Two-photon micropatterning of amines within an agarose hydrogel

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The ability to create three-dimensional micropatterns within polymeric materials is applicable in a wide number of fields, from photonic bandgaps to tissue engineering. We are particularly interested in three-dimensional chemical patterning of soft materials with a view towards their use in regenerative medicine. To this end, we created three-dimensional micropatterns of amines within an agarose hydrogel using two-photon patterning. Agarose was first modified with caged amines, using a derivative of 6-bromo-7-hydroxycoumarin, which upon two-photon excitation cleaved the coumarin molecule thereby yielding primary amine-functionalized agarose. Three-dimensional micropatterns were achieved because the excitation/deprotection reaction was limited to the focal volume of the two-photon laser absorbance. The three-dimensional amines serve as reactive sites for further water-based chemistry and may also render agarose cell adhesive in those amine-containing volumes.

Introduction

Micropatterning has become a rapidly expanding field in areas such as microelectronics, photonics, tissue engineering and microfluidics.[1,2] The ability to create complex patterns on the micron scale is crucial for the design of future materials. Photolithography is one of the more common methods to create micropatterns, although these techniques have been limited to the generation of two-dimensional (2D) structures. For biomaterials applications, for example, amines have been patterned on polymeric scaffolds for the immobilization of oligonucleotides,[3] proteins[4] and carbohydrate microarrays.[5]

Since the creation of patterns using photolithography is governed by the excitation of molecules with a laser, it is possible to increase the spatial resolution and decrease the excitation volume by utilizing two-photon irradiation. For two-photon excitation, a molecule must absorb two or more photons simultaneously in order to reach the excited state, requiring a high intensity of light. This intensity can be achieved using a pulsed laser that is focused through a microscope lens where the photons being emitted are equal to half the energy required for excitation. In this case excitation is limited to the focal point of the laser since the absorption of two or more photons depends non-linearly on the light intensity.[6] Therefore two-photon lithography provides the spatial control needed for 3D patterning thereby overcoming the limitations associated with traditional 2D photolithography. A variety of different 3D microstructures have been produced using this technique including conductive metal/polymer hybrid devices,[6] as well as micro-chains and springs.[7] Typically a laser is used to control the polymerization (or crosslinking) of polymers through the excitation of a radical initiator.

In contrast to the methods discussed above, the micropatterning described herein involves chemical modification of hydrogel scaffolds by the two-photon uncaging of functional groups. This technique results in minimal changes to the material's mechanical and structural properties while modifying the local chemical environment through the placement of specific functional groups.[8–11] Using the spatial control associated with two photon irradiation, we now demonstrate, for the first time, how primary amines can be three-dimensionally patterned within agarose hydrogels. The natural polymer agarose was chosen as the scaffold since it is a transparent three-dimensionally networked hydrogel that has hydroxyl groups available for chemical modification and is itself non-adhesive to cells, thereby allowing this functionality to be dialed in through chemical modification. Three-dimensional (3D) micropatterning of amine groups in agarose is desirable because the amine functional group is stable in water, serving as a site itself for either cellular interaction or further modification with cell-specific molecules.

Herein we describe the modification of agarose scaffolds with coumarin-caged amines that are deprotected upon irradiation with a pulsed laser to yield primary amines. 6-Bromo-7-hydroxycoumarin was chosen as the photolabile group since it is known as a photocage for amines and is two-photon active.[12] By selectively positioning the focal point of the pulsed laser, the location, volume and concentration of free amines within the agarose scaffold can be three-dimensionally controlled. Two-photon patterning of amines could prove useful in materials engineering by supplying the spatial control and chemical modifications needed for the construction of complex materials, by either covalent or non-covalent (*i.e.* electrostatic) interactions.

Results and discussion

In order to create 3D micropatterned amine-functionalized hydrogels, agarose was first chemically modified with coumarin-caged amines, dissolved in water and then cast into a mold. By cooling the ultra-low gelling temperature agarose to 4 °C for 2 h, a hydrogen-bonded crosslinked gel resulted which was then

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patterned with a pulsed laser, yielding distinct chemical volumes of amine groups.

Synthesis of aminocoumarin 3

The coumarin caged amine was synthesized for attachment to agarose according to Scheme 1. 6-Bromo-7-hydroxymethylcoumarin[12] **1** and *tert*-butyl 2-aminoethylcarbamate[13] **2** were synthesized according to published literature procedures. Compound **3** was synthesized by reacting 6-bromo-7-hydroxymethylcoumarin with carbonyl diimidazole followed by the addition of *tert*-butyl 2-aminoethylcarbamate in dichloromethane. The product was purified by reverse phase preparative HPLC. Compound **3** was deprotected in a 1 : 10 solution of trifluoroacetic acid and dichloromethane to yield aminocoumarin, compound **4**.

Modification of agarose with amine-protected coumarin

Aminocoumarin modified agarose was synthesized, as shown in Scheme 2, by activating agarose with carbonyl diimidazole prior to reaction with aminocoumarin **4** (from Scheme 1). The modified agarose was purified by dialysis and lyophilized to yield a white solid, **5**.

Degree of substitution of aminocoumarin agarose

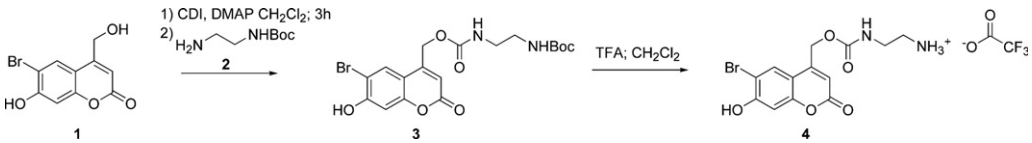
The amount of aminocoumarin bound to agarose was determined by measuring the absorbance of the coumarin moiety at 370 nm: 0.100 mol of aminocoumarin was bound per mol of agarose monomer as calculated relative to a standard curve. To determine whether aminocoumarin was covalently bound or physically adsorbed to agarose, a control experiment was conducted where **3** and agarose were co-dissolved in DMSO and allowed to react as described for the covalent modification except in the absence of the coupling agent carbonyl diimidazole. Unbound aminocoumarin was removed by dialysis prior to measuring the absorbance at 370 nm where it was determined that 0.0037 mol of aminocoumarin was present per mole of agarose monomer. By subtracting the physically adsorbed aminocoumarin (0.0037) from the total aminocoumarin (0.100) measured, we determined that there were 0.0963 moles of aminocoumarin per mole of agarose monomer covalently immobilized, yielding a degree of substitution of 9.63%.

Photo-uncaging of aminocoumarin agarose

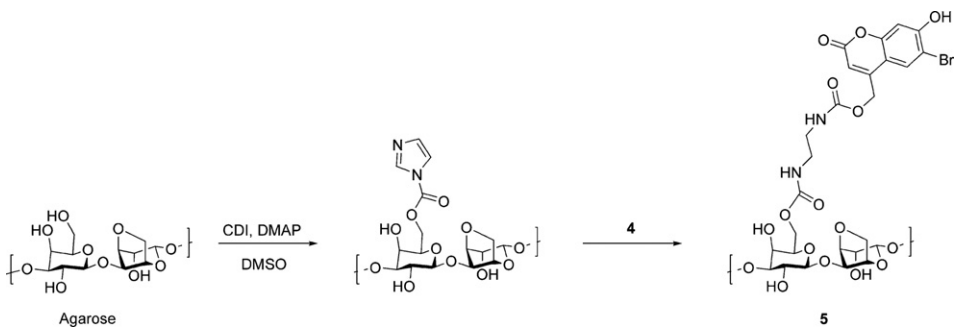
To demonstrate that amines are photocaged within the agarose hydrogels, samples of aminocoumarin agarose were irradiated with UV light and compared to samples that were not irradiated. The excitation of 6-bromo-7-hydroxycoumarin caged amines results in the cleavage between the carbon and oxygen producing carbamic acid, which then undergoes decarboxylation to yield a primary amine (Scheme 3).[14] The fluorogenic probe 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA) was used to confirm the production of amines after photoirradiation of aminocoumarin agarose. CBQCA with sodium cyanide forms a fluorescent complex with primary amines, having excitation and emission wavelengths of 465 nm and 560 nm, respectively.[15] A 100 μL solution of aminocoumarin agarose (10 mg mL^{-1}) was irradiated with a UV lamp under long wavelength (365 nm) for 30 min. After irradiation a solution of CBQCA in DMSO and sodium cyanide was added to the aminocoumarin agarose solution and left at room temperature for 30 min. Fig. 1 shows that the irradiated samples produce a stronger fluorescent signal than non-irradiated samples indicating the production of primary amines after irradiation.

Two-photon irradiation of aminocoumarin agarose hydrogels (5)

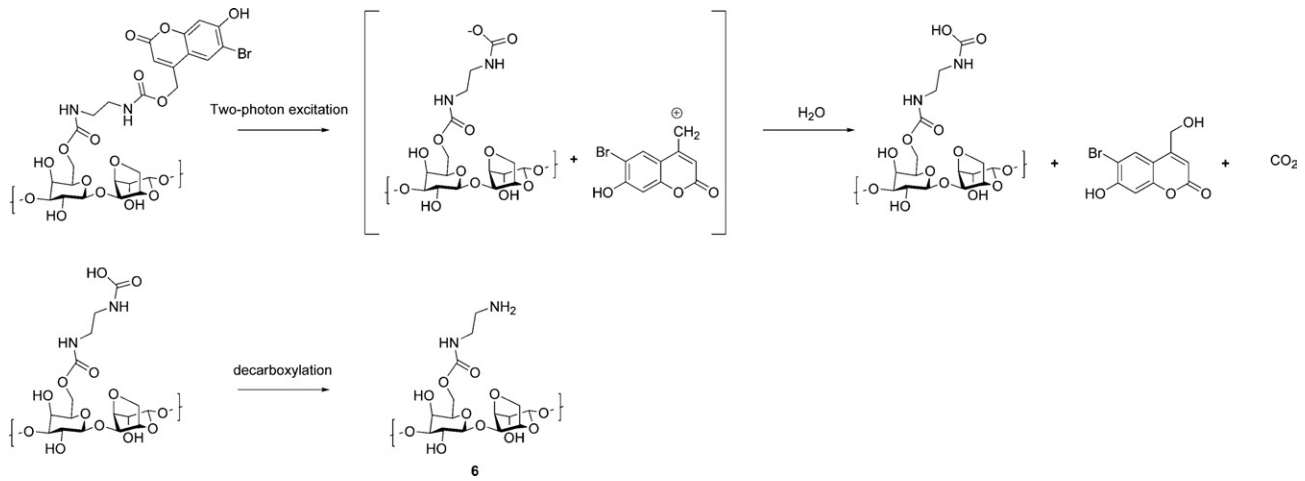
To demonstrate three-dimensional patterning, a 50 μL 1 wt% agarose hydrogel of **5** was irradiated using a femtosecond Ti–sapphire pulsed laser on a Leica TCS SP2 confocal microscope equipped with a 20×/0.5 NA objective. An excitation wavelength of 740 nm for two-photon activation was selected since the one-photon maximum absorbance of aminocoumarin



Scheme 1 Synthesis of aminocoumarin.



Scheme 2 Synthesis of aminocoumarin agarose.



Scheme 3 Photoinduced deprotection of agarose amines.

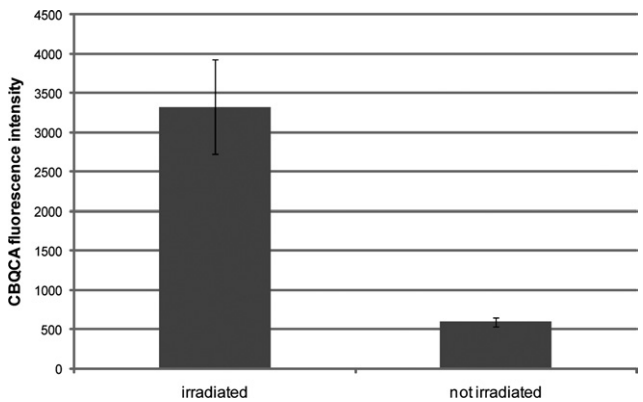


Fig. 1 Detection of primary amines using CBQCA after the irradiation of an aminocoumarin agarose solution. Samples irradiated with a UV lamp at long wavelength (365 nm) showed significantly greater fluorescence when compared to samples that were not irradiated. The increase in fluorescence from the irradiated samples confirms the production of primary amines upon irradiation.

agarose occurs at 370 nm; two-photon excitation requires each photon to be half the energy or twice the wavelength as those for one-photon irradiation. The focal point of the pulsed laser was directed 40 μm below the surface of the gel and a region of interest (ROI) of 50 μm × 50 μm was selected. The laser will only irradiate in the ROI. The ROI was then scanned 3 times with the pulsed Ti–sapphire laser with an offset of 75% and a gain of 65%.

The gel was then viewed using coumarin fluorescence with the Ti–sapphire laser set to low intensity with an offset of 75% and a gain of 0%, thereby ensuring little further aminocoumarin deprotection at the low intensity laser setting. Fig. 2e shows a dark box 50 μm by 50 μm at a depth of 40 μm. The dark region indicates the lack of coumarin fluorescence and therefore the deprotection of aminocoumarin agarose.

To demonstrate three-dimensional patterning, the percentage yield for coumarin photocleavage was calculated as a function of depth by measuring the change in coumarin fluorescence within the hydrogel using the confocal microscope (Fig. 2a). The patterned region, a 50 μm by 50 μm box, was selected and the change in coumarin fluorescence was measured every micron from the top of the gel to 100 μm below the surface of the gel. Fig. 2a shows that the cleavage of coumarin begins around 20 μm, reaches a maximum at 40 μm and decreases back to close to zero at 60 μm. Coumarin cleavage occurs wherever two-photon absorption is possible. The maximum of coumarin cleavage occurs at 40 μm since the laser was focused at that depth for two-photon irradiation; therefore the highest concentration of photons is at a depth of 40 μm. Moving away from the centre of the focal point, the probability of two-photon absorption decreases thus lowering the amount of coumarin cleaved. Confocal micrographs at depths of 20, 30, 40, 50 and 60 μm are shown in Fig. 2. No box is visible at depths of 20 and 60 μm because less than 5% deprotection occurred. A box is clearly visible at the point of maximum (33%) coumarin cleavage at 40 μm below the surface of the gel. At 30 and 50 μm below the surface of the gel, the box is still visible where 17% deprotection occurred. Therefore a box was patterned with approximate dimensions of 50 × 50 × 40 μm within the aminocoumarin agarose hydrogel, the depth of the box was determined from Fig. 2a where coumarin cleavage occurs between 20 μm to 60 μm. 40 μm represents the minimum size of the pattern in the *z*-direction (*i.e.* depth); however, boxes with a larger *z*-dimension (>40 μm) can be created by irradiating the gel at multiple depths.

The molar amount of free amines in the patterned region was calculated using the coumarin cleavage yield and the substitution rate of aminocoumarin on agarose calculated above. Fig. 2b demonstrates that the amount of free amines in the box varies in the picomole range for a given depth.

A second pattern of a series of boxes was patterned with 25 μm between each box. The amount of irradiation per box was controlled by the number of scans per box, which can be used to control the concentration of amine functionality. The first box, on the left in Fig. 3, was scanned 7 times with two scans added to each subsequent box.

After the irradiation was complete, the gel was imaged for the fluorescence of the coumarin moiety at 450 nm on the confocal microscope. A decrease in fluorescence was observed in the areas that were irradiated, resulting in a pattern of dark boxes (Fig. 3). The dimensions of the individual boxes were determined to be

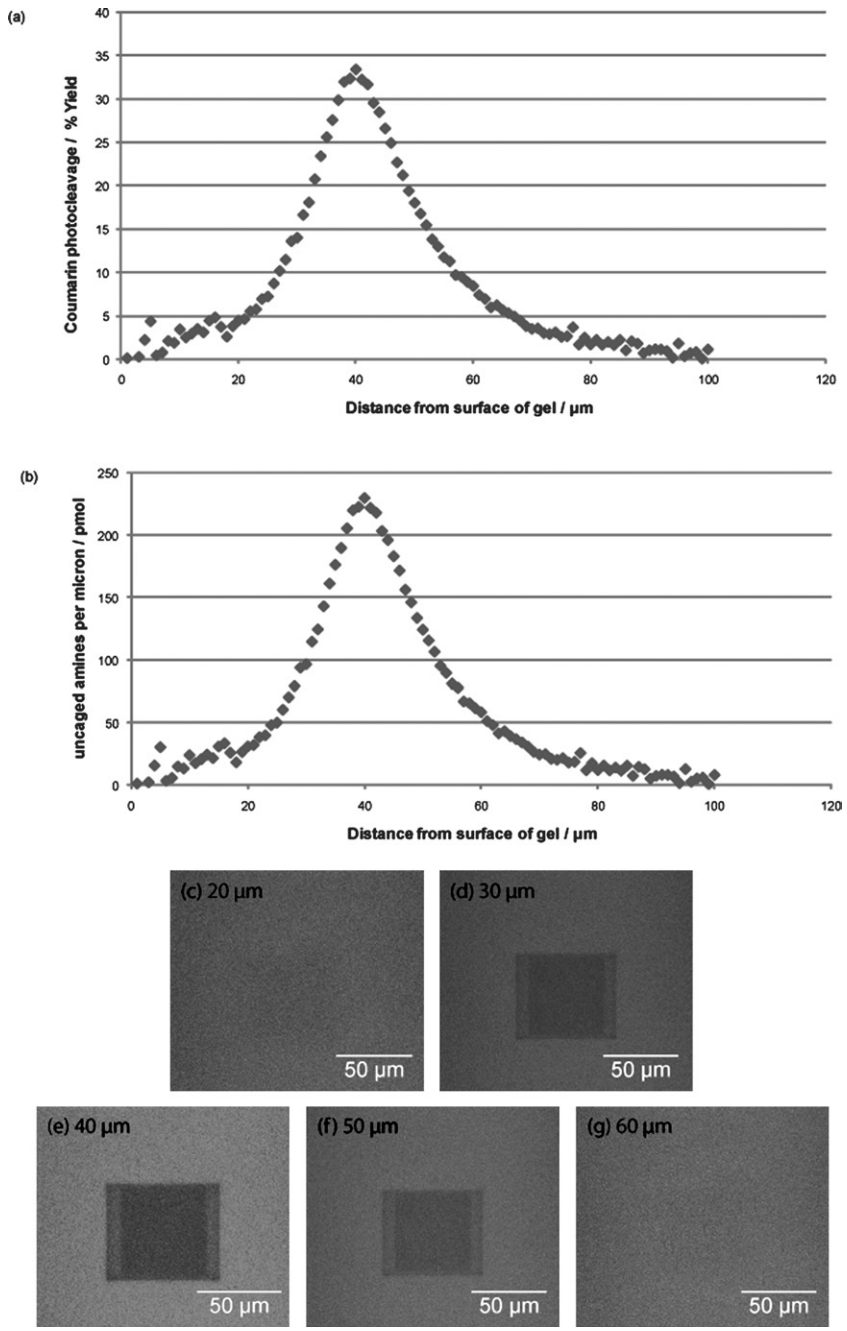


Fig. 2 A 50 by 50 μm box was patterned 40 μm below the surface of the gel. The yield of reaction (percent of coumarin photocleavage and pmol of amines) was determined by measuring the decrease in coumarin fluorescence within the patterned region. The change in fluorescence intensity of coumarin was measured over the patterned region through the first 100 μm. The box was scanned three times with the pulsed Ti–sapphire laser set to 740 nm. (a) The yield of coumarin deprotection by two-photon irradiation was then calculated as a function of depth by comparing the change in coumarin fluorescence in the patterned region to a non-patterned region. (b) The amount of amines in pmol as a function of depth within the patterned region. Confocal micrographs of coumarin fluorescence are shown at: (c) 20 μm, (d) 30 μm, (e) 40 μm, (f) 50 μm and (g) 60 μm below the surface.

~75 μm wide, ~75 μm long and ~40 μm high, which corresponded to the volume of the gel that was irradiated with the pulsed laser. The decrease in fluorescence results from the coumarin moiety being either photobleached or cleaved from the agarose to produce free amine.

To confirm the presence of amines within the box patterns, CBQCA with sodium cyanide was added to the gel. To enhance the reaction of agarose amine groups with CBQCA, triethylamine was also added to increase the pH of the gels and thus the reactivity of the primary amines. The fluorescence of CBQCA within the gel was then visualized using a HeCd laser at 442 nm (Fig. 4) and confirmed the presence of amines in the patterned volumes. The low CBQCA fluorescence intensity reflected the picomolar amount of uncaged amine groups present (Fig. 2b). While the concentration of amine groups is low, it is sufficiently high for biomaterial applications where femtomolar

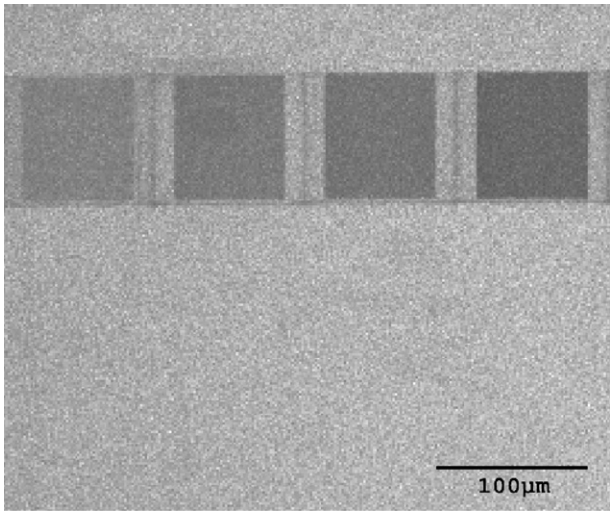


Fig. 3 Confocal image of patterned aminocoumarin agarose hydrogel visualized using the fluorescence of coumarin. A series of boxes was patterned into the hydrogel using two-photon excitation. The first box on the left was scanned 7 times, the second 9 times, the third 11 times and the fourth 13 times. As the number of scans increased, the fluorescence observed decreased due to greater photocleavage of coumarin. The fine lines located between the boxes are due to the laser scanning on the confocal microscope. The microscope scanned the region bordered by the fine lines but only irradiated in the region of the boxes by modulating the laser intensity; however, the intensity of the laser outside of the boxes is still sufficient to produce the fine lines observed. (The image was enhanced for clarity only using Photoshop.)

concentrations of peptides have been shown to promote a cellular response.[16] Notwithstanding the weak fluorescent signal from CBQCA, the irradiation of aminocoumarin hydrogels with a pulsed laser resulted in the selective deprotection and micropatterning of defined volumes of amine groups in agarose. The reaction of these amine groups with CBQCA demonstrates the capacity of these amine groups for further modification and is useful for imaging.

Conclusions

A coumarin caged amine was synthesized and immobilized onto agarose gels, which upon two-photon excitation resulted in cleavage of the coumarin moiety yielding primary amines. Using a pulsed laser, spatially defined volumes of micropatterned amine cubes were patterned into hydrogels of the modified agarose. Using fluorescent CBQCA, we proved the success of the uncaging chemistry while demonstrating the capacity of these amine groups for further modification. This first demonstration of 3D micropatterned volumes of amine functional groups within transparent polymeric hydrogels is currently being explored for cell guidance in the context of tissue engineering and regenerative medicine.

Experimental

Materials

All reagents were used as received unless otherwise noted. Agarose type IX-A, carbonyl diimidazole, dimethylaminopyridine, triethylamine and sodium cyanide were purchased for Sigma-Aldrich (Oakville, ON, Canada). Dichloromethane and trifluoroactic acid were purchased from Caledon Labs (Georgetown, ON, Canada). CBQCA was purchased from Invitrogen Inc. (CA, USA).

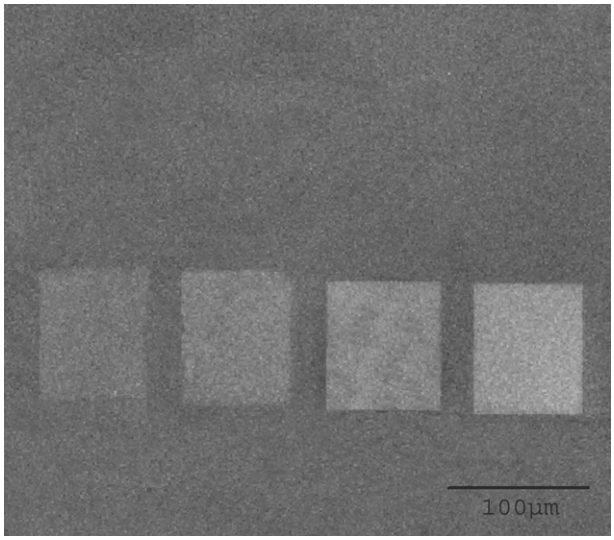


Fig. 4 Confocal image showing the presence of amines within the patterned regions, the boxes correspond to those in Fig. 3. The amine reactive fluorescent probe CBQCA was used to detect the uncaged amines. The bright boxes represent the fluorescence from the CBQCA amine complex. The box on the left was irradiated with 7 scans and each subsequent box (to the right) was scanned two more times. (The image was enhanced for clarity only in Photoshop.)

Methods

Synthesis of 3. A solution of compound **1** (500 mg, 1.84 mmol), carbonyl diimidazole (359 mg, 2.21 mmol) and dimethylaminopyridine (450 mg, 3.68 mmol) in 150 mL of dichloromethane was stirred under nitrogen in the dark at room temperature for 3 h. *tert*-Butyl 2-aminoethylcarbamate **2** (354 mg, 2.21 mmol) was then added and stirred for an additional 24 h. The solution was washed with 100 mL of a 10% citric acid solution, distilled water and brine. After drying over magnesium sulfate the solution was concentrated to yield a light yellow solid. The product was purified by reverse phase preparative HPLC using a gradient mixture of acetonitrile to water (10%–80%) to yield compound **3** as an off-white solid (240 mg, 28.5%). Mp 170 °C. ^{1}H NMR (400 MHz, DMSO-d_6): 1.35 (s, 9 H), 3.00 (m, 4 H), 5.24 (s, 2 H), 6.18 (s, 1 H), 6.87 (m, 1 H), 6.89 (s, 1 H), 7.51 (m, 1 H), 7.86 (s, 1 H), 11.47 (s, 1 H). ^{13}C NMR (400 MHz, acetone-d_6): δ 27.9, 40.4, 41.4, 61.2, 78.6, 103.8, 106.2, 109.7, 111.7, 128.8, 150.6, 154.7, 155.8, 157.4, 159.7. ESI-MS (M^+): 456.1 (calc: 456.05). El. Anal. Found: C, 47.0; H, 4.4; N, 6.2. Calc. for $C_{18}H_{21}BrN_2O_7$: C, 47.3; H, 4.6; N, 6.1%.

Synthesis of 4. 200 mg of **3** was stirred in 10 mL of a 10 : 1 (v/v) mixture of dichloromethane and trifluoroacetic acid for 24 h. The solution was concentrated, redissolved in water and lyophilized yielding an off-white solid (347 mg, 100%). Mp 184 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.07 (t, 2 H, $J = 5.85$ Hz), 3.39 (t, 2 H, J

= 5.91 Hz), 4.99 (s, 2 H), 6.08 (s, 1 H), 6.57 (s, 1 H), 7.47 (s, 1 H). ^{13}C NMR (400 MHz, acetone-d_6): δ 38.6, 47.5, 61.5, 103.8, 106.2, 109.3, 111.3, 128.4, 150.5, 154.7, 156.0, 157.7, 159.7. ESI-MS (M^+): 357.0082 (calc: 357.0080). El. Anal. Found: C, 37.9; H, 3.1; N, 6.0. Calc. for $C_{15}H_{14}BrF_3N_2O_7$: C, 38.2; H, 3.0; N, 6.0%.

Synthesis of aminocoumarin agarose 5. A solution of agarose (250 mg) and carbonyl diimidazole (35 mg, 0.21 mmol) in 100 mL of DMSO was stirred under nitrogen for 3 h. Compound **4** (50 mg, 0.14 mmol) was added and the solution was stirred for an additional 24 h. The solution was dialyzed (MW cut-off of 3500) and lyophilized to yield a white solid (yield 190 mg, 76%).

Photo-uncaging of aminocoumarin agarose with UV light. A 100 μL solution of aminocoumarin agarose (10 mg m^{-1}) was irradiated with a UV lamp under long wavelength (365 nm) for 30 min. After irradiation 50 μL of a 1 mg mL^{-1} solution of CBQCA in DMSO and 50 mM sodium cyanide solution in TES pH 8.5 was added to the aminocoumarin agarose solution and left at room temperature for 30 min. Non-irradiated samples were prepared in the same way as the irradiated samples except that they were not exposed to UV light. The fluorescence was then measured using a fluorescent plate reader with an excitation and emission wavelength of 465 nm and 560 nm, respectively.

Two-photon irradiation of aminocoumarin agarose hydrogels. One weight percent agarose hydrogels were irradiated with a confocal microscope equipped with a femtosecond Ti–sapphire laser, 20×/0.5 NA objective and an electronic stage. To view the hydrogels using coumarin fluorescence, the laser was set to 740 nm with an offset of 75% and a gain of 0% and a scanning speed of 400 Hz using the Leica confocal software. The focal point of the laser was positioned within the gel by moving the stage. A region to be patterned was selected by creating a region of interest using the Leica confocal software. The intensity of the laser was increased by setting the offset to 75% and the gain to 65%, and the region of interest was scanned. In order to visualize the pattern the intensity of the laser was lowered by setting the offset to 75% and the gain to 0%. The depth profile was created by taking a picture every micron for the first 100 μm below the surface of the gel. The intensity of coumarin fluorescence was then measured for each picture in the position of the patterned square and was compared to a non-patterned region. The change in coumarin fluorescence was then plotted as a function of depth to give the yield for amine deprotection (Fig. 2).

Preparation of 1 wt% aminocoumarin agarose hydrogels for amine visualization with CBQCA. 70 μL of a 10 mg mL^{-1} solution of CBQCA in DMSO and 132 μL of a 50 mM solution of sodium cyanide in water was added to 600 μL of 1.35 wt% solution of **5** in water. 50 μL of this solution was pipetted into ~70 μL chambers on a glass slide and placed at 4 °C for 2 h for gelation. The patterns were created as mentioned above. The amine patterns were visualized using an excitation wavelength of 442 nm (HeCd laser) and an emission wavelength of 560 nm.

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