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Polymer Microstructures through Two-Photon Crosslinking

*David Schwärzle, Xiaoqang Hou, Oswald Prucker, and Jürgen Rühe**

Two-photon crosslinking of polymers (2PC) is proposed as a novel method for the fabrication of freestanding microstructures via two-photon lithography. During this process in the confocal volume, two-photon absorption leads to (formal) C,H-insertion reactions, and consequently to a strictly localized crosslinking of the polymer. To achieve this, the polymer is coated as a solvent-free (glassy) film onto an appropriate substrate, and the desired microstructure is written by 2PC into this glass. In all regions outside of the focal volume where no two-photon process occurs, the polymer remains uncrosslinked and can be washed away during a developing process. Using a self-assembled monolayer containing the same photoreactive group allows covalent attachment of the forming freestanding structures to the substrate, and thus guarantees an improved stability of these structures against shear-induced detachment. As the two photon process is carried out in the glassy state, in a simple way, multilayer structures can be used to write structures having a varying chemical composition perpendicular to the surface. As an example, the 2PC process is used to build a structure from both protein-repellent and protein-adsorbing polymers so that the resulting 3D structure exhibits spatially controlled protein adsorption.

Two-photon lithography (2PL) allows the fabrication of freestanding, truly 3D microstructures with a resolution of the finest structures well in the upper nanometer range.[1–6] Many spectacular microstructures with very fine and detailed features such as a miniature Eiffel tower, a charging bull, or a microscopic propeller have been generated.[5,7,8] As this process allows the generation of freestanding 3D microstructures, two-photon lithography has become a powerful tool for the fabrication of complex structures including photonic crystals and micro-lab-on-chip devices for optical applications or biomedical systems.[9–14]

The 2PL systems reported so far are essentially all based on two-photon polymerization (2PP). To this, a low energy (high wavelength) laser beam (typically λ = 780 nm) is focused into a photoresist solution consisting of a dye, which acts as a photoinitiator and a monomer. Only within a small volume close to the focal point is the light intensity high enough to induce a two-photon process in which the molecule is lifted to an intermediate state by the first adsorbed photon, and reaches an excited state through the absorption of a second photon. The excited dye molecule then initiates a free radical polymerization reaction, which leads to a local solidification of the photoresist.[15] In cases when a bifunctional monomer is added to the polymerization mixture, a crosslinking polymerization is performed, which leads directly to the formation of a locally crosslinked structure. The so-formed microstructures can be developed through washing away of the still unreacted monomer using a suitable solvent, which removes the monomer but does not dissolve the polymer. As the polymer formation process is strongly locally confined, it is possible to produce microstructures with a resolution below 100 nm through 2PP processes.[16]

However, the 2PP process has also some limitations. One problem, common for any bulk polymerization process, is that it might be difficult to remove residual monomer completely after polymerization. Monomer could become entrapped in the formed structures and complete removal might prove to be difficult. Upon prolonged use then it might slowly leach out. While the polymers are usually not bioavailable and therefore are usually considered unproblematic, the presence of residual monomer, might be of concern if the microstructures are used in a biological environment as monomers are due to their high reactivity almost by definition non-biocompatible compounds.

Additionally, as the polymerization is performed in liquid monomer only microstructures out of one type of polymer or copolymer can be fabricated in one step. The generation of microstructures consisting of two or more different materials requires two or more subsequent 2PP reactions including washing steps after each process step.[17]

Here, we present a novel method for the fabrication of microstructures using two-photon crosslinking (2PC) based on a photoactive copolymer. The main difference to standard two-photon lithography processes is, hat the photoreactive unit excited by the two-photon process is already part of a polymer chain instead of a dye in a liquid monomer solution. In the 2PC case, the excited dye can undergo an intersystem crossing to a biradicaloid triplet state. This biradical can react with neighboring aliphatic CH-bonds, which are ubiquitously present in the polymer, through hydrogen abstraction and recombination. In total, this reaction represents a formal C,H insertion and the overall process is called C,H-insertion crosslinking (CHic).[18] Only very few connections between the different polymer chains are needed to achieve complete crosslinking, and hence

D. Schwärzle, X. Hou, Dr. O. Prucker, Prof. J. Rühe
Department of Microsystems Engineering
University of Freiburg
79110 Freiburg, Germany
E-mail: ruehe@imtek.de

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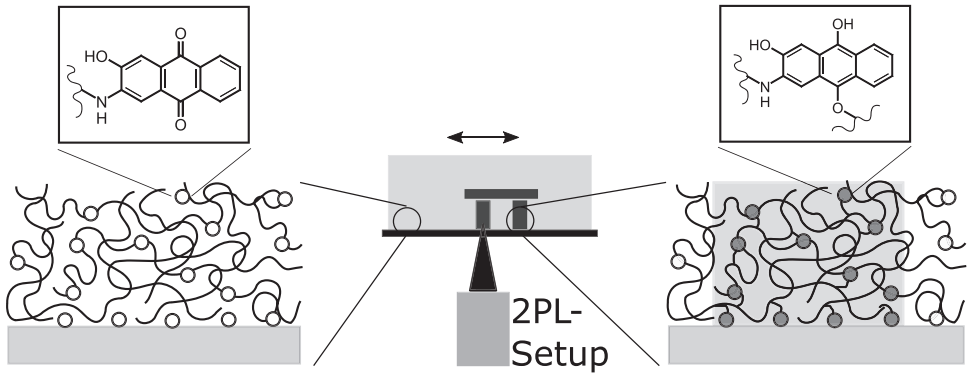

Figure 1. Schematic depiction of microstructure formation through two-photon crosslinking. On the left side the polymer film containing the photoactive anthraquinone units is shown. The right side shows a cross section through a microstructure formed by two-photon crosslinking and the surrounding unmodified photopolymer. The anthraquinone dye is activated by two-photon absorption and forms covalent bonds between neighboring polymer chains.

the gel point is quickly reached in the focal volume. In all other areas the gel point is not reached, the polymer remains soluble and can be washed away during a short solvent exposure.[19] **Figure 1** shows the overall procedure.

As photoreactive copolymer we have chosen a functionalized polymer containing 9,10-anthraquinone groups. 2-Amino-3-hydroxyanthraquinone has an extended π-system, which is highly planar and contains an electron-donating substituent. This compound can be readily transferred to a acrylamide monomer and then copolymerized with dimethyl acrylamide and other (meth)acrylates through a standard free-radical polymerization. The respective concentrations of the comonomers were typically chosen such that a dye content in the copolymer of around 3–5 mol% was established (**Figure 2**a).

To demonstrate that the anthrachinone crosslinker can be activated with a femtosecond laser in the near infrared (NIR) region, the fluorescence after excitation was measured with a two-photon microscope (Figure 2c). The highest fluorescence emission is observed during excitation with a wavelength around 800 nm. This wavelength is quite advantageous as the 2PL setup employed here uses a Ti:sapphire laser, and the emission wavelength was set to 780 nm. Although the fluorescence emission does not provide direct information about the crosslinking process, it allows to conclude that significant two-photon absorption occurs.

Next, model reactions were carried out, which use the copolymer and crosslink it through light induced CHic-reaction during irradiation at λ = 365 nm. After irradiation with UV-light for a given period of time, the gel content was measured by recording the thickness before and after solvent extraction (see Figure 2e). The half-life time of P(DMAA-*co*-AAHAQ) at λ = 365 nm is reached after an irradiation with $t_{1/2}$ = 20.5 J cm^{-2} (see Figure 2d). This dose is rather low compared to the output of the laser of the 2PC system, which is in the setting used for the experiments presented here a laser intensity of 25 mW, a writing speed of 10 mm s^{-1}, a line distance of 0.3 µm and an energy density of ≈830 J cm^{-2}. It should be specifically noted that crosslinking occurs in the focal volume even while the polymer is in the solid state, e.g., the polymer is deposited to form of a solvent-free (glassy) film and the desired microstructure can be written into this glass. In all other regions the polymer remains uncrosslinked and can be washed out during the developing process with an appropriate solvent for the polymer. The efficiency of the CHiC process has been recently studied by Körner et al. on a similar polymeric system for a one-photon process.[19] It was found that the efficiency right at the percolation point can assume values even higher than 1 as finite clusters of high-molecular weight polymer chains which have been formed, but which are not bound to the just formed infinite network, can become entrapped in the infinite network. At the later stages of the photochemical process the efficiency factor drops. As the polymers used in this study have degrees of polymerization around 300–400 and a crosslinker contents of 3–5% the number of crosslinker units per chain is sufficient to guarantee that practically all polymer chains activated by the light become part of the infinite network and the gel point is rapidly reached.

A critical factor for obtaining intact microstructures, however, is to prevent debonding/delamination of the formed (somewhat swollen) microstructures from the surface, especially during the washing step. To avoid any delamination it is necessary to establish strong bonds between the substrate and the microstructures. To ensure this, a silane containing an anthrachinone chromophore similar to that in the polymer was synthesized in a simple two step procedure. The silane was subsequently deposited on a glass slide and in a standard base catalyzed (NEt_3) process a self-assembled monolayer is formed. The resulting anthraquinone group containing monolayer can photochemically react with adjacent polymer chains and link them to the substrate by the same CHic reaction as described above for the crosslinking process (see **Figure 3**). This way a covalent bond is established between the substrate and the forming polymer network.

Some examples of microstructures generated using the described techniques are shown in **Figure 4** together with the image of the CAD file used to generate the structures. The scanning electron microscopy (SEM) images demonstrate that freestanding structures can be obtained following the described strategy. Strong shear during extraction of the structures did not remove the structures from the surface due to the covalent attachment of the polymers to the surface. Reference experiments performed on samples prepared without

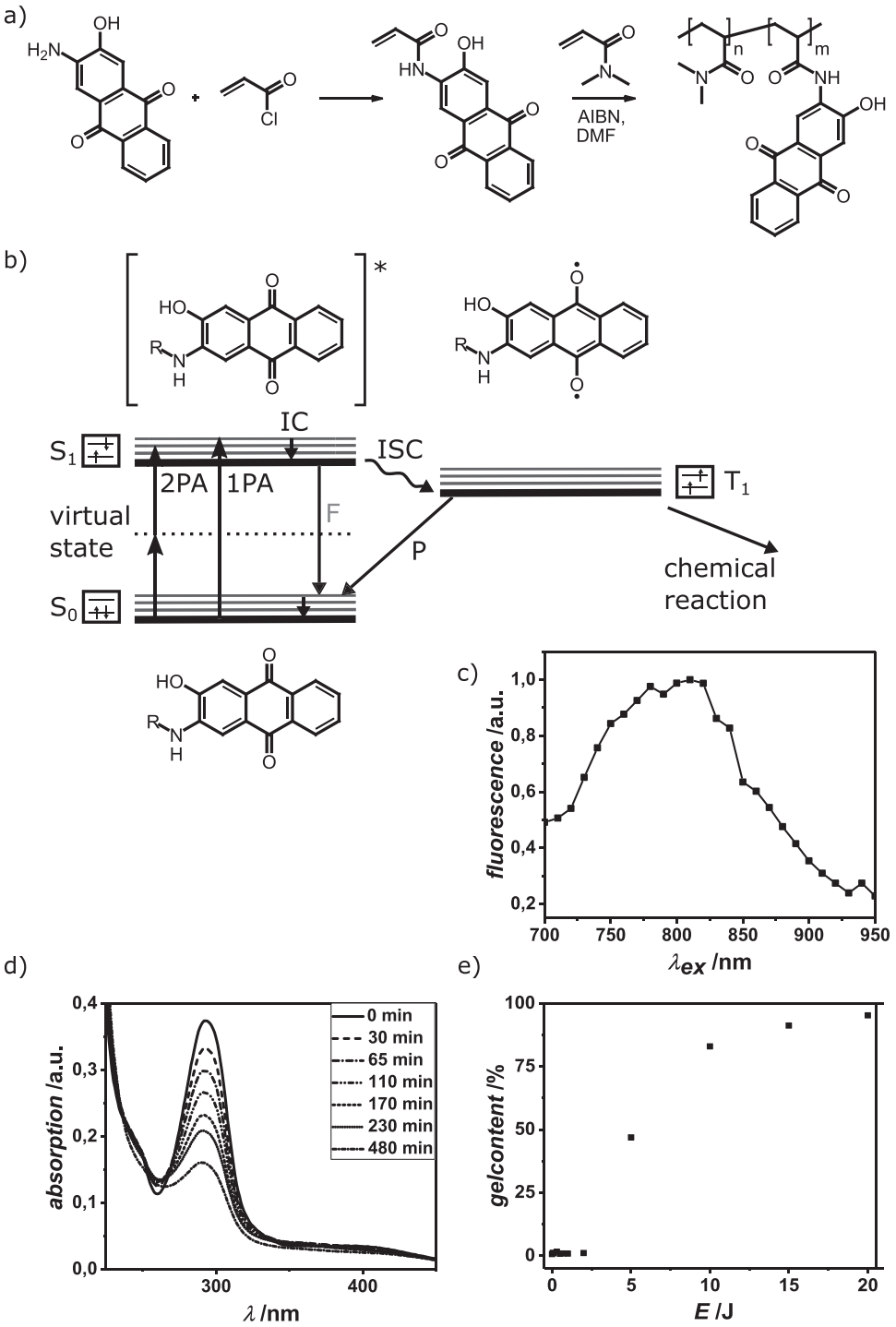

Figure 2. a) Synthetic route for the photoactive acrylamide-3-hydroxy-2-anthrachinone (AAHAQ) monomer and the following reaction of the crosslinker containing copolymer P(DMAA-AAHAQ). b) Jablonski-scheme with one- and two-photon absorption from the S_0-state to the excited S_1-state. After the intersystem crossing to the T_1-state, the molecule is in a highly reactive biradicaloid triplet state. c) Normalized fluorescence (λ = 510–530 nm) of a ≈3-µm-thick P(DMAA-10%AAHAQ) film on a glass slide after excitation with laser light of different wavelengths, measured with a two-photon microscope. d) UV–Vis absorption spectra of a P(DMAA-5%AAHAQ) film. The decay of the maximum at λ = 290 nm is caused by the irradiation of UV light with a wavelength of λ = 365 nm and an intensity of 2.4 mW cm^{-2} for different periods of time. e) Gel content for P(DMAA-5%AAHAQ) films after irradiation with λ = 365 nm at different energy doses.

the silane monolayer showed that the structures generated were damaged or even completely washed off during solvent exposure.

In addition to these rather simple structures we have also generated microstructures that cannot be easily made following conventional 2PP methods. One such example is shown

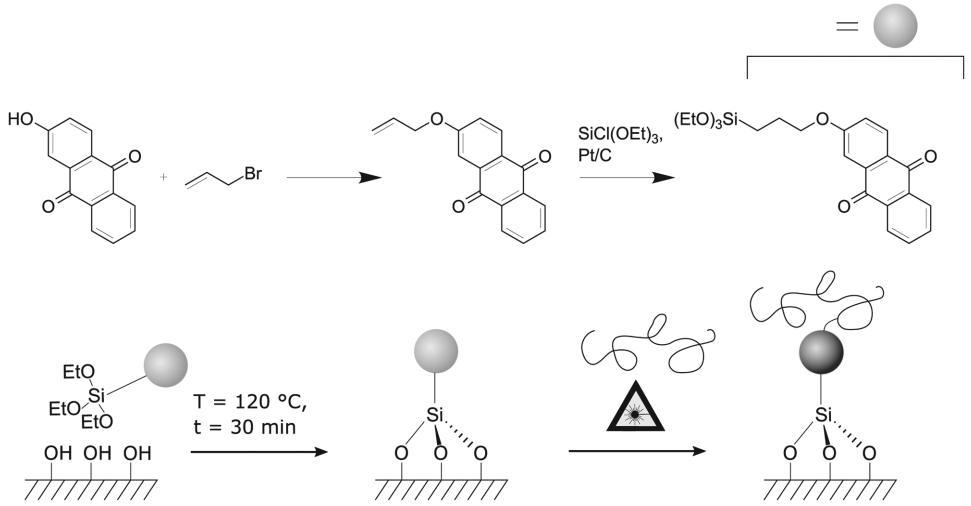

Figure 3. Synthetic route for the photoreactive silane (TEA-silane) and the functionalization of a glass substrate with this silane. Also shown is the covalent attachment of a polymer strain to the functionalized substrate caused by illumination with laser light.

in Figure 4c. Here in the first step a glassy bilayer is formed consisting of two different copolymers through two subsequent spin coating processes from orthogonal solvents. The upper layer consists of a hydrophobic copolymer based on poly(*n*-butyl acrylate) P(nBA-*co*-AAHAQ), the lower of P(DMAA-*co*-AAHAQ). Into this bilayer film a simple structure is written in by the two-photon crosslinking process, which is designed to form a cone with a step-like appearance (Figure 4c). This way the microstructure was written across the interface between the two materials. Because both materials possess crosslinker units and C—H-groups, they do not just form a covalent network with themselves but also across the interface with the other polymer which leads to a stable and covalent binding between the two layers during the microstructure fabrication. When the microstructure is viewed from the top in a microscope rings are visible and indicate the presence of different terrace levels on the cone.

From previous studies it is known that the hydrogel material is, because of the high degree of swelling of crosslinked hydrogels in aqueous media, very protein repellent ("entropic shielding"), whereas the *n*-butyl acrylate polymer represents an attractive surface for such biomolecules.[18,20,21] We have utilized this differences in behavior to visualize the presence of the two materials. For that, the samples were exposed to Alexa Fluor647 labeled IgA from goat antihuman serum. These proteins only adsorb to the PnBA located in the top part of the cones accordingly only these regions show a bright fluorescence emission in the micrographs shown in Figure 4c. The protein repellent lower parts of the cones remain dark.

These examples demonstrate that two-photon crosslinking, 2PC is an attractive tool in the 2PL family to generate polymeric 3D and self-supporting microstructures where the structures are directly written into a glassy layer of the polymer.

The use of a functionalized substrate containing a self-assembled monolayer which carries essentially the same moieties which induce the crosslinking reaction, allows a covalent attachment of the forming microstructures to the substrate. This leads to an improved stability against detachment, especially when the structures are exposed to shear and/or flowing liquids. This stability can be observed, even when the liquid represent a good solvent for the polymer material.

As 2PC is performed in the glassy state of the photoresist material, easily multilayered assemblies can be employed for the formation of the microstructures, which consist of different polymers in the *z*-direction. Here we have shown as a demonstration case the fabrication of a microstructure consisting out of two different polymeric materials in just one two-photon lithography step. A 2PC process in the glassy state allows also the generation of microstructures in composite materials as we will show in a follow up publication.

Experimental Section

Materials: 2-Amino-3-hydroxyanthraquinone was purchased from TCI, Germany. Alexa Fluor647-conjugated AffiniPure Goat Anti-Human Serum IgA was purchased from Jackson ImmunoResearch. All other chemicals where purchased from Sigma Aldrich, Germany. Dimethyl acrylamide and *n*-butylacrylate was purified trough filtration with basic aluminum oxide and distillation. 2,2′-Azobis(2-methylpropionitrile) was recrystallized from ethanol. All other chemicals and solvents were used as received.

Instrumentation: ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker DPX 250 spectrometer. The Fourier-transform infrared (FTIR) spectra were recorded on a Nicolet 730 FTIR spectrometer under nitrogen at a resolution of 2 cm^{-1}. The size exclusion chromatography measurements were carried out with an Agilent 1100 from PSS and narrow polydispersity poly(methylmethacrylate) PMMA was used as the standard. The SEM-images were taken with a Zeiss DSM 962 system. The UV–vis spectroscopy was carried out using a Cary 50 UV–Vis spectrometer from Varian. The thickness measurements for the gel contents of the samples were carried out on a Nanofilm EP3 ellipsometer from Accurion. The fluorescence measurement after two-photon absorption was carried out with an LSM 510 META microscope from Zeiss with a tunable two-photon laser from Coherent (Chameleon Vision1).

Synthesis of 2-Hydroxyanthraquinone-Triethoxysilane (TEA-Silane): Freshly distilled triethoxysilane (30.0 mL, 26.7 g, 162.0 mmol) was mixed with Pt on coal (30 mg, 10% Pt) and 2-allyloxyanthraquinone (1.5 g, 5.7 mmol). While stirring the reaction mixture was heated to

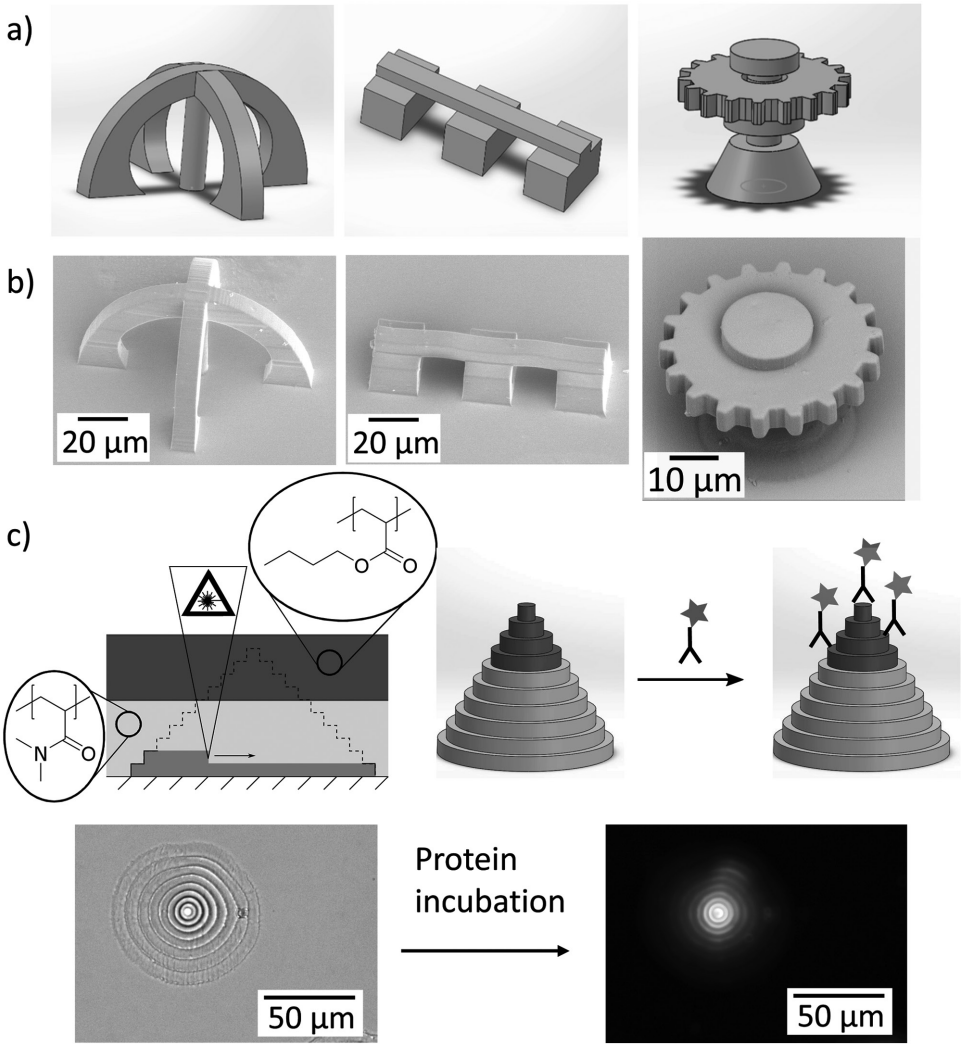

Figure 4. a) CAD design and b) scanning electron microscopy images of microstructures produced with P(DMAA-*co*-AAHAQ) through two-photon lithography. c) Schematic depiction and optical microscope image of a pyramidal microstructure consisting out of two materials. A bilayer consisting of P(DMAA-*co*-AAHAQ) and P(nBA-*co*-AAHAQ) was produced and then microstructured via 2PC. The material on top of the pyramid is protein attractive, whereas the lower part of the pyramid is protein repellent. After incubation with a fluorescently labeled protein, IgA from goat antihuman serum, only the top of the pyramid shows a strong fluorescence emission.

reflux overnight. The residual triethoxysilane was removed through distillation and the product was used without further purification.

Synthesis of Acrylamido-3-Hydroxy-2-Anthraquinone (AAHAQ): 2-Amino-3-hydroxyanthraquinone (2.39 g, 10.0 mmol, 1 eq) was dissolved in dioxane (100 mL) and under cooling to 0 °C and stirring a solution of chloroacrylic acid (0.68 mL, 0.72 g, 8.0 mmol, 0.8 eq) in dioxane (20 mL) was added dropwise. Then the cooling was removed and the reaction mixture was heated to reflux for 6 h under stirring. The solid material was filtered and recrystallized out of ethanol to obtain a yellow product (1.97 g, 6.7 mmol, 84%).

^{1}H-NMR (250 MHz, DMSO-d_6, δ): 5.80 (dd, J = 2 Hz, 10 Hz, 1H, C1), 6.35 (dd, J = 2 Hz, 17 Hz, 1H C1), 6.87 (dd, J = 10 Hz, 17 Hz, 1H, C2), 7.63 (s, 1H, C_{Ar}), 7.82–7.94 (m, 2H, C_{Ar}), 8.10–8.22 (m, 2H, C_{Ar}), 9.05 (s, 1H, C_{Ar}), 9.81 (s, 1H, OH), 11.71 (s, 1H, NH).

^{13}C-NMR (250 MHz, DMSO-d_6, δ): 112.51 (C3), 119.90 (C6), 126.71 (C5), 127.34 (C11), 127.49 (C14), 128.84 (C17), 130.77 (C13), 132.51 (C12), 133.09 (C1), 133.96 (C16), 134.15 (C8), 134.88 (C9), 135.17 (C2), 153.38 (C4), 164.92 (C15), 182.36 (C7), 182.75 C10).

FTIR (KBr, cm^{-1}): 3331 (N—H stretch); 3140 (br, O—H stretch); 2955, 2922, 2852 (C—H stretch); 1680 (acryloyl C=O); 1668, 1625 (AQ C=O); 1656 (C=C stretch); 1579, 1546, 1533 (ar C=C).

Synthesis of P(DMAA-co-AAHAQ): The polymer was synthesized by statistical free-radical copolymerization of dimethyl acrylamide (0.98 mL, 0.94 g, 9.5 mmol) (DMAA) with AAHAQ (0.15 g, 0.5 mmol). The polymerization was carried out at 60 ± 1 °C for 16 h in dimethylformamide under nitrogen and was initiated with 0.5% α,α'-azobisbutyronitrile (AIBN). The reaction mixture was degassed through three freeze and thaw cycles. The reaction was stopped through precipitating the mixture in diethyl ether. The polymer was purified through repeated dissolving in methanol and precipitating in diethyl ether, the typical yielding was ≈43% of a yellow powder. The amount of AAHAQ in the copolymer was calculated from the ^{1}H-NMR spectra to be around 4 mol% and the molecular weight M_w was measured to be 68 000 g mol^{-1} with a polydispersity of 2.3.

Sample Preparation: Glasslides (22 × 22 mm, 170 µm thick) were spin coated with a solution of TEA-silane in toluene (c = 10 mg mL^{-1}, 0.1 mL) and heated to 120 °C for 30 min. On this slides were a drop of a P(DMAA-*co*-AAHAQ) solution in methanol (c = 200 mg mL^{-1}, 10 µL) was added and annealed at 90 °C.

The microstructures were fabricated with a Photonic Professional GT system from Nanoscribe using a 63x oil objective with a laser power of 25 mW and a laser speed of 10 mm s^{-1}. The developing of the

microstructures was performed in *iso*-propanol or in case of the bilayer structure *n*-butanol for 30 min. Afterward the microstructures where characterized by taking SEM images.

Protein Repellency Test: The microstructures were incubated in a solution of an Alexa Fluor647-conjugated AffiniPure Goat Anti-Human Serum IgA in phosphate buffer saline (PBS) for 1 h. Afterward the samples were washed with PBS and examined with a fluorescence microscope.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

crosslinking, multilayered microstructures, photoreactive polymers, two-photon lithography

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[1] J. Fischer, M. Wegener, *Laser Photonics Rev.* **2013**, *7*, 22.
[2] S. Maruo, J. T. Fourkas, *Laser Photonics Rev.* **2008**, *2*, 100.
[3] K. S. Lee, R. H. Kim, D. Y. Yang, S. H. Park, *Prog. Polym. Sci.* **2008**, *33*, 631.
[4] H. B. Sun, S. Kawata, *Adv. Polym. Sci.* **2004**, *170*, 169.
[5] S. Kawata, H. B. Sun, T. Tanaka, K. Takada, *Nature* **2001**, *412*, 697.
[6] L. Piedad, C. Gomez, A. Spangenberg, X. Ton, Y. Fuchs, F. Bokeloh, J. Malval, B. Tse, S. Bui, D. Thuau, *Adv. Mater.* **2016**, *28*, 5931.
[7] *Nanoscribe "News and Reviews"* (Ed: M. Hermatschweiler) **2013**.
[8] B. H. Xia, J. Wang, Y. Tian, Q. Chen, X. Du, Y. Zhang, *Adv. Mater.* **2010**, *22*, 3204.
[9] K. K. Seet, V. Mizeikis, S. Juodkazis, H. Misawa, *Appl. Phys. A* **2006**, *82*, 683.
[10] B. Sun, *Lab Chip* **2013**, *13*, 1677.
[11] T. Gissibl, S. Thiele, A. Herkommer, H. Giessen, *Nat. Photonics* **2016**, *10*, 554.
[12] M. Malinauskas, H. Gilbergs, A. Žukauskas, V. Purlys, D. Paipulas, R. Gadonas, *J. Opt.* **2010**, *12*, 035204/1.
[13] S. Baudis, D. Bomze, M. Markovic, P. Gruber, A. Ovsianikov, R. Liska, *J. Polym. Sci., Part A: Polym. Chem.* **2016**, *54*, 2060.
[14] F. Klein, B. Richter, T. Striebel, C. M. Franz, G. Von Freymann, M. Wegener, M. Bastmeyer, *Adv. Mater.* **2011**, *23*, 1341.
[15] R. H. Kim, K.-S. Lee, *Macromol. Symp.* **2010**, *298*, 25.
[16] L. Li, R. R. Gattass, E. Gershgoren, H. Hwang, J. T. Fourkas, *Science* **2009**, *324*, 910.
[17] T. K. Claus, B. Richter, V. Hahn, A. Welle, S. Kayser, M. Wegener, M. Bastmeyer, G. Delaittre, C. Barner-Kowollik, *Angew. Chem. Int. Ed.* **2016**, *55*, 3817.
[18] R. Toomey, D. Freidank, J. Rühe, *Macromolecules* **2004**, *37*, 882.
[19] M. Körner, O. Prucker, J. Rühe, *Macromolecules* **2016**, *49*, 2438.
[20] C. K. Pandiyarajan, O. Prucker, B. Zieger, J. Rühe, *Macromol. Biosci.* **2013**, *13*, 873.
[21] M. Eichhorn, C. Stannard, K. Anselme, J. Rühe, *J. Mater. Sci.: Mater. Med.* **2015**, *26*, 108.

