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Formulating An Ideal Protein Photoresist For Fabricating Dynamic Microstructures With High Aspect Ratios And Uniform Responsiveness

Chee Leng Lay,^{§, †} Yih Hong Lee,[§] Mian Rong Lee,[§] In Yee Phang,^{†, *} Xing Yi Ling^{§, *}

[§]Division of Chemistry and Biological Chemistry, School of Physical and Mathematical Sciences, Nanyang Technological University, 637371, Singapore. Email: xyling@ntu.edu.sg

[†]Institute of Materials Research and Engineering, Agency for Science, Technology and Research (A*STAR), 2 Fusionopolis Way, Innovis, #08-03, 138634, Singapore. Email: phangiy@imre.a-star.edu.sg

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The physical properties of aqueous-based stimuli-responsive photoresists are crucial in fabricating microstructures with high structural integrity and uniform responsiveness during two-photon lithography. Here, we quantitatively investigate how various components within bovine serum albumin (BSA) photoresists affect our ability to achieve BSA microstructures with consistent stimuli-responsive properties over areas exceeding $10^4 \mu\text{m}^2$. We unveil a relationship between BSA concentration and dynamic viscosity, establishing a threshold viscosity to achieve robust BSA microstructures. We also demonstrate the addition of an inert polymer to the photoresist as viscosity-enhancer. A set of systematically optimized processing parameters is derived for the construction of dynamic BSA microstructures. The optimized BSA photoresists and processing parameters enable us to extend the microstructures from 2D to 3D, culminating in arrays of micropillars with aspect ratio > 10 . Our findings foster the development of liquid stimuli-responsive photoresists to build multi-functional complex three-dimensional (3D) geometries for applications such as bio-implantable devices or adaptive photonic systems.

1. Introduction

Photoresists are central to lithographical fabrication of micro/nanostructures. In two-photon lithography, photoresists with efficient two-photon polymerizability and considerable viscosity are critical in enabling the precise fabrication of well-defined three-dimensional (3D) structures.¹ In particular, aqueous-based BSA photoresists are widely used as 3D tissue scaffolds and replicas,^{2, 3} cell micropatterns for cell culture and microanalysis,⁴⁻⁸ and bioelectronic components.⁹ BSA photoresists also demonstrate tremendous potential in generating smart stimuli-responsive microactuators¹⁰ and dynamic tunable micro-optical components.¹¹⁻¹³ However, stimuli-responsive hydrogel structures reported to date show only single or a few microstructures for demonstration.¹⁰⁻¹³ It remains challenging to fabricate a sizable number of free-standing stimuli-responsive microstructures exhibiting uniform responsiveness over areas exceeding $10^4 \mu\text{m}^2$.¹⁴ In addition, high aspect ratio stimuli-responsive 3D microstructures have yet been demonstrated, with most reported BSA-based microstructures having aspect ratios below 5.¹⁵⁻¹⁷ High-aspect-ratio hydrogel microstructures are expected to significantly improve sensitivity in cell behavioral studies,¹⁵ and detection limits of sensors,^{18, 19} as well as promote fabrication of smart wetting/non-wetting surfaces.^{20, 21} This knowledge gap arises from the inconsistent physical properties of the liquid photoresist (such as monomer concentration and viscosity), often leading to structural distortion during the layer-by-layer two-photon-induced polymerization process and creating non-uniform responsiveness in the fabricated microstructures.^{1, 6, 22} These limitations have impeded the full realization of the aforementioned smart material platforms.

To improve the structural integrity of stimuli-responsive microstructures, it is essential to create a standardized quantitative protocol for the analysis of aqueous-based photoresists. Aqueous-based photoresists normally have low viscosity and suffer from mechanical vibration-induced drifts, leading to structural distortion and inconsistent

responsiveness in the fabricated microstructures.^{1, 22} The two main approaches typically used to fabricate stable stimuli-responsive microstructures are predominantly qualitative.^{6, 7, 23} The first approach uses an additional solvent evaporation step to concentrate the dilute premixed photoresists into a more viscous fluid.⁶ This spontaneous evaporation process can lead to batch-to-batch photoresist inconsistency, and in turn impact the material properties of fabricated microstructures. In the second approach, hydrogel-coated substrates⁷ or extra support frames^{23, 24} are used to secure the geometrical alignment of the microstructures by immobilizing crosslinked protein microstructures within networks of a 3D hydrogel or around stiff support frames. These hydrogel networks or frames prevent the drift-induced shifting of the written structures within the photoresists during laser writing. However, this approach does not resolve the issue of low photoresist viscosity. Furthermore, incorporating additional processing steps comes at a price of increasingly tedious and time-consuming fabrication procedures, limiting the adaptation of such approaches for large-area production of 3D microstructures. As such, quantitative analyses on the physical properties of liquid photoresists are important and useful to predict the ability to achieve dynamic yet stable stimuli-responsive microstructures.

Here, we present a quantitative analysis on how various components within the photoresist and fabrication parameters affect our ability to achieve BSA microstructures with consistent stimuli-responsive properties over areas exceeding $10^4 \mu\text{m}^2$. We establish a relationship between dynamic viscosity of the photoresist and concentration of BSA in the photoresist. This relationship enables us to gauge the success of fabricating microstructures with consistent mechanical properties. In addition, we introduce an inert high molecular weight polymer to the photoresist as a viscosity enhancer. We also develop a set of optimized photoresist formulation (photoresist solvent composition, threshold concentration for BSA, and photoinitiator) and fabrication parameters (laser power and laser dwell time) to achieve

microstructures with uniform pH-responsive swelling over areas exceeding $10^4 \mu\text{m}^2$. We also extend the type of microstructures fabricated from 2D to 3D, creating micropillar arrays with aspect ratios > 10 . Our findings on photoresist formulation can potentially be adapted to other aqueous-based photoresists in deriving optimal photoresist properties for efficient two-photon direct laser writing.

2. EXPERIMENTAL SECTION

2.1 Materials. Lyophilized bovine serum albumin (BSA) was supplied by Biowest. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; 99.5+ %), rose bengal (95 %), powdered sodium chloride (NaCl; 99+ %) and phosphate buffered saline tablets (PBS), glutaraldehyde (50 wt% in water) and polyvinylpyrrolidone with average molecular weight of 360 K was purchased from Sigma Aldrich. Dimethyl sulfoxide (DMSO; AR grade), hydrochloric acid (37 %), and sodium hydroxide pellet (> 97 %) were purchased from Goodrich Chemicals. The chemicals were used without further purification. Milli-Q water (> 18.0 M Ω .cm) was purified with a Sartorius arium® 611 UV ultrapure water system.

2.2 Preparation of BSA Protogel Precursor Solution. BSA powder (2.9 g, 3.8 g and 4.6g) were dissolved in solution (9 mL) containing HEPES buffer solution (82 v/v%, having 20 mM HEPES and 100 mM NaCl) and DMSO (18 v/v%), separately. Rose bengal solution (8.5 mM) was added to the as-prepared BSA solution to make up a total volume of 10 mL. Concentration of rose bengal was changed when regulating experimental conditions.

2.3 Fabrication of BSA Microstructures. Fabrication of the protogel structures were performed using the Nanoscribe® Photonic Professional. The system is equipped with an erbium-doped, femtosecond laser source with a center wavelength of 780 nm, pulse repetition rate of 80 MHz and pulse length of 100 fs. BSA microstructures were designed using a computer-aided design (CAD) software, 3ds Max®. Parameters of the structures were defined by the Nanoslicer and DeScribe softwares. Laser power, laser scan speed and line distance are programmed using DeScribe software. NanoWrite software controls both the movement of piezo-driven nanopositioning scanning sample stage as well as emitting power of the laser as programmed using the Nanoslicer and DeScribe softwares. All structures were written on square glass substrates (width = 22 mm and thickness $\approx$ 0.13 to 0.17 mm) with 0.2

μm line distance in x, y and z directions. Photoresist containing BSA and rose bengal was deposited on a glass substrate. Photopolymerization started from the interface between photoresist and glass substrate which caused fabricated structures to be firmly attached to the glass substrate. Photoresist at locations exposed to the laser undergoes polymerization and crosslinking on glass substrate. After fabrication, the substrate was soaked in 20 mM PBS buffer to remove excess unpolymerized photoresist, and subsequently washed with water. The fabricated microstructures remained fixed on glass substrates after development.

For structural integrity study, free-form cross-shaped (“+”) microstructures with dimensions of $20\ \mu\text{m} \times 20\ \mu\text{m} \times 10\ \mu\text{m}$, ($l \times w \times h$) were fabricated from different BSA photoresists containing various BSA concentrations and PVP (refer to section 3.2 Effects of BSA Concentration and PVP Addition on Properties of BSA Microstructures). On the other hand, all individual circular microstructures fabricated for systematic studies on operating conditions and on pH-responsiveness against prolong fabricating time has diameter of $5\ \mu\text{m}$ and $3\ \mu\text{m}$ in height. To construct an array of 80 free-standing cross-shaped microstructures (individual structure had dimension of $20\ \mu\text{m} \times 20\ \mu\text{m} \times 8\ \mu\text{m}$, $l \times w \times h$), photoresist containing BSA (460 g/L) and PVP (10 g/L) was used (refer to section 3.4 Large-Area Fabrication of BSA Microstructures). In section 3.5 on fabrication of high-aspect-ratio micropillars, the lateral width of the BSA micropillars was $1.41\ \mu\text{m}$, the height (h_p) ranged from 2 to $24\ \mu\text{m}$, and the spacing between the micropillars was $20\ \mu\text{m}$. Rose bengal concentration, laser power and laser scan speed were varied during tuning experimental conditions to obtain two-photon lithography operating windows. Otherwise, 8.5 mM of rose bengal was mixed with BSA photoresists for microfabrication operating at laser power of 12 mW and laser scan speed of $30\ \mu\text{m/s}$.

2.4 Atomic Force Microscopy (AFM). AFM measurements were carried out using a JPK Nanowizard[®]3 Bioscience AFM equipped with JPK Vortis SPM Control controller with

XYZ closed-loop feedback (JPK instrument, Berlin) on an inverted microscope. Silicon cantilevers from Budget sensor (model: Multi-75-Al-G with 30 nm Aluminium reflected coating) were used for the quantitative imaging (QI) mode and force spectroscopy. The samples were first incubated in the pH 5 aqueous solution, where AFM tip was approached to the sample and QI measurement was used to measure the morphology of the samples. Force spectroscopy was carried out later on the sample on various location and the force spectra were collected. The force spectra are averaged from the multiple force curves. The spring constants of the cantilevers were calibrated according to thermal noise method, which were found to be in the range of 4 – 6 N/m. The sensitivity of the probe was subsequently measured on a silicon wafer. All data conversions were done on the JPK Data Processing software. The modulus was obtained by fitting the extended part of the force-penetration curves with simple Hertz model (JPK data analysis software). Individual force curve was fitted and tabulated into the graph.

2.5 Scanning Electron Microscopy (SEM) Imaging. To prepare sample for SEM imaging, BSA microstructures were fixed in glutaraldehyde solution (2.5 v/v%) for two hours, followed by rinsing with three aliquots of ethanol with increasing serial concentrations (0 %, 25 %, 75 %, 95 % and 100 %). The samples were then freeze-dried after immersing in 1-butanol. Samples were sputter-coated with platinum particles at 40 mV for 30 s using a JEOL JFC-1600 AUTO FINE COATER. Coated surfaces were viewed using a JEOL 7600F SEM operating at accelerating voltage of 4.5 kV and beam current of 5 in SEI mode.

2.6 Materials Characterization. Refractive indexes (n_D) of solutions were measured using a thermostatically controlled Abbe refractometer model, Atago-3T operates with D-line (589 nm) light source and has temperature controlled at $(24 \pm 1) ^\circ\text{C}$. For every sample, the measurements were repeated 5 times and mean value was taken in each calculation. Dynamic

viscosities (μ) of solutions were measured at atmospheric pressure and $(25 \pm 0.2) ^\circ\text{C}$ using Brookfield LVDV-II+ PRO with rotational speed of the cone plate increased from 0 to 200 rpm with increment of 10 rpm at every 30 seconds interval. Bright field optical microscopy was performed using an Olympus BX51BD microscope. Absorption spectra of rose bengal were obtained on a Agilent Cary 60 UV-Vis spectrophotometer. Raman imaging was obtained using the Ramantouch microspectrometer (Nanophoton Inc, Osaka, Japan) equipped with an Apochromat NIR water immersion objective from Nikon ($60\times$ magnification, NA 1.0). The wavelength and power of excitation laser were set at 532 nm and 0.13 mW, respectively, and Raman acquisition was performed with an exposure time of 0.2 s per line with scanning distance of 500 nm and 800 nm in x-y plane and x-z plane, respectively. All data for the physical characterization of BSA microstructures were averaged using at least 10 measurements.

2.7 Data Analysis. Measurements of swelling percentage of the BSA microstructures of as-fabricated BSA microstructures were conducted using ImageJ. Swelling percentage was obtained from equation $[(A_{\text{pH}11} - A_{\text{pH}5}) / A_{\text{pH}5}] \times 100 \%$ as $A_{\text{pH}5}$ and $A_{\text{pH}11}$ are areas measured at pH 5 and pH 11, respectively. Energy dose per area (E_d) was calculated via equation $(P \times t) / A$, where P is laser power, t is laser dwell time and A is laser lateral spot size.¹⁷ Laser dwell time is calculated by dividing point distance (distance between virtual irradiated points fixed at 100 nm) with laser scan speed, hence faster laser scan speeds corresponding to shorter dwell times.¹⁷

3. RESULTS AND DISCUSSION

3.1 Parameters and Dispersion Compositions. To formulate an ideal photoresist capable of fabricating well-defined BSA microstructures with excellent structural integrity, we investigate the influence of dynamic viscosity, BSA monomer concentration, and photoinitiator (rose bengal) concentration within the photoresist on the laser writing process (Scheme 1). Fabrication parameters such as laser power and laser dwell time on the protein microstructure integrity are also investigated (Scheme 1).

We first create a photoresist which is optically transparent at the laser excitation wavelength (Figure 1), corresponding to 780 nm for our system, to prevent one-photon absorption from affecting the two-photon laser writing process. A typical photoresist comprises 82 v/v% aqueous solution containing HEPES buffer solution and 18 v/v% dimethyl sulfoxide (DMSO) as the solvent for BSA and rose bengal. Instead of using a pure aqueous system, we use DMSO to increase the vapor pressure of the aqueous/DMSO mixture,⁶ reduce bubble formation during the laser writing process, and induce protein unfolding.^{25, 26} 18 v/v% DMSO is the solubility limit of BSA, beyond which BSA aggregation occurs, leading to decreased optical transparency at 780 nm (Figure 1).^{26, 27}

3.2 Effects of BSA Concentration and PVP Addition on Properties of BSA Microstructures. Next, we study the influence of BSA concentration on the structural integrity of the fabricated BSA microstructures. BSA is the largest molecule in our photoresist with a molecular weight of 66.5 kDa. Variations in its concentration will directly affect both the extent of photo-polymerization during laser writing¹⁵ and the dynamic viscosities of the photoresists,^{6, 24, 28} both of which will determine the eventual quality of fabricated microstructures. However, reported minimum concentration of protein monomers required for successful microfabrication is varied across different research groups and effect of photoresist's viscosity on structural properties is not investigated. For structural integrity

analyses, we fabricate free-standing cross-shaped (“+”) BSA microstructures supported by a substrate-attached square support column (Figure 2A). Cross-shaped microstructures can clearly reveal how drift-induced shifting triggered by the piezo-stage movements affects the quality of the fabricated BSA microstructures. Photoresists with BSA concentrations ranging from 290 g/L to 460 g/L are used, with 460 g/L being the solubility limit of BSA in our solution system (Figure 2A).

The structural integrity of the cross-shaped BSA microstructures increases with increasing BSA concentration (Figure 2Ai-iii). At BSA concentration of 290 g/L, only the substrate-attached column can be formed (Figure 2Ai). Increasing the BSA concentration to 380 g/L produces highly distorted free-standing cross-shaped microstructures (Figure 2Aii). We only observe the distinct cross-shaped geometry without significant structural distortion at BSA concentrations of 460 g/L (Figure 2Aiii). Although dependence of protein matrix porosity on BSA concentration was reported,¹⁵ our experiments here directly verify that sufficient BSA needs to be present in the photoresist to enable adequate polymerization and crosslinking for the fabrication of well-defined BSA microstructures.

In addition, we compare the dynamic viscosity of the various BSA photoresists to elucidate the relationship between structural integrity and BSA concentration. Dynamic viscosity is a measure of a fluid’s resistance to flow when shear force is applied, and is dependent on the concentration of the solutes in the fluid. In our fixed-beam moving-sample setup, sample stage movements during the laser writing process may give rise to stage vibration-induced drifting. Such drifting can cause parts of the fabricated microstructure to shift and/or float away from the designated positions, especially in low viscosity photoresists.^{1, 6, 22} Thus, the photoresist has to be sufficiently viscous to prevent structural distortion due to such drifts. For the photoresists containing 290 g/L, 380 g/L and 460 g/L of BSA, the dynamic viscosities are (8.2 ± 0.5) cP, (18.5 ± 0.2) cP and (36.6 ± 0.5) cP,

respectively (Figure 2B). An approximately 2-fold increase in the dynamic viscosity of the photoresist is observed with BSA concentration is increased from 290 g/L to 380 g/L and from 380 g/L to 460 g/L.

BSA microstructures with improved structural integrity can also be fabricated by introducing polyvinylpyrrolidone (PVP) (10 g/L, $M_w = 360$ K) as a viscosity-enhancing agent into our photoresist (Figure 2Aiv–vi). PVP is widely used as a viscosity-enhancing agent in food,^{29, 30} medical formulations,^{31–34} and conventional lithography and photography.³⁰ In addition, PVP is chemically inert, highly polar, non-ionic, and does not compromise the photo-polymerization reactions occurring within the photoresist during the laser writing process (Figure S1, S2).^{30, 34} In comparison with the pure BSA photoresist, PVP further increases the dynamic viscosities by an average of 1.9 fold, which are (15.9 ± 0.3) cP, (35.1 ± 2.3) cP and (68.6 ± 0.7) cP for the photoresists containing 290 g/L, 380 g/L and 460 g/L of BSA, respectively. Enhanced structural integrity in the presence of PVP is clearly evident in the case of photoresist with BSA concentration of 380 g/L. The individual suspending arms of the cross-shaped microstructure are fully formed as dynamic viscosity of the photoresist increases from (18.5 ± 0.2) cP to (35.1 ± 2.3) cP after the addition of PVP (Figure 2Av; Figure 2B). Here, we quantitatively demonstrate how PVP modulates the viscosity of our photoresists. From the experiments, we establish a minimum dynamic viscosity range of 35 to 37 cP for the construction of well-defined microstructures, corresponding to photoresists containing 380 g/L BSA with 10 g/L PVP and/or 460 g/L BSA (Figure 2A,B).

By changing the BSA concentration, we are also able to tune the swelling capability of substrate-attached cylindrical microstructures (Figure 2C; Figure S2). The pH-responsiveness of the cylindrical microstructures is determined by their swelling percentage (refer to section 2.7 Data Analysis). For microstructures fabricated from photoresists containing 290 g/L, 380 g/L, and 460 g/L of BSA, the swelling percentages are (32 ± 2) %, (45 ± 2) %, and (58 ± 2) %, respectively.

(34 ± 2 %), and (36 ± 1 %), respectively (Figure 2C, blue bars). A slightly larger swelling extent is observed for microstructures fabricated at higher BSA concentrations, likely arising from the greater repulsive forces generated by the higher amount of negatively charged carboxyl groups within the BSA microstructures.²² A similar trend of swelling percentage is also observed for the BSA photoresists containing PVP, which are (30 ± 1 %), (29 ± 2 %) and (33 ± 1 %) for microstructures fabricated from photoresists containing 290 g/L, 380 g/L and 460 g/L of BSA, respectively (Figure 2C, red bars).

On the whole, the presence of PVP slightly lowers the swelling percentages of the microstructures. The difference in microstructure swelling capability in the absence and presence of PVP arises from the mechanical property disparity of these microstructures. To quantitatively evaluate the mechanical property of the microstructures fabricated from liquid photoresists containing 460 g/L BSA and 460 g/L BSA with 10 g/L PVP, we use atomic force microscopy (AFM)-based nano-indentation measurements at pH 5. A steeper force-to-penetration gradient is observed from the microstructure fabricated in the presence of PVP, giving rise to a larger Young's modulus (Figure 2D,E). The presence of long chain PVP introduces additional physical entanglement that hinders the swelling of the BSA microstructures. This leads to a slight increase in the stiffness of the PVP-fortified BSA microstructures and also reduces the swelling capability of these microstructures. The mechanical analysis further evidences good structural integrity of our fabricated BSA microstructures. Our measured elastic modulus matches well with the literature value of 0.03 to 3 MPa, indicating structurally stable albumin hydrogels have been formed.⁴

Notably, our collective experiments on the variation of BSA concentration and the addition of PVP are the first to quantitatively correlate fabricated microstructure quality with the dynamic viscosity of an aqueous-based photoresist. A direct correlation between these two entities demonstrates the importance of improving the dynamic viscosity of the liquid

BSA photoresists to realize the fabrication of mechanically robust and well-defined 3D microstructures. From here on, we will base our subsequent discussion on photoresists containing 460 g/L BSA and its counterpart with 10 g/L PVP.

3.3 Effect of Rose Bengal Concentration and Laser Processing Parameters. To further enhance the ability to fabricate microstructures with optimal pH-swelling response, we investigate the interplay between rose bengal concentration and laser power using photoresists containing 460 g/L BSA. Rose bengal acts as the photoinitiator in our photoresist. Its concentration is proportional to the amount of free radicals generated upon laser irradiation (Figure S3), directly affecting the polymerization and crosslinking extent of BSA during fabrication. We establish a contour plot based on the experimental results to characterize the correlation between microstructure swelling percentages and variations in both rose bengal concentration as well as laser power (Figure 3A). The rose bengal concentration ranges between 4 – 12 mM and the laser power ranges between 4 – 24 mW. Circular microstructures are fabricated for this part of the study (Figure 3B; Figure S3A).

For successful microstructure fabrication, the threshold rose bengal concentration is 8 mM with a minimum laser power of 10 mW (Figure 3A). This condition enables us to achieve BSA microstructures with a maximum swelling of 40 %. At rose bengal concentrations < 8 mM, significantly higher laser powers are required to generate the minimum amount of initiating rose bengal molecules for the polymerization and cross-linking of BSA monomers (Figure 3A; Figure S3A). In contrast, at rose bengal concentration > 8 mM, lower laser powers are able to generate microstructures with good pH-swelling capabilities. In both cases, the swelling percentages are in the range of 17 – 40 % (Figure 3A). However, no well-defined microstructures can be fabricated at rose bengal concentrations ≤ 4 mM regardless of laser power (Figure 3A,C; Figure S3A). The damage threshold laser power in our system is 20 mW (energy dose per area (E_d) = 18.2 kJ/cm²; refer to section 2.7 Data

Analysis and Figure S3 for equation), whereby internal burning and microstructure explosion are observed at this laser power (Figure 3A,B; Figure S2; Figure S3).¹⁷ In addition, rose bengal does not alter the dynamic viscosity of the BSA photoresists since its molecular mass is < 1 kDa and is present in much smaller quantities (Figure S4).

Higher rose bengal concentration and greater laser power implies that more initiating molecules are generated to promote polymerization and crosslinking of BSA molecules within the laser focal volume (Figure 3C).^{5, 15, 35} We employ equation (1) to theoretically quantify the effect of rose bengal concentration and laser power on the number of initiating rose bengal molecules (N_{RB}) generated.

$$N_{RB} = \frac{1}{2} \frac{g_p N \phi \delta}{\tau f} \left(\frac{\lambda}{hc} \right)^2 \left(\frac{P}{A} \right)^2 t \quad (1)$$

where P is laser power, t is laser dwell time, g_p describes pulse shape of Fourier-transform-limited pulse, τ is pulse bandwidth, f is laser repetition rate, λ is excitation wavelength, h is Planck's constant, c represents speed of light in vacuum, A is laser lateral spot size, N is the number of two-photon absorbing molecules, ϕ is quantum efficiency for conversion of excited states to polymerization initiating species, and δ is two-photon absorption cross-section of photoinitiator at the excitation wavelength (Figure S3).³⁶

Equation (1) describes an ideal scenario where every dye molecule is efficiently converted to a photochemical reaction initiator after undergoing an absorption event within the laser focal volume. It is also assumed that the laser lateral spot size (A) does not change significantly with respect to the properties of the photoresist and processing parameters.³⁶ By correlating our experimental contour plot (Figure 3A) and calculated number of initiating molecules generated against rose bengal concentration and laser power (Figure 3C), the minimum of initiating molecules required for successful microfabrication at 8 mM rose bengal and laser power of 10 mW is 7.8×10^{21} radicals/cm³.

The impact of laser dwell time on the swelling behavior of the microstructures is also investigated (Figure 3D). We vary the laser dwell time from 10.0 ms to 1.4 ms by changing the laser scan speed from 10 $\mu\text{m/s}$ to 70 $\mu\text{m/s}$ during the laser writing process. With constant laser power of 12 mW, the microstructures are burnt at laser dwell time of 10 ms due to excessive laser exposure (Figure S5A). At laser dwell time of 10 ms, the calculated energy dose per area is 33.0 kJ/cm^2 which is far beyond the damage threshold of our system (18.0 kJ/cm^2 , Figure S5B). As laser dwell time decreases from 5.0 ms to 2.0 ms, the swelling percentages increase from approximately 21 % to 43 %. Further decrease in laser dwell time from 2.0 ms to 1.4 ms, the swelling percentage approaches a plateau around 45 % (Figure 3D). The fabricated microstructures are not well-constructed as laser dwell time decreases below 2.0 ms (Figure S5A). A shorter dwell time minimizes the interaction between laser beam and BSA photoresist. As laser dwell time is decreased to 1.4 ms, the energy dose per area is reduced to 4.7 kJ/cm^2 (Figure S5B). The efficiency for the two-photon polymerization process can be reflected by the number of initiating molecules generated, as it reduces from 3.6×10^{22} radicals/ cm^3 at 10.0 ms to 5.1×10^{21} radicals/ cm^3 at 1.4 ms (Figure S5B). Lesser photo-induced crosslinking events during microfabrication produce microstructures with greater swelling ability upon pH change.¹⁶ However, sufficient laser dwell time is critical to enable the localized radical generation ($\geq 7.8 \times 10^{21}$ radicals/ cm^3) for effective photopolymerization and BSA cross-linking. Therefore, the optimal laser scan speed is determined to be 30 $\mu\text{m/s}$ for our system based on a laser power of 12 mW, corresponding to a dwell time of 3.3 ms. The swelling properties of protein microstructures demonstrated in our work plays an important role in identifying and explaining influences of the properties of BSA photoresist (dynamic viscosity of photoresist, concentration of BSA, and concentration of rose bengal) and operating parameters (laser power and laser dwell time) on structural behaviors of the as-fabricated BSA microstructures. Moreover, besides viscosity and

refractive index, swelling percentage of the fabricated microstructures also provides information on property consistency of the BSA photoresists and the fabricated microstructures. With our finding on threshold viscosity for aqueous-based BSA photoresists, the threshold viscosity can potentially contribute to the development of other aqueous-based stimuli-responsive hydrogel photoresists.

3.4 Large-Area Fabrication of BSA Microstructures. Based on the results presented, we employ BSA photoresists with 8.5 mM rose bengal concentration and higher laser powers of 12 mW for the subsequent fabrication of microstructures with consistent structural properties. Having established an ideal photoresist formulation and optimized the fabrication parameters, we demonstrate the application of our setup to create large-area microstructures with consistent swelling properties. 160 circular microstructures are fabricated over an area of $\sim 0.06 \text{ mm}^2$ from BSA photoresist containing 460 g/L BSA and 10 g/L PVP (Figure 4A). Throughout the microstructure fabrication duration of 4.4 h, the dynamic viscosities and refractive indices of the BSA photoresist remain virtually unchanged, with less than 10 % fluctuation observed (Figure 4B). All 160 microstructures display identical swelling percentages of $(33 \pm 2) \%$ (Figure 4C,D). The consistent physical and chemical properties of our photoresist formulation indicate its competence for fabrication of microstructures with arbitrary shapes beyond the stated number of the cylindrical structures, area and processing time. In contrast, circular microstructures constructed from BSA photoresist containing 290 g/L BSA exhibit highly inconsistent swelling behaviors, varying from $(31 \pm 2) \%$ to $(64 \pm 1) \%$, under the same fabrication process (Figure S6). Our results collectively highlight the importance of developing a stable photoresist in achieving large-area consistent stimuli-response and the use of dynamic viscosity as a good measure of photoresist quality.

To demonstrate the consistency of the photoresist formulation in fabricating free-standing microstructures with long-ranged reproducible structural integrity and uniform responsiveness, we construct a 0.035-mm^2 ordered array of 80 free-standing cross-shaped (“+”) BSA microstructures (Figure 4E,F). All fabricated free-standing cross-shaped microstructures are well-fabricated (Figure 4Ei,F). Each microstructure exhibits $(35 \pm 1) \%$ swelling in area at pH 11 (Figure S7). SEM results confirm the reproducibility of fabricating free-standing cross-shaped microstructures using photoresist containing 460 g/L BSA and 10 g/L PVP (Figure 4F; Figure S7). The distinct edges of the microstructures further highlight the structural integrity of suspending arms against stage motion induced drift in photoresist (Figure S7). The SEM result further verifies the structural integrity of our microstructures. The roughened surface on the microstructures arises from the growth of multiple nucleation points during the two-photon induced radical polymerization for microfabrication.^{15, 17}

3.5 Fabrication of High-Aspect-Ratio Micropillars. We also highlight the ability to fabricate high aspect ratio microstructures using the same photoresist containing 460 g/L BSA and 10 g/L PVP. To demonstrate this capability, we fabricate an ordered array of square micropillars. The width of the micropillars is kept constant at $1.41 \mu\text{m}$, and the height is varied from 2 to $24 \mu\text{m}$ to generate micropillars with aspect ratio ranging from 1.4 to 17.0 (Figure 5A). The actual height of the micropillars in their wet state is obtained by determining their full-width half-maximum (FWHM) Raman intensity profiles in pH 5 solution (Figure 5B).

SEM characterization shows the successful fabrication of BSA micropillars with height of $16 \mu\text{m}$ and aspect ratio of 11.3 (Figure 5C). Notably, the free-standing BSA micropillars are mechanically robust and sufficiently stiff to maintain their structural integrity even after freeze-drying (Figure 5A, Inset: bottom). The measured FWHM height of the micropillars from the Raman intensity profiles show micropillars with heights of $16 \mu\text{m}$

(Figure 5B). At larger aspect ratios, the micropillars begin to bend or topple (Figure S8), giving rise to smaller FWHM heights measured from the Raman intensity profiles (Figure 5A). We observe a similar trend when comparing FWHM heights of micropillars fabricated from photoresist containing 460 g/L BSA (Figure S9). To date, only non-responsive microstructures, such as acrylate- and epoxy-based photoresists have been utilized for construction of high-aspect-ratio microstructures (aspect ratio > 5).^{20, 21, 37-39} Whereas responsive hydrogel-based photoresists are used to produce either microstructures with aspect ratios < 5 ¹⁵⁻¹⁷ or horizontally-laid high-aspect-ratio rod-like microstructures,^{10, 22} via two-photon lithography. Therefore, our high aspect ratio BSA micropillars can enhance the usefulness of BSA microstructures reported thus far for potential applications in creating smart wetting/non-wetting surfaces and in cell behavioral studies.

4. CONCLUSION

In conclusion, we have successfully developed an ideal aqueous-based photoresist to fabricate stimuli-responsive microstructures with consistent responsive behaviors over large areas. We introduce dynamic viscosity as a quantitative gauge of photoresist quality, and demonstrate how individual components of the photoresist impact the structural integrity of the fabricated microstructures. Fabrication parameters such as laser power and dwell time are also optimized to enable us to successfully extend the 2D microstructures into 3D ones with aspect ratios > 10 . Our results illustrate the importance in understanding complex process-structure-property relationships, contributing to the development of aqueous-based photoresists containing stimuli-responsive monomers. Our quantitative approach in photoresist formulation bring us closer to the realization of large-area multi-functional three-dimensional (3D) microstructures, with potential applications as adaptive microlenses, responsive microactuators, photonic integrated systems or implantable biocompatible micro-devices.

ASSOCIATED CONTENT

Supporting Information

The supporting information is available free of charge on the ACS Publications website at <http://pubs.acs.org>.

Further information including effect of BSA and PVP on rose bengal's UV-Vis absorption, influence of concentrations of BSA and rose bengal, average laser power, laser dwell time and presence of PVP on two-photon polymerizability of BSA photoresists, effect of rose bengal on the dynamic viscosity and refractive index of BSA photoresists, and characterization on structural integrity of free-standing microstructures and high aspect ratio micropillars via Ramantouch microspectrometer and SEM.

AUTHOR INFORMATION

Corresponding Author

In Yee Phang. Email: phangiy@imre.a-star.edu.sg

Xing Yi Ling. Email: xyling@ntu.edu.sg

Note:

The authors declare no competing financial interests.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Scheme 1. Factors affecting structural integrity of BSA microstructures fabricated using two-photon lithography. The factors relate to the properties of BSA photoresist, including dynamic viscosity of photoresist, concentration of BSA, and concentration of rose bengal, as well as operating parameters such as laser power and laser dwell time.

Figure 1. Effect of HEPES buffer solution-to-DMSO ratio on BSA solubility. **(Top)** Digital images of as-prepared BSA solutions with different HEPES buffer solution-to-DMSO ratio. **(Bottom)** Changes in optical transmittance of the various BSA solutions at the laser excitation wavelength of 780 nm. The concentration of BSA used for all solutions is 460 g/L.

Figure 2. Effects of BSA concentration and polyvinylpyrrolidone (PVP) addition on the structural integrity, swelling capability, and nanomechanical properties of BSA microstructures. **(A)** Optical microscopy images of free-standing BSA microstructures made from BSA photoresists with (i - iii) no PVP added and (iv – vi) with 10 g/L PVP. (vii, viii) Schematic illustration of how drifts in photoresists affect the structural integrity of microstructures. **(B)** BSA concentration-dependent plot of the dynamic viscosity of various BSA photoresists. Dotted line indicates minimum dynamic viscosity required to construct well-defined microstructures. **(C)** (i) Substrate-attached circular microstructures and (ii) their corresponding swelling percentages. Plots of **(D)** force-penetration depth and **(E)** Young's modulus obtained from AFM nanoindentation measurements conducted at pH 5 for the circular BSA microstructures fabricated using 460 g/L BSA photoresist with and without 10 g/L PVP.

Figure 3. Effect of rose bengal concentration, laser power, and laser scan speed on the swelling capabilities of BSA microstructures. **(A)** Contour plot of swelling percentages with respect to laser powers and rose bengal concentration variations. Each colored zone represents a range of swelling percentage. **(B)** Optical microscopy images of BSA circular microstructures at pH 5 and pH 11, representing different regions labeled in **(A)**. **(C)** Calculated number of initiating rose bengal molecules against average laser power at different rose bengal concentrations. The region within the two dotted lines is optimal for microfabrication using BSA photoresists with respect to **(A)**. **(D)** Graph illustrating the effects of laser scan speed and corresponding laser dwell time on swelling capability of microstructures written with laser power of 12 mW on photoresists containing 460 g/L BSA and 8.5 mM of rose bengal, with and without addition of 10 g/L PVP.

Figure 4. Large-area fabrication of BSA microstructures. **(A)** Optical microscopy images of an array of BSA circular microstructures fabricated over 4.4 hours. **(B)** Monitoring the dynamic viscosity (μ) and refractive index (n_D) of sample solutions upon complete fabrication of each row of eight microstructures. Average standard deviation of refractive index is 0.0035. **(C)** Swelling percentage of fabricated microstructures. **(D)** Optical microscopy images of selected circular microstructures written at different times. **(E)** Optical microscopy images of an array of 80 free-standing cross-shaped BSA microstructures immersed in (i) pH 5 and (ii) pH 11 solution. **(F)** SEM micrograph of the same array.

Figure 5. Fabrication of high aspect ratio micropillars. **(A)** Measured height of micropillars at various aspect ratios is obtained via determining the full-width half-maximum (FWHM) intensity profiles of their x-z Raman images at pH 5 (Figure 5B). Inset: **(Top)** Schematic illustration of micro-pillars. **(Bottom)** Enlarged SEM micrograph of a representative BSA micropillar. **(B)** Two-dimensional (x-z) Raman image and **(C)** SEM micrograph of an array of micropillars (height (h_p) = $16 \mu\text{m}$; aspect ratio = 11.3) obtained in pH 5 solution and after freeze-drying, respectively.

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Formulating An Ideal Protein Photoresist For Fabricating Dynamic Microstructures With High Aspect Ratios And Uniform Responsiveness

Chee Leng Lay, Yih Hong Lee, Mian Rong Lee, In Yee Phang, Xing Yi Ling

Supporting Information

Formulating An Ideal Protein Photoresist For Fabricating Dynamic Microstructures With High Aspect Ratios And Uniform Responsiveness

Chee Leng Lay,^{§, †} Yih Hong Lee,[§] Mian Rong Lee,[§] In Yee Phang,^{†, *} Xing Yi Ling^{§, *}

[§]Division of Chemistry and Biological Chemistry, School of Physical and Mathematical Sciences, Nanyang Technological University, 637371, Singapore. Email: xyling@ntu.edu.sg

[†]Institute of Materials Research and Engineering, Agency for Science, Technology and Research (A*STAR), 2 Fusionopolis Way, Innovis, #08-03, 138634, Singapore. Email: phangiy@imre.a-star.edu.sg

Figure S1. Effect of BSA and PVP on rose bengal's absorption spectrum. (A) UV-Vis absorption curve of (i) pristine 8.5 mM rose bengal solution and (ii – vii) 8.5 mM rose bengal in different BSA photoresists. 2 μ L of sample solutions were diluted in 3 mL of solvent containing 82 v/v% HEPES buffer solution and 18 v/v% DMSO prior to UV-Vis measurements. Relative absorbance is obtained by dividing absorbance at specific wavelength over maximum absorbance. Absorption peaks (peak 1 at $\lambda = 518$ nm ; peak 2 at $\lambda = 556$ nm) of rose bengal are slightly red-shifted (peak 1' at $\lambda = 527$ nm ; peak 2' at $\lambda = 564$ nm) when rose bengal molecules are mixed with BSA. The presence of PVP has insignificant effect on rose bengal's absorption. (B) Dependence of absorption spectra on rose bengal concentration measured by diluting sample solution containing 8.5 mM rose bengal, 460 g/L BSA and 10 g/L PVP to obtain rose bengal concentration range from 3 μ M to 167 μ M. (C) Graph extracted from (B) to illustrate the linear relationship between absorbance at 390 nm as a function of rose bengal concentration, where absorbance = $0.00252 \times [\text{RB}]$ with $R^2 = 0.9988$.

Increasing BSA concentration and addition of PVP do not affect photochemical polymerization and cross-linking processes of the photoresists since the BSA photoresists have similar UV-Vis absorption spectra and laser power threshold, before and after increasing BSA concentration and adding PVP (Figure S1; Figure S2). Linear concentration

dependence of absorbance on rose bengal concentration at 390 nm is also independent of BSA concentration and presence of PVP (Figure S1B,C). Our results are in consistent with findings reported independence of rose bengal-sensitized photodynamic crosslinking of proteins on addition of acrylamide copolymers^{1,2} or dye-labelled long-chain dextran.³

Figure S2. Effect of average laser power, BSA concentration and presence of PVP on photo-polymerizability of BSA photoresists using two-photon lithography. Optical microscopy images of microstructures taken at pH 5. Microstructures are fabricated from different BSA photoresists over laser power ranging from 4 to 24 mW, with constant scan speed of 30 $\mu\text{m/s}$. Concentration of rose bengal in each BSA photoresist is fixed at 8.5 mM. Swelling capability of microstructures fabricated from different BSA photoresists at 12-mW laser power is compared in Figure 2C.

Figure S3. Effect of average laser power and rose bengal concentration on efficiency of two-photon induced polymerization of BSA photoresists. (A) Optical microscopy images illustrating influence of laser power and concentration of rose bengal on polymerizability of BSA photoresists when subjected to two-photon lithography. Microstructures are fabricated from BSA solutions containing 460 g/L BSA, rose bengal with concentration of 4 mM to 12 mM, 82 v/v% HEPES buffer solution and 18 v/v% DMSO and are written at laser power ranging from 4 to 24 mW, with constant scan speed of 30 $\mu\text{m/s}$. Images are taken at their original state in pH 5 solution after development. (B) Calculated energy dose per area versus applied average laser power. Regions exposed to energy dose per area $\geq 18.2 \text{ kJ/cm}^2$ experience internal burning and microstructure explosion (Figure 3; Figure S3A).

The following equations are used to calculate (i) number of initiating rose bengal molecules within focal volume⁴ and (ii) energy dose per area.⁵ The equations describe the ideal scenario where every dye molecule is efficiently converted to a photochemical reaction trigger after undergoing an absorption event within the laser focal volume.⁴ It also assumes that the laser lateral spot size (A) has insignificant change with respect to properties of the photoresist and processing parameters.⁵

(i) Number of initiating rose bengal molecules within focal volume (N_{RB})

P = Laser power (W)

t = Laser dwell time (s)

g_p = 0.664 (Parameter describes pulse shape of Fourier-transform-limited pulse)

τ = Pulse bandwidth = $1 \times 10^{-13} \text{ s}$

f = Laser repetition rate = $8 \times 10^7 \text{ s}^{-1}$

λ = Excitation wavelength = $7.8 \times 10^{-5} \text{ cm}$

- h = Planck's constant = 6.626×10^{-34} J s
 c = Speed of light in vacuum = 3×10^{10} cm/s
 A = Laser lateral spot size
 $= \pi \times [(0.61 \times \lambda) / (\text{Numerical aperture of objective lens})]^2$
 $= \pi \times [(0.61 \times 7.8 \times 10^{-5}) / 1.4]^2$
 $= 3.629 \times 10^{-9}$ cm²
 N = Number of two-photon absorbing molecules (molecules/cm³)
 ϕ = Quantum efficiency for conversion of excited states to polymerization initiating species
 $= 1$ (rose bengal molecules have quantum efficiency > 98 %) ^{3, 6}
 δ = Two-photon absorption cross-section of rose bengal at the excitation wavelength
 $= 10 \times 10^{-50}$ cm⁴ s/photon

As a demonstration by using $P = 1.2 \times 10^{-2}$ W, $t = 0.0033$ s and $N = 5.117 \times 10^{18}$ molecules/cm³:

$$\begin{aligned}
 N_{RB} &= 0.5 \times [(g_p N \phi \delta) / (\tau f)] \times [\lambda / (h c)]^2 \times (P / A)^2 \times t \\
 &= 0.5 \times [(0.664 \times 5.117 \times 10^{18} \times 1 \times 10 \times 10^{-50}) / (1 \times 10^{-13} \times 8 \times 10^7)] \times [7.8 \times 10^{-5} / \\
 &\quad (6.626 \times 10^{-34} \times 3 \times 10^{10})]^2 \times (1.2 \times 10^{-2} / 3.629 \times 10^{-9})^2 \times 0.0033 \\
 &= 1.192 \times 10^{22} \text{ radicals/cm}^3
 \end{aligned}$$

(ii) Energy dose per area (E_d)

As a demonstration by using $P = 1.2 \times 10^{-2}$ W and $t = 0.0033$ s,

$$\begin{aligned}
 E_d &= (P \times t) / A \\
 &= (1.2 \times 10^{-2} \times 0.0033) / 3.629 \times 10^{-9} / 1000 \\
 &= 11.0 \text{ kJ/cm}^2
 \end{aligned}$$

Figure S4. Effect of rose bengal on the dynamic viscosity and refractive index of BSA photoresists. (A) Dynamic viscosity and (B) refractive index of BSA sample solutions with or without rose bengal. Addition of rose bengal has little interference on physical properties of BSA sample solutions.

Figure S5. Influence of laser scan speed on fabricating BSA microstructures. (A) Optical microscopy images of microstructures fabricated over laser scan speeds ranging from 10 to 70 $\mu\text{m/s}$ at constant average laser power of 12 mW. BSA photoresists containing 460 g/L BSA and 8.5 mM rose bengal, (i) without PVP and (ii) with addition of 10 g/L PVP. Images are taken at their original state in pH 5 solution after development. (B) Graph illustrates the dependence of calculated energy dose per area and number of initiating rose bengal molecules in focal volume on laser dwell time. Theoretical data assume rose bengal molecules within the focal volume are all excited upon irradiation. Well-defined microstructures can be fabricated between laser dwell time of 2.0 ms and 5.0 ms.

Laser scan speed is defined as the speed of focused laser beam travels between irradiated spots, which is inversely proportional to laser dwell time. Laser dwell time is calculated by dividing point distance (distance between laser irradiated spots) over laser scan speed.⁵ For consistency, point distance is fixed at 100 nm in all experiments.

To understand the effect of laser scan speed on two-photon induced polymerization of BSA photoresists containing 460 g/L BSA and 460 g/L BSA with 10 g/L PVP, circular microstructures are fabricated from both BSA photoresists over laser scan speed ranged from 10 $\mu\text{m/s}$ to 70 $\mu\text{m/s}$ (Figure 3D; Figure S5A). At very low laser scan speed such as 10 $\mu\text{m/s}$, microstructures are burnt due to prolonged laser exposure induced accumulation of thermal energy within the confined volume interacts with focused laser beam (laser dwell time = 10 ms; energy dose per area = 33.0 kJ/cm^2) (Figure S5A,B). On the other hand, fast laser scan speed minimizes interaction between laser beam and BSA photoresist, and hence less rose bengal molecules are photo-initiated for subsequent photochemical polymerization and crosslinking reactions. As such, microstructures fabricated at too high laser scan speed are not well-constructed. Using equations mentioned in Figure S3, we obtain data points for plotting Figure S5B.

Figure S6. Photoresist properties and responsiveness of BSA microstructures against fabricating time. (A) Optical microscopy images of an array of BSA microstructures fabricated over 4.4 hours. (B) Refractive index (n_D) of sample solutions determined at time of writing completion for each row of eight microstructures. Average standard deviation of refractive index is 0.0040. (C) Swelling percentage of fabricated microstructures when immersed in pH 11 solution. Swelling percentage is calculated by dividing the area difference observed between pH 11 and pH 5 over the original area measured at pH 5. (D) Optical microscopy images of selected circular microstructures fabricated at different times. BSA circular microstructures are fabricated from BSA photoresist containing 290 g/L BSA and 8.5 mM of rose bengal with laser power of 12 mW at 30 $\mu\text{m/s}$ scan speed. The BSA photoresist is exposed to ambient condition while fabricating microstructures to observe the effect of water evaporation on properties of fabricated microstructures.

It is observed that BSA photoresist containing 290 g/L BSA needs approximately two-hour of evaporation before obtaining microstructures with considerable percentage of swelling and structural integrity (Figure S6). Microstructures constructed from BSA

photoresist containing 290 g/L BSA with as-discussed processing parameters (laser power = 12 mW, laser scan speed = 30 $\mu\text{m/s}$) display significant difference in location- and time-dependent physical appearance and swelling behaviours over the 4.4 hours. Since the BSA photoresist has too low BSA monomers to be polymerized and crosslinked within the volume of focused laser beam during microfabrication, leading to microstructures fabricated at initial stage with poor integrity (Figure S6D, 1.7 min to 80 min). As water content decreases over time due to evaporation, there is gradual increment in both concentration of BSA and rose bengal from 0 min to 120 min, as reflected from refractive index of the BSA photoresist (refractive index of BSA photoresist changes from 1.3814 at 0 min to 1.4170 at 120 min). As shown in Figure S6B, refractive index of BSA photoresists increases with increasing BSA concentration. The swelling percentage of BSA microstructures also raises from $(31 \pm 2)\%$ to $(48 \pm 2)\%$. It is an indication that more BSA molecules are polymerized and crosslinked within the laser focal volume. After the swelling percentage reaches its maximum at 70 % when BSA photoresist is almost dried out at 240 min, the swelling percentage drops to 34 % at the end of fabrication. The BSA photoresist becomes highly concentrated with BSA, rose bengal and salt when drying, causing the photoresist turn into a solid film around 240 min. Although microstructures fabricated after 240 min have good structural integrity, their response to pH stimulus regress to the swelling percentage of microstructures with poor integrity fabricated between 0 min and 80 min (Figure S6C). This phenomenon is opposed to results obtained from BSA photoresists containing 460 g/L BSA and PVP-incorporated counterparts which are two-photon lithography processable without extra evaporation step (Figure 4).

Besides manually measuring dynamic viscosity and refractive index of the BSA photoresist at different time interval, a sub-system of Nanoscribe called “Definite Focus” from Carl Zeiss automatically detects the change in refractive index between glass substrate and BSA photoresist at a specific position and generate a value termed “interfacial strength” before the focused laser beam starts writing a new circular microstructure at a specific position. Interfacial strengths measured at 160 locations throughout the fabrication constantly change with fabrication time. The displayed interfacial strength at time 0 s of first position has an initial value of 1766 @ 50 and a final value of 429 @ 1 at the 160th position before the last microstructure was written. On the other hand, for photoresist containing 460 g/L BSA and 10 g/L PVP, no significant deviation is observed for interfacial strengths measured at 160 locations throughout the fabrication. The displayed interfacial strength at time 0 s of first

position is 1423 @ 33 and 1593 @ 29 at the 160th position before the last microstructure is written (Figure 4).

These promising results emphasize the feasibility of monitoring dynamic viscosity and refractive index of the liquid photoresists for reproducibility evaluation if fabricated microstructures or patterns have consistent material properties and uniform responsiveness.

Figure S7. Free-standing microstructures with reproducible consistent responsiveness and structural integrity. (A) SEM micrograph (50° tilted view) of an enlarged representative BSA cross-shaped microstructure among 80 free-standing cross-shaped BSA microstructures obtained after de-swelling in pH 5 solution and freeze-drying (Figure 4). (B) Average area and swelling property of individual free-standing cross-shaped microstructures of the array. BSA photoresist contains 460 g/L BSA, 10 g/L PVP and 8.5 mM rose bengal. Microstructures are written with laser power of 12 mW at 30 μm/s scan speed.

Figure S8. Mechanically unstable micropillars. BSA micropillars fabricated from photoresist containing 460 g/L BSA and 10 g/L PVP with aspect ratio of **(A)** 14.2 (height (h_p) = 20 μm) and **(B)** 17.0 (height (h_p) = 24 μm). (i) Full-width half-maximum (FWHM) intensity profiles of x-z Raman images at pH 5 and (ii) SEM micrographs of the micropillars (40° tilted view).

Figure S9. Fabrication of high aspect ratio micropillars. (A) Actual height of micropillars of various aspect ratios is determined via measuring the full-width half-maximum (FWHM) intensity profiles of their x-z Raman images at pH 5 (Figure S9Bi – Ci). (B - C) (i) Two-dimensional (x-z) and (ii) three-dimensional Raman images of an array of micropillars (height (h_p) = 16 μm ; aspect ratio = 11.3) made of BSA sample solutions containing (B) 460 g/L BSA and (C) 460 g/L BSA with 10 g/L PVP to illustrate integrity of the micropillars in pH 5 solution.

To explore the feasibility of fabricating high-aspect-ratio protein micropillars using two-photon lithography, we fabricate micropillars with aspect ratio up to 11.3 from BSA photoresists containing either 460 g/L BSA or 460 g/L BSA and 10 g/L PVP. The measured actual heights of these micropillars fabricated from both of the photoresists display similar trend where height of micropillars measured in pH 5 solution constantly increases with as-designed aspect ratio and starts tapering off at aspect ratio of 11.3 ($h_p = 16 \mu\text{m}$) (Figure S9A). The micropillars are not stiff enough to withstand weight of structures as pillar height is further increased to 20 μm and 24 μm (Figure S8).

Deviation in measured FWHM heights and theoretical as-designed height of micropillars might be attributed to vibrational movement of the motorized stage in z-direction resulting in poor axial sectioning resolution of the Raman imaging along x-z plane in our photoresist. As such, the system might not be sensitive enough to detect minute changes in the height of micropillars. Nevertheless, Raman imaging and FWHM measurements verify

high structural integrity of the as-fabricated micropillars which survived from soaking and rinsing in PBS buffered solution for two days during development and Raman imaging.

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