black squares) after a single multiphoton patterning scan within a sample of **4** followed by washing. The red line represents a Gaussian fit with $\sigma = 4.4$ μm.

solution of AF488-Mal. This observation implies that multiphoton irradiation creates stable agarose-bound thiol groups that remain reactive for prolonged periods, an important and new finding which we confirmed by treating these patterned gels with a large excess of 3-maleimidopropanoic acid prior to introduction of AF488-Mal. In the latter experiment no fluorescence was observed, likely due to the rapid reaction of all available thiol groups with the nonfluorescent maleimide derivative. Overall, the stability of the "latent"patterned image in our system (see Figure 1) provides a method for immobilization of molecules that are themselves photoreactive or easily bleached. This stands in marked contrast to previously reported gel-patterning methods that rely on radical cross-linking[5a] in which the molecule to be immobilized must be present during irradiation in order to interact with the highly reactive photogenerated radicals.

As a further demonstration of the versatility of our "pattern-writing medium", we attempted to form patterns in gelled samples of **4** containing equal concentrations of the fluorescent "dummy"molecules sulforhodamine 101 and tetramethylrhodamine cadaverine alongside the thiol-reactive AF488-Mal described above. After washing, confocal fluorimetry demonstrated that the resulting fluorescent patterns consisted only of AF488, confirming that immobilization in the patterned gel is the result of direct thiol−maleimide coupling and not nonspecific entrapment. Along similar lines, we found that fluorescent patterns could only be formed when the photogenerated thiol groups were covalently bound to the agarose gel matrix: irradiation of plain agarose hydrogels in the presence of soluble **3** and AF488-Mal did not yield any visible patterns.

To demonstrate the degree of 3D control available in our system, we patterned a gelled sample of **4** containing the fluorescent AF488-Mal dye in a single focal plane about 100 μm below the gel surface. Confocal microscopy was then used to measure the fluorescence resulting from the immobilized AF488 dye as a function of axial distance from the initial patterning focal plane (Figure 4). The resulting

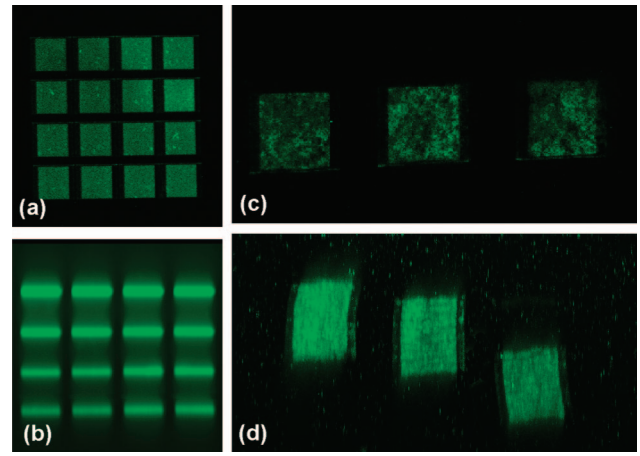


Figure 5. (a) Top and (b) side views of a 4 × 4 × 4 array of patterned squares (60 μm per side) of AF488-Mal with 50-μm interlayer spacing. (c) Top and (d) side views of continuous volumes (ca. 150 μm in height) of AF488-Mal-modified hydrogel created by stacking 100 × 100 × 18 μm patterned squares at small z-spacing increments.

curve is roughly Gaussian in shape, as expected,[18] and reflects the probability of multiphoton absorption by the agarose-bound Bhc groups at varying axial distances from the patterning focal plane The curve shown in Figure 4has a standard deviation $\sigma = 4.4$ μm, indicating that ∼95% of the area under the curve (representing ∼95% of the immobilized AF488 dye molecules) lies within 9 μm of the patterning focal plane. This value provides a quantitative measure of the feature sizes accessible in the z direction in our system, suggesting that within the ∼1 mm axial working range available for patterning, individual features can be spaced as close as 18 μm (2 × 9 μm) apart and still remain distinct.[19] As this distance is comparable to the size of a mammalian cell, our goal of creating cell-sized 3D channels is clearly achieved using this system.

We exploited the relatively thin nature of patterns created in individual planes to create fully 3D patterns by stacking flat shapes at predetermined intervals in the z direction. The hardware scripting function of the Leica TPS SP2 microscope automates formation of arrays of flat shapes (such as the 4 × 4 × 4 array of flat squares shown in Figure 5a and b) by changing the stage position according to user-selected values. Similarly, we created continuous 'solid' shapes of immobilized dye, such as cubes (Figure 5c and d), by patterning flat shapes on top of one another at z intervals of less than 18 μm. In both cases, use of scripts written in-house

(18) Zipfel, W. R.; Williams, R. M.; Webb, W. W. *Nat. Biotechnol.* **2003**, *21*, 1369.

(19) This figure is somewhat larger—though within the same order of magnitude—than what would be expected based on microscope resolution alone, likely due the quasi-fluid nature of the agarose or local heating effects near the focal point of the laser. In general, the axial resolution available in multiphoton patterning is proportional to $1/NA^2$, where NA is the numerical aperture of the overfilled objective lens being used. Feature sizes can thus always be made smaller by choosing a lens of higher NA; however, high-NA lenses are invariably restricted to very short working distances that limit the ability to make features deep within a sample. As a result, while submicrometer feature sizes are achievable for objects under 50 μm in height (such as the "microbulls"and photonic devices of ref 8), we selected an objective of moderate NA for this study (Leica 20×/0.5 HC PL Fluotar) to provide a compromise between axial resolution and working distance. This particular objective enables us to form 3D patterns up to 1 mm below the surface of gel samples.

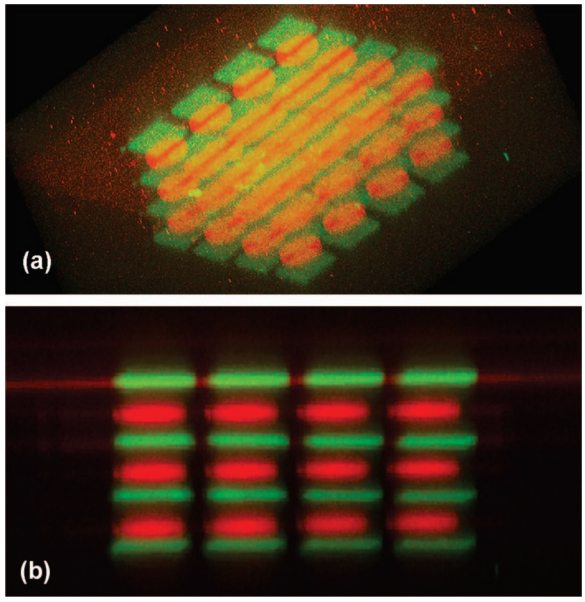


Figure 6. (a) Oblique and (b) side views of the 4 × 4 × 4 array of 3D patterned squares from Figure 5a,b, over-patterned with a second 4 × 4 × 3 array of circles (ca. 50 μm diameter) of the red fluorescent dye AF546-Mal.

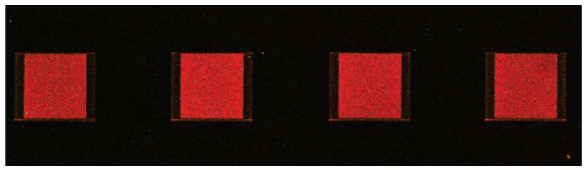


Figure 7. Squares (75 μm per side) formed by patterning a nonfluorescent, maleimide-modified biotin derivative approximately 200 μm below the surface of a hydrogel of **4** followed by blocking and staining the gel with AF546-labeled streptavidin.

(available from the authors on request) allowed us to create intricate 3D patterns within 10–15 min, requiring no operator intervention beyond the pattern spacing parameters. We further extended our technique by subjecting previously patterned gels of **4** to an additional patterning cycle using the red-fluorescent Alexa Fluor 546 Maleimide (AF546-Mal) dye; this allowed us to create 'two-color' patterns, derived from two distinct patterned molecules, with excellent 3D precision (Figure 6).

As a proof-of-principle for the 3D immobilization of biologically relevant molecules inside gels, we patterned gelled samples of **4** (1% in PBS) containing maleimide-derivatized biotin (1 mg/mL Biotin-PEO_2-Maleimide, Pierce) using the techniques described above. After washing and blocking (1% BSA in PBS) the gel samples, a small quantity of AF546-labeled streptavidin—which binds to biotin with very high affinity—was added to visualize the immobilized biotin. The fluorescent streptavidin derivative was found to bind selectively to the flat regions that had been patterned with biotin (Figure 7).

Molecular biology techniques frequently make use of the strong biotin−streptavidin interaction to attach biomolecules to one another or to surfaces, and a large number of biomolecule maleimide derivatives are commercially available or are easily synthesized. Because our gel-patterning system makes use of the easily prepared **4** and increasingly commonplace multiphoton microscope systems, this patterning technique will be particularly useful in cell guidance and tissue engineering research.

Conclusion

We synthesized a new bromohydroxycoumarin thiol derivative and used it to prepare a modified agarose derivative that yields gel-bound thiols on multiphoton excitation. Using a confocal microscope equipped with a Ti−Sapphire laser source as a patterning platform, cell-sized volumes of free thiol can be created hundreds of micrometers below the surface of gelled agarose samples and modified with biomolecules or fluorescent dyes without causing hydrogel cross-linking. The photochemical behavior of coumarin−sulfides and the tissue-engineering applications of photochemically patterned hydrogels are currently being investigated in our laboratory.

Experimental Section

All NMR spectra were collected on a Varian Mercury 400 spectrometer and referenced to the resonances of residual protonated solvents (for acetone-d_6: ^{1}H resonance at 2.05 ppm, ^{13}C resonance at 29.9 ppm). Mass spectra were recorded on an AB/Sciex QStar mass spectrometer with ESI source. IR spectra were measured on a Nicolet Avatar 370 MCT FT-IR spectrophotometer. All chemicals were purchased form Sigma-Aldrich and used as received unless otherwise specified.

Synthesis of 2. A solution of compound **1**[12] (1.33 g, 4.6 mmol), DBU (800 μL), and *N*-(*tert*-butoxycarbonyl)aminoethanethiol (0.88 mL, 5.2 mmol) in THF (50 mL) were heated at reflux overnight in the dark under a nitrogen atmosphere. After cooling to room temperature, the reaction mixture was evaporated to dryness, taken up in EtOAc, and washed with dilute aqueous HCl and brine. The organic phase was separated, dried over anhydrous $MgSO_4$, and evaporated to an orange-colored oil which was chromatographed on silica gel (6% MeOH in CH_2Cl_2) to provide a clear, viscous oil, which solidified on standing (yield 1.00 g, 52%). ^{1}H NMR (acetone-d_6, ppm): 1.40 (s, 9H), 2.68 (t, 2H, J = 6.8 Hz), 3.31 (m, 2H), 3.99 (s, 2H), 6.16 (br s, 1H), 6.33 (s, 1H), 6.92 (s, 1H), 8.04 (s, 1H), 9.99 (s, 1H). ^{13}C NMR (acetone-d_6, ppm): 28.7, 32.1, 32.4, 40.6, 104.6, 106.7, 113.3, 130.6, 151.9, 155.9, 157.9. HRMS: calcd for[$C_{17}H_{20}NO_5SBr.Na$] 452.0137, found 452.0149.

Synthesis of 3. A solution of **2** (780 mg, 1.8 mmol) in CH_2Cl_2 (10 mL) was treated with CF_3COOH (1 mL), and the reaction mixture was stirred in the dark for 4 h. The reaction mixture was evaporated to dryness, re-suspended in water, and lyophilized to provide an off-white powder in quantitative yield, pure enough for further use. Analytically pure samples could be obtained by preparative reverse-phase HPLC (C_{18} column, 10/90 to 90/10 MeCN/H_2O gradient over 80 min with constant 0.1% TFA). ^{1}H NMR (acetone-d_6, ppm): 3.10 (t, 2H, J = 7.6 Hz), 4.09 (t, 2H, J = 7.0 Hz), 4.09 (s, 2H), 6.37 (s, 1H), 7.01 (s, 1H), 8.00 (s, 1H). ^{13}C NMR (acetone-d_6, ppm): 32.4, 47.7, 104.6, 106.7, 113.2, 130.4, 151.8, 155.9, 158.3, 160.4, 168.0. FT-IR (KBr pellet, cm^{-1}): 3420, 3087, 1709, 1604, 1382, 1270, 1203, 875, 850, 722. UV−vis: λ_{max} = 375 nm (ϵ = 14600 M^{-1} cm^{-1}) in 0.1 M HEPES, pH 7.0. HRMS: calcd for[$C_{12}H_{12}NO_3SBr.H$] 329.9794, found 329.9808.

Preparation of 4. Type IX agarose (Ultralow gelling temperature, Sigma) (0.80 g) was dissolved in 40 mL of hot DMSO. After cooling to room temperature, 1,1-carbonyldiimidazole (0.36 g) in 4 mL of DMSO was added, and the reaction mixture was stirred under nitrogen for 2 h. Coumarin **3** in DMSO (60 mg in 1 mL)

was then added dropwise followed by 5 drops of Et_3N, and the solution was stirred overnight under nitrogen. After dilution to ca. 200 mL with water, the agarose solution was dialyzed against deionized water for 5 days in the dark with frequent water changes. The solution remaining after dialysis was lyophilized in the dark to yield a spongy, white fluorescent solid ($\lambda_{abs} = 375$ nm). The degree of substitution was determined to be 0.2% based on A_{375} values for solutions of **4** of known concentration.

Analysis of Fluorescein Immobilization (Figure 2). A solution of **4** in PBS was irradiated in a Rayonet UV reactor ($\lambda_{ex} = 350$ nm peak emission from eight tubes), and aliquots were removed at set time intervals. Aliquots were mixed with excess fluorescein-5-maleimide, allowed to gel at 4 °C, and washed extensively to remove unbound dye. The luminescence of the immobilized dye was measured on a Molecular Devices Gemini fluorescence plate reader to generate the data presented in Figure 2. Statistical significance was validated using the JUMP software packaged based on a one-way ANOVA followed by Tukey's Honest Significant Difference test with alpha = 0.05. Data points that are significantly different from one another are indicated with asterisks in Figure 2.

3D Hydrogel Patterning and Visualization Procedures. In a typical experiment, a solution of **4** (0.1% in hot 0.1 M PBS) containing 0.5 mg/mL AF488-Mal was pipetted into a shallow, open container with a glass bottom (small rubber "O-rings"glued onto microscope cover slips are convenient for this purpose). After cooling in a refrigerator for ~2 h to ensure complete gelation, gel samples were mounted on the stage of a Leica TPS SP2 confocal microscope equipped with a Leica 20×/0.5 HC PL Fluotar objective lens and a Spectra-Physics Mai Tai broadband Ti−Sapphire laser, tuned to 740 nm. After focusing the laser to a plane in the interior of a gel at low power, the Leica software was used to define a "region of interest"(ROI), the laser power was increased through software controls to the maximum available (~100 mW average power), and a single scan was performed at a scan rate of 400 Hz (corresponding to 0.01–0.03 mm^2/s, depending on the size of the ROI). 2D arrays and 3D volumes were built up by automatically incrementing stage positions between scans using microscope control scripts written in-house using the Leica/Visual Basic software interface (contact the authors for sample scripts). After patterning, gel samples were immersed in buffer and gently agitated for several hours (for small gels) or days (for thicker gels) to remove reaction byproducts and unbound dye. Gels were then imaged by epifluorescence or standard confocal techniques.

Measurement of Axial "Thickness"of Patterned Regions. A pattern created by the procedure described above was imaged in the *z*-stack mode of the Leica TPS SP2 confocal microscope using standard techniques. A plot of AF488 fluorescence vs *z* position was fit to a Gaussian distribution with standard deviation $\sigma = 4.4$ μm. On the basis of the statistical principle that approximately 95% of the area underneath a Gaussian distribution lies within 2σ of the mean, it can be concluded that 95% of the immobilized AF488 lies within a region of thickness 4σ, or approximately 18 μm.

Acknowledgment. We thank R. Wylie, Dr. N. Leipzig, Prof. R. J. D. Miller, and Prof. M. A. Winnik for helpful discussions, T. T. Yu for synthetic assistance, and Matregen Corp. and Ontario Centres of Excellence for financial support.

Supporting Information Available: Animated 3D confocal reconstructions of actual patterned AF488-Mal volumes within a hydrogel of **4** (AVI video files); complete ref 8a. This material is available free of charge via the Internet at http://pubs.acs.org.

CM071158M