Two-photon lithography for reconfigurable microstructures

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**NANYANG
TECHNOLOGICAL
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**TWO-PHOTON LITHOGRAPHY
FOR
RECONFIGURABLE MICROSTRUCTURES**

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School of Physical and Mathematical Science**

2018

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Abstract

Two-photon lithography is a promising three-dimensional (3D) laser writing technique to construct miniature structures at the sub-micrometer to micrometer length scale. Moreover, two-photon lithography can achieve structures with spatial resolution down to 120 nm, and this lithographic technique is highly flexible in constructing arbitrary shapes and architectures. However, two-photon lithography is limited by the typical use of epoxy-based and acrylate-based photoresists, and hence the developed structures are typically stationary microstructures. There is currently no stable aqueous-based stimuli-responsive hydrogel photoresists for the construction of shape-shifting microstructures. The objectives of my thesis aim to render acrylate microstructures a new function as plasmonic anti-counterfeiting substrates with two-tier of security features (Chapter 2), and to formulate aqueous-based stimuli-responsive hydrogel photoresists for constructing shape-shifting biomimetic microstructures (Chapter 3 to Chapter 5). In Chapter 3, we create a formulation for a protein-based photoresist, which allows stable and consistent fabrication during two-photon laser writing process. This is a major breakthrough in generating aqueous-based polymer photoresists for construction three-dimensional (3D) of suspending and high-aspect-ratio ($\text{HAR} \geq 10$) hydrogel microstructures. By using the established protein photoresist, we also accomplish the first Kagome arrays of pH-responsive dynamically shape-shifting microstructures (Chapter 4). The shape-shifting microstructures undergo reversible shape change from polygons to circles against external pH, hence grant reconfigurability to the Kagome arrays. In Chapter 5, two-photon polymerizable aqueous-based N-isopropylacrylamide (NIPAAm) photoresist is formulated. Effects of processing parameters are systematically investigated for the construction of structurally stable temperature-responsive microstructures. Lastly, in Chapter 6, I outline my four-year PhD

research project and present an outlook on significant progress on future design of multi-functional “smart” microstructures.

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Chapter 1

Introduction

1.1 Abstract

The demand for crafting three-dimensional (3D) stimuli-responsive hydrogel microstructures by laser technology has fostered the development of 3D microfabrication. Among various lithography techniques, femtosecond laser assisted two-photon polymerization receives tremendous compliments. This technology involves user-friendly and flexible fabrication procedures, as well as possesses capability on generating microstructures of arbitrary shapes and architectures with sub-micrometer resolution. Currently reported photoresists and strategies used to generate reconfigurable micrometer-scale features via two-photon lithography are merely satisfactory due to limitations in available processable photoresists. We need to strive for photoresists of stimuli-responsive hydrogels and innovative fabrication strategies to empower two-photon lithography as a sustainable microfabrication technique. In this chapter, I will briefly introduce the principles and mechanism of photopolymerization via two-photon lithography, followed by a brief review on applications of two-photon polymerization. At the end of this chapter, I will identify two challenges encountered by current two-photon polymerization on generating functional and/or biomimetic microstructures, and subsequently describe the objectives and scope of my dissertation.

1.2 Photopolymerization

Polymerization is a process which converts small unsaturated molecules (monomers) in liquid state to macromolecular chains or solid-state polymer networks. When light is used to trigger polymerization process of monomers, it is known as photopolymerization.^[1]

^{2]} The wavelength of irradiation used for photopolymerization ranges from ultraviolet to infrared. Since the fabrications of photopolymers in 1960s, photopolymerization processes have been popularly adopted in the industries. As compared with many other conventional approaches, photopolymerization is an economically feasible and versatile process. Photosensitive materials can be cured on demand to achieve polymers with tunable properties by spatially controlling location of the irradiation and by temporally manoeuvring the irradiation intensity.^[3, 4]

Photopolymerization is normally initiated by light-sensitive compounds (known as photoinitiators) which generate active moieties (free radicals or cations) upon irradiating by light (Figure 1.1). In photo-induced free radical polymerization, the photoinitiator undergoes dissociation to form either primary radicals or secondary radicals by reacting with a second species via hydrogen abstraction.^[5-7] Cleavage of chemical bonds such as C–C, C=O, C–S, and C–Cl occurs during photoinitiator dissociation.^[4] The photoinitiators used can be classified into Norrish type I and type II. Type I photoinitiators can be dissociated into radicals upon absorption of light. On the other hand, type II photoinitiators require assistance from a co-initiator such as an amine or an alcohol. For Norrish type II system, the co-initiator becomes active radical after donating a hydrogen atom to the photoinitiator. Hydrogen-abstraction-governed radical photopolymerization involves aromatic ketones undergoing hydrogen abstraction from a proton donor molecule to generate a donor radical and a ketyl radical upon irradiation. It is followed by

polymerization triggered by the H-donor radical, while the ketyl radical couples to the growing polymer chain.^[8, 9]

Figure 1.1. General mechanism of photo-induced free radical polymerization. Adapted from Ref. 4 with permission from The Royal Society of Chemistry.^[4]

1.3 Two-photon Absorption

In 1931, Maria Göppert-Mayer theoretically explained the phenomenon of two-photon absorption.^[10, 11] After about three decades with the emergence of lasers, the two-photon phenomenon was then experimentally verified by Kaiser and Garrett in 1961 when two-photon-excited fluorescence was measured in a europium-doped crystal.^[12, 13] Kawata's group are the pioneer in utilizing two-photon absorption for microfabrication since 1997.^[14]

Two-photon absorption has several properties which differ from conventional single-photon absorption. Firstly, in single-photon absorption, a light-sensitive molecule only absorbs one photon as it undergoes electronic transition from its ground state to excited state (Figure 1.2). In contrast, two-photon absorption is a process where a molecule is excited from its ground state to higher-energy excited state via simultaneous absorption of two photons which generally have the same wavelength in the infrared to near-infrared range (Figure 1.2).^[15-17] During the simultaneous two-photon absorption process, one photon is absorbed to a virtual intermediate state, followed by a second photon only when the first absorbed photon is still residing at the virtual intermediate state (lifetime is ~ 5 fs).^[18] Consequently, successful two-photon absorption processes require high-intensity ultrafast lasers for excitation.

Secondly, two-photon absorption is defined as a third-order nonlinear optical phenomenon, while single-photon absorption is a linear event. For single-photon absorption, the linear polarization of an optical material under illumination can be expressed as $P = \epsilon_0 \chi E$, while for two-photon absorption it is represented by $P = \epsilon_0 \chi E + \epsilon_0 \chi^3 E^3$. In these equations, E represents magnitude of the applied electromagnetic field, ϵ_0 is the permittivity of free space, P denotes the degree of polarization, while χ and χ^3 represent the 1st and 3rd order nonlinear susceptibilities, respectively.^[19]

Figure 1.2. Schematic illustration of the one-photon and two-photon absorption processes exemplified for near-infrared (780 nm) photons.

The difference in polarization expressions shows contrast between single-photon and two-photon absorption behaviors. The Beer-Lambert law verifies that intensity of two-photon irradiation falls off much faster with respect to distance, and therefore can be focused to a small volume at the laser focus plane (Figure 1.3).^[17] The Beer-Lambert law differentiates one-photon absorption as $I(x) = I_0 e^{-\alpha c x}$, while it becomes $dI = -I^2 \beta c dx$, for two-photon absorption, where $I(x)$ is transmitted light intensity as a function of path length from focal plane x , I_0 denotes initial light intensity, α and β represent linear and nonlinear absorption coefficient and c is concentration. Thirdly, two-photon absorption can be spatially focused to pinpoint resolution (Figure 1.3). Since two photons are absorbed simultaneously in an event, the two-photon absorption probability depends quadratically on the intensity of the incident irradiation (two-photon absorption is proportional to I_0^2). The intensity of the femtosecond two-photon laser drops with the square of x , so I is proportional to x^{-2} . Therefore, the two-photon absorption probability relates inversely to x^{-4} (Figure 1.3). On the other hand, since one-photon absorption depends linearly on I , while

its light intensity decreases with the square of the distance and hence one-photon absorption is proportional to x^{-2} .^[17]

Figure 1.3. (A) Illumination with one-photon beam (532 nm) from the right of the cuvette induces fluorescence along the entire light path. By illuminating the two-photon wavelength (1057 nm) from the left, pinpoint-like fluorescence is observed only at the focal spot, as indicated by the arrow. (B) Difference in mechanisms between single-photon polymerization and two-photon polymerization. Reproduced from Ref. 20 with permission from Springer Nature. Copyright 2009.^[20]

Lastly, in one-photon absorption, intensity of irradiation at the focal plane is significantly reduced as the irradiation is screened by photosensitive molecules along its travel path. In contrast, two-photon absorption can be observed perfectly at the focus plane since intensity of the radiation is at its maximum only at the focal point, resulting in weak radiation absorption by photosensitive molecules along the light propagating path. Therefore, two-photon absorption offers better penetration of irradiation into targeted

absorbing photosensitive molecules for accurate photopolymerization at designated regions (Figure 1.4).

Figure 1.4. (Left) During one-photon absorption, absorption of irradiation is only restricted to the surface of a photosensitive material, resulting a 2D patterns through photopolymerization. (Right) Two-photon absorption renders the focused laser beam with improved material penetration. Hence, three-dimensional structures can be fabricated without interference. Adapted from Ref. 21 with permission from the Springer Nature: Springer. Nanoelectronics and Photonics: From Atoms to Materials, Devices, and Architectures by Anatoli Korkin and Federico Rosei. © Springer Science+Business Media, LLC 2008 (2008).^[21]

1.4 Lithography via Two-photon Polymerization

Direct-write two-photon polymerization, a nonlinear polymerization technique, involves the confinement of two-photon excitation processes that occur within a tightly focused near-infrared femtosecond laser to fabricate three-dimensional (3D) patterns in a photosensitive monomer/oligomer liquid (photoresist). During two-photon polymerization, a photoresist will only polymerize at location where the probability of two photons interacting with the photoresist's molecules simultaneously is the highest, namely at the laser focal point (Figure 1.5).^[15, 22] Since the two excitation photons must interact at the laser beam waist to induce polymerization, this phenomenon causes the effective laser volume to reduce to below diffraction limit as a function of the number of photons

involved, and as a function of the cross-sectional intensity.^[21] This peculiar characteristic of two-photon polymerization enables the efficient fabrication of 3D architectures with no topological constraints at resolution in sub-micrometer- to micrometer scale.

Figure 1.5. Two-photon induced chemical reactions occur only within the focal volume and pattern forms along the trajectory of the laser. The residue of the near-infrared laser radiation moves through the photoresist without causing photopolymerization. Inset illustrates a two-photon absorption process. Reprinted from Ref. 21 with permission from the Springer Nature: Springer. Nanoelectronics and Photonics: From Atoms to Materials, Devices, and Architectures by Anatoli Korkin and Federico Rosei. © Springer Science+Business Media, LLC 2008 (2008).^[21]

During two-photon polymerization, since a large portion of the laser intensity is concentrated at the laser focal point at a predetermined location within a photoresist, the rest of the laser beam just passes through the photoresist without interaction—provided the photoresist is transparent to the near-infrared laser.^[21] In a subsequent processing step unexposed regions of the photoresist remain unpolymerized (liquid-like) and can be removed by suitable solvents, while leaving behind the polymerized and solidified exposed structures on the substrate (Figure 1.6).

Figure 1.6. Two-photon induced polymerization of a photoresist at predesigned locations to create microstructures which are revealed after development. Reprinted from Ref. 23 with permission from the Royal Society of Chemistry.^[23]

In most schemes illustrating photo-induced chemical reaction pathways for two-photon polymerization, three typical steps are usually involved and are discussed in Section 1.2 (Figure 1.1). The two-photon absorption rate of a photoinitiator molecule during two-photon polymerization can be described as $W = \delta I^2$ (Equation 1.1), where δ represents two-photon absorption cross-section of a photon-absorbing molecule and I is intensity of the irradiating laser.^[15] From Equation 1.1, it is observed that the two-photon polymerization process depends quadratically on the laser intensity and is directly proportional to two-photon absorption cross-section of a photon-absorbing molecule.^[15] Since most dye-based photoinitiators have extremely low two-photon absorption cross-section—typically in the order of 10^{-52} to 10^{-58} m⁴s/photon, the near-infrared femtosecond laser pulses must have very high peak power to coincide with the photoinitiator molecules.^[15, 24]

To achieve two-photon polymerization for all photoresists, a polymerization threshold of specific laser energy dose and laser dwell time must be achieved to initiate polymerization.^[21, 25] When laser energy dose is below the polymerization threshold, no polymerization events occur. Yet, if the energy dose is too high, bubbling at the irradiating site is observed and leads to explosion in the written microstructures (Figure 1.7). Due to

the presence of polymerization threshold, precise manipulation over the applied laser energy dose can be used to control physical properties of the fabricated microstructures. Experimental data shows that lateral and axial dimensions or size of volume pixel (in short “voxel”) of a photoresist to be polymerized at the laser focal point grow with increment in laser power and laser dwell time (or exposure time) (Figure 1.8).^[25-27]

Figure 1.7. Effect of laser intensity on the diameter of laser focus point and fabricating efficiency. Reprinted from Ref. 21 with permission from the Springer Nature: Springer. Nanoelectronics and Photonics: From Atoms to Materials, Devices, and Architectures by Anatoli Korkin and Federico Rosei. © Springer Science+Business Media, LLC 2008 (2008).^[21]

Figure 1.8. (A) Size of voxel generated at laser focal spot. (Left) SEM micrographs, and (right) voxel size against laser exposure time. (B) Dependence of volume voxels on the inverse of scan speed at different laser powers. It is assumed that the voxel has an ellipsoid of rotation. Reprinted from Ref. 26 with permission from the AIP Publishing for American Institute of Physics.^[26]

Besides laser energy dose and dwell time, the structural resolution of two-photon polymerization is governed by several factors such as laser characteristics, properties of microscope objective lenses and material composition. For multi-photon lithography, spatial resolution becomes better with the increase in the order of process non-linearity. During simultaneous absorption of multiple photons, the effective wavelength of the laser beam being absorbed by the photoresist is equal to λ / N , where λ and N are the wavelength of the irradiating laser and the number of photons involved in the multiphoton absorption process, respectively. Therefore, the laser beam waist is expressed by:

$$\omega_o = \frac{\lambda}{NA\sqrt{N}\pi} \quad (1.2)$$

where NA is numerical aperture of the objective lens and the dependence of $\frac{1}{\sqrt{N}}$ is attributed to multiphoton absorption which is proportional to N^{th} order of the laser intensity (I^N). It is noteworthy that the laser beam waist is inversely related to the number of photons needed for the photochemical reactions which in turn renders higher spatial resolution.^[15] Moreover, the laser beam width decreases (or spatial resolution increases) with laser beam of shorter wavelength and objective lens of higher numerical aperture.

For a microscope objective with a round aperture, a point source forms an Airy pattern in the image plane. The intensity peak at the centre produces maximum amplitude which resembles a Gaussian distribution (Figure 1.9).^[15, 21] The oscillating amplitude then dramatically decreases away from the centre. Since there is a polymerization threshold to initiate two-photon polymerization, the insignificant side peaks do not interfere with two-photon lithography. Hence, the lateral (r) and axial (z) values at the focus point of a laser beam with diffraction-limited resolution emitting from a microscope objective can be calculated using the following equation, where n_i is refractive index of photoresist:^[28]

$$r = 0.61 \frac{\lambda}{NA} \quad \text{and} \quad z = 2 \frac{\lambda n_i}{NA^2} \quad (1.3)$$

Figure 1.9. Spatial distribution of laser beam intensity for two-photon absorption. Reprinted from Ref. 1,5 with the permission of AIP Publishing.^[15]

As shown in Figure 1.8, the actual polymerized volume of photoresist within the laser focal point is strongly influenced by both laser energy dose and laser dwell time, and the lateral (ω) and axial (h) dimensions of the polymerized photoresist volume can be deduced as:

$$\omega = \omega_0 \sqrt{\ln \frac{I}{I_{th}}} \quad \text{and} \quad h = 2z_R \sqrt{\left(\frac{I}{I_{th}}\right)^{\frac{1}{2}} - 1} \quad (1.4)$$

where I , I_{th} and z_R represent the applied intensity, intensity at polymerization threshold and Rayleigh range, respectively. Experimental findings are in agreement with the mathematical formulae (Figure 1.8). It is also observed that with increasing laser intensity the axial dimension of a polymerized photoresist becomes elongated much faster than its lateral dimension (Figure 1.8).^[26] Additionally, objective lens with higher NA grants better control on lateral and axial spatial resolution (Figure 1.10).

Figure 1.10. Dependence of voxels on numerical aperture (NA) of objective lenses. (Left) Side-view SEM micrographs of voxels formed with same laser power but with objective lenses of different NA. (Right) Exposure time-dependent lateral and longitudinal voxel sizes at different NAs. Reprinted from Ref. 29 with permission from the AIP Publishing for American Institute of Physics.^[29]

The last factor affecting two-photon polymerization is material composition. Material composition relates to feasibility of a photoresist being polymerized under irradiation and hence properties of final products. Effects of photoinitiators' concentration and optical properties, as well as concentration of monomers/oligomers on two-photon processability of a photoresist have been scrutinized.^[23, 25, 27] Optical properties of photoinitiators directly impact polymerization threshold, while their concentration determine the number of initiating radicals generated under laser irradiation and hence possibility of a polymerization event to occur.^[23, 24, 27] Role of monomer/oligomer concentration influences refractive index of a photoresist, degree of polymerization and crosslinking density of polymerized solids, as well as viscosity and fabricating efficiency of a photoresist.^[23, 30-32] Moreover, functional groups anchored on a monomer/oligomer determine behaviour of the as-fabricated polymer structures towards environmental changes.^[25, 31, 33] Consequently, robustness and versatility of two-photon lithography is hindered by optical setups and photochemistry of photoresists.

1.5 Applications

1.5.1 Polymeric Photonic Crystal

A photonic crystal is an optical structure containing periodic dielectric nano-/micrometer scale features which affect electromagnetic properties of propagating light. Photonic crystals' internal feature dimension, topology and contrast in dielectric constant are changeable to achieve on-demand optical properties.^[34, 35]

Gorb and co-workers constructed lamellar nanostructure with ridge shape similar to the Morpho butterfly via two-photon lithography (Figure 1.11).^[36] They found that the artificial angle-dependent structural colors can be observed using spectroscopy due to interference of light between “branches” of nanometer scale. The range of observation angle is from 0 ° to 60 °. It is noted that the micrometer-sized primary structures do not contribute to structural color formation. Wavelength and intensity of the scattered irradiation can be tuned by controlling density of the lamellar microstructures.

On the other hand, Bityurin and co-workers had employed two-photon lithography and electroless plating to construct a dielectric 3D spiral structures with 600 nm intralayer periodicity by using metal-binding hybrid material (Figure 1.12).^[37] The metal is silver and monomer in photoresist is 2-(dimethylamino)ethyl methacrylate. The final spiral photonic nanostructures have a smooth metallic surface and exhibit well-defined diffraction spectra, confirming promising fabrication quality and internal periodicity.

Figure 1.11. (A)(i) and (B)(i) are SEM micrographs of the as-fabricated photonic structures of different structural dimensions. The microscope images in (iii) demonstrate the color formation observed from (A)(ii) and (B)(ii). Reproduced from Ref 36 with permission from Spring Nature.^[36]

Figure 1.12. Spiral photonic crystal structures. (a) An array of dielectric spirals. (b) An array of metalized spirals. (c) Detail of a spiral with lateral resolution is in the order of 100 nm. (d) The diffraction pattern generated when the structure is illuminated with white light. Reproduced from Ref 37 with permission from Hindawi.^[37]

1.5.2 Printable Microelectronics

Svendsen *et al.* proposed a versatile approach on fabricating printable microelectronics. The microelectronic structures can be produced by first generating polymer structures, followed by deposition of metallic films (e.g. 10 nm of titanium with 100 nm of gold). Through cyclic voltametry, the composite electrodes show that they are electrochemically active. It is a good indication of the metalized surfaces, and electrical connections are suitable for electrochemical sensing.^[38] Besides this step-by-step fabrication method, Schmidt and co-workers formulated a two-photon processable pyrrole-based organic photoresist.^[39] An array of conducting polypyrrole wires were written in between silver contacts on a glass substrate. This indicates benefit of two-photon lithography on on-demand fabrication with high precision. The arrays of polypyrrole wires are proved to deliver controlled release of drug molecules. By using as-mentioned approaches to produce microelectronic devices, two-photon lithography also relieves the fabrication of microelectronic parts from stringent laboratory conditions (i.e. high vacuum, low humidity).^[38]

Figure 1.13. (A) A photograph of the sample after lift-off. (B) SEM micrograph of interconnections of (A). (C) Enlarged SEM micrograph taken at 45 ° for the fabricated structures with the interconnections after the lift-off. Reproduced from Ref. 38 with permission from Elsevier B.V..^[38]

Figure 1.14. (A) Optical microscope image of written polypyrrole wires. The white outline represents the scan area for AFM images. Scale bar = 10 mm. (B) AFM images of polypyrrole wires in dry (top) and hydrated (bottom) states. Reproduced from Ref. 39 with permission from the Royal Society of Chemistry.^[39]

1.5.3 Biological Applications

In order to understand cell growth, proliferation, and differentiation mechanisms of neural cell lines, 3D woodpile architectures made of (2-(hydroxymethyl)-2-[[[(1-oxoallyl)oxy]methyl]-1,3-propanediyl diacrylate) (also known as IP-Dip) are fabricated via two-photon lithography.^[40] It is observed that the surface of acrylate-based woodpile microstructures promotes high-density of cell colonization throughout the microstructures. It is evidenced by the presence of cell-generated, long and thick neurotic extensions on the IP-Dip woodpile structures.

Besides commercially available photoresists, biocompatible static microstructures made of proteins are also explored. Proteins are biocompatible macromolecules which can be used for determining cellular activities through constructing microstructures for cell micropatterning.^[41] Sun and co-workers reported on the construction of arbitrary 2D/3D micro/nanostructures made of aqueous diversiform-regenerated-silk-fibroin-centric inks via two-photon lithography.^[42] The silk-based microstructures exhibit strong mechanical properties such that they retain their structures even after development.^[42] To further demonstrate the highly controlled positioning accuracy and spatial resolution of two-photon lithography, Kaeher *et al.* fabricated 3D microchamber using two-photon lithography in the presence of cells.^[43] It is workable owing to excellent depth penetration ability and highly focused laser spot of two-photon irradiation. It is found that cells not only retain their environmental sensing ability, but also proliferate with the microchamber made of bovine serum albumin.^[43] It is suggested that this in-situ direct laser writing approach is useful for cellular behavioural study for multiple generations.

Figure 1.15. (A)(i) SEM micrograph of the 3D scaffold and its (ii) enlarged features. (iii) Dense cells network found on the external shell of a 3D scaffold. Reproduced from Ref 40 with permission of John Wiley and Sons.^[40] (B) SEM micrographs silk-based 3D micro/nanosculptures. Reproduced from Ref 42 with permission from Creative Commons Attribution 4.0 International License.^[42] (C)(i) Microscopy images of the isolation of a single *S. cerevisiae* cell trapped in in-situ written BSA microstructures, followed by (ii) cell growth after keeping entire structure in incubator. Reproduced from Ref 43 with permission of the American Chemical Society.^[43]

1.5.4 Microrobots

Microrobots made of various materials and shapes have been actively designed for undergoing functional tasks. These microrobots are normally fabricated via two-photon lithography since this technique allows construction of microstructures of arbitrary architectures. This is then followed by coating the written polymeric microstructures with ferromagnetic metallic films. It is foreseen that these microrobots can travel into remote regions of the body such as the peritoneal cavity,^[44] hepatic arteries,^[45] and the gastrointestinal tract^[46] to perform targeted delivery, removal of occlusions, collection of biological samples and regulation of local fluid flows for enhanced circulation.

Duan *et al.* had fabricated 3D smart micromachines through a combination of two-photon polymerization of polymer and electroless plating of nickel-phosphorous alloy to achieve high mechanical performance and remotely controllable capability (Figure 1.16).^[47] The polymer photoresist compose of dipentaerythritol hexaacrylate and poly(methacrylic acid), while benzil and 2-benzyl-2-(dimethylamino)-4'-morpholinobutyrophenone are photosensitizer and photoinitiator, respectively. The authors also investigate the effects of composition of the deposited nickel-phosphorous layer at different plating timings affecting the mechanical properties and translational movement efficiencies of the micromachine. Furthermore, the vast usage of two-photon lithography has led to the development of similar magnetic-controllable micromachines of different materials and designs.^[44, 48]

Nelson *et al.* introduced magnetic-responsive microtransporters which are fabricated with commercially available SU 8 photoresist by two-photon lithography (Figure 1.17).^[49] Subsequently, these microtransporters are selectively coated with nickel-titanium bilayers via physical vapour deposition. Microtransporter comprises of two parts: a microfluidic channel and a helical microswimmer. A pumping system is designed based on an

Archimedean screw-pump to uptake and unload targeted molecules in a dose-dependent manner. The authors suggested that the size of rotating shaft (or helical swimmer) must be compromised between rotating efficiency and mechanical properties to enhance controllable loading/release performance. These microtransporters are fabricated for the selective drug delivery of medical particles and biomaterials, hence can be applied to specific microfluidic system. With assistance of magnetic force in rotational and translational movements, these microtransporters are considered self-driven in collection, transportation and controlled release.

Figure 1.16. (a) Model of the designed micromachine. (b) Polymer micromachine after electroless plating with a thin metal layer. (c, d) Enlarged images based on (b) at different sections. (e to h) Translational motion of the micromachine which moves from left side to right side. Reproduced from Ref. 47 with permission from the American Chemistry Society.^[47]

Figure 1.17. (a) Schemes of the loading and unloading mechanism of the micromachine. (b) Loading of 3- μm polystyrene microbeads into the micromachine via remotely actuating rotation of the screw to generate current flows that goes through the capsule. (c) Summary of the sequential release process. All scale bars are 50 μm . Reproduced from Ref. 49 with permission from John Wiley and Sons.^[49]

1.5.5 Shape-shifting Microstructures

Nowadays, the word “biomimicry” fills the minds of materials engineers and architects with eager to design innovative materials based on structures and functions from our nature resources. Nature inspires us with its advanced functionalities which can be discovered from living things covered with environmental cues-responsive soft tissues. For instance, octopus is capable in morphing by changing shape, color, and texture of its body upon presence of predators.^[50] Stomach of puffer fish is well-known to be expandable up to three times its original body volume by uptaking water into its stomach when the pufferfish detects danger around it.^[51] On the other hand, Venus flytrap is a carnivorous plant that self regulates its mechanically sophisticated leaves of snap-buckling instability and it is capable in object recognition to catch and engulf insects by instant closure of its leaves.^[51, 52] Therefore, by implementing 3D structural morphing mechanisms of these

smart living creatures, we can then construct and actively control structural changes in soft elastic matters (e.g. hydrogels with stimuli-responsive moieties) to achieve on-demand novel functionalities.

Hydrogels are hydrophilic 3D polymeric networks filled with aqueous solutions.^[53] There are many approaches to form hydrogels with functional groups. Photopolymerization is one of the most popular methods since it renders spatial and temporal control on gel network formation degree which is very important to achieve stimuli-responsive shape-shifting structures.^[4] A few groups have employed two-photon lithography to construct miniature hydrogel structures that are responsive to environmental cues and undergo shape-shifting, by precise control on network formation degree (it is known as crosslinking density) via manipulating laser dwell time, laser power or the crosslinked distance between neighbouring layers along light propagating direction in z-axis (it is termed z-layer distance).^[12, 31, 33, 54]

Duan *et al.* constructed ion-responsive microcantilevers using two-photon lithography. These microcantilevers consist of hydrophilic acrylamide molecules and ion-responsive 2-acrylamido-2-methylpropane sulfonic acid (Figure 1.18).^[31] To render the microcantilevers undergoing shape-shifting under the influence of sodium chloride salt (NaCl), they instilled different crosslinking density regions within an individual microcantilever through manipulating laser dwell time. Crosslinking density can be increased by extending laser dwell time since longer interaction between laser and the photoresists promotes more photochemical reactions. They observed that regions with higher crosslinking density exhibits greater swelling owing to the extension and coiling of polymer chains in response to external stimuli. Hence, asymmetric swelling in the acrylamide hydrogel induces microcantilevers to bend.

Similarly, Shear *et al.* also scrutinized the effects of laser dwell time and laser power during lithography on the swelling efficiency of microstructures made of bovine serum albumin (BSA) (Figure 1.19).^[33] BSA is a hydrophilic macromolecule that possesses pK_a at around 4.1, hence it is responsive to changes in environmental pH. Moreover, Shears and co-workers encounter similar discoveries as Duan *et al.*, except the fact that their highly crosslinked BSA regions exhibit lesser swelling towards pH changes in the environment.

Figure 1.18. Dependence of the microcantilever length on the bending behaviour (a) in water and (b) in 1 M NaCl. Reproduced from Ref. 31 with permission from the Royal Society of Chemistry.^[31]

Figure 1.19. (a) pH responsive BSA microstructures that open at pH 3 and close upon addition of 250 μM sodium sulphate. (b) The curling extent of the microstructures as a resulting from the scanning speed of the laser, where faster scanning speeds result in greater curling extent. (c) A segmented rod fabricated with different scanning speeds and the subsequent curling extent of each segment at pH 3. Scale bars are 10 μm . Reproduced with permission from Ref. 33. Copyright 2008 National Academy of Sciences.^[33]

Ling *et al.* also constructed stimuli-responsive BSA-based microstructures (such as Venus flytrap) using two-photon lithography (Figure 1.20).^[54] They discovered that by changing z-layer distances at different regions within the microstructures during fabrication, microstructures can still undergo shape-shifting via bending due to swelling mismatch within the BSA hydrogel microstructures. The authors also determined dependence of elasticity of the microstructures on z-layer distance and found that Young's modulus decreases with increase in z-layer distance, owing to decrease in crosslinking density of the BSA hydrogel.

Figure 1.20. Fabrication of a dynamic and reversible 3D trap-like structure. (A, B) The trap possesses four dual-layer structures which appear to be upright and “opened” at pH 5 under examination of (A) optical microscopy and (B) 3D Raman imaging. (C, D) Changing the pH of the solution from 5 to 11 results in the inward bending of the four dual-pillar structures towards one another to “close”. All scale bars are 10 μm . Reproduced from Ref. 54 with permission from John Wiley and Sons.^[54]

1.6 Motivation and Objectives

For sustainable development, developing innovative ideas and exploring new solutions are necessary for existing technologies to generate high quality miniature structures. The nature of two-photon absorption grants its lithography technique versatility in fabricating architectures with unconstrained topology.^[12, 16, 22, 55] Innovative three-dimensional (3D) functional microstructures, constructed via two-photon lithography have also gained immense positive reviews as the next-generation miniature tools, regardless if they are either stationary or stimuli-responsive.^[16, 22, 55]

Despite its capability to construct well-defined microstructures, two-photon lithography suffers from the limited choice of processable photoresists. Most of the stationary functional microstructures are made of commercially available acrylate-, epoxy- or ceramic-based photoresists, with future applications foreseen. Moreover, reported stimuli-responsive microstructures are made from physically unstable liquid-like hydrogel photoresists.^[30, 31] As a result, stimuli-responsive microstructures fabricated via two-photon lithography are in primitively modest shapes and undergo simple bending.^[31, 56] In contrast, stimuli-responsive structures made from other lithographic techniques—but structure sizes range from millimetres to centimetres—are capable to undergo sophisticated shape change towards stimuli.^[57-62]

The objectives of my thesis are (1) to construct plasmonic anti-counterfeiting substrates with two-tier of security features out of conventional acrylate microstructures to instill new idea to the applications of acrylate-based materials (Chapter 2); and (2) to systematically develop stable aqueous-based stimuli-responsive hydrogel photoresists for achieving shape-shifting biomimetic microstructures (Chapter 3 to Chapter 5). We adopt five reported rules on formulating stable aqueous-based hydrogel photoresists containing

stimuli-responsive moieties. The rules suggest that a precursor photoresist (1) must be two-photon polymerizable, (2) have to be transparent in the near-infrared wavelength to prevent one-photon absorption and generation of excessive heat, (3) can be cured rapidly within the confined laser focal spot, (4) can produce mechanically stable structures with resistance against development, and (5) has optimum viscosity to avoid structural deformation during lithography.^[55]

By employing above-mentioned rules, in Chapter 3, we are able to formulate a promising aqueous-based protein photoresist that is stable for large-area two-photon lithography. Furthermore, by fine-tuning the viscosity of aqueous-based polymer photoresists, 3D suspending and high-aspect-ratio ($\text{HAR} \geq 10$) hydrogel microstructures are constructed. Notably, we also accomplish shape-shifting microstructures by demonstrating reversible shape changes from polygons to circles in different pH environment. These geometrically shape-shifting microstructures exhibit as the building blocks for a dynamic transformative Kagome array towards change in environmental pH (Chapter 4). In Chapter 5, with former built-up knowledge on preparing two-photon polymerizable aqueous-based protein photoresist, we proceed to formulate two-photon processable aqueous-based N-isopropylacrylamide (NIPAAm) photoresist. The effects of processing parameters for the construction of structurally stable temperature-responsive microstructures are thoroughly surveyed. Lastly, in Chapter 6, a summary of my PhD research project and an outlook discussion are provided to further illustrate on the potential fabrication approaches to realize bio-inspired microstructures with agile environmental adaptation.

1.7 References

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Chapter 2

Aluminum Nanostructures with Strong Visible-range SERS Activity for Versatile Micropatterning of Molecular Security Labels

2.1 Abstract

Regarded as one of the promising substituent for noble metals, the application of aluminum (Al)-based nanostructures for visible-range plasmonics, especially for surface-enhanced Raman scattering (SERS), currently suffers from inconsistent local electromagnetic field distributions and/or inhomogeneous distribution of probe molecules. Herein, we successfully fabricate structurally uniform visible-range SERS-active Al nanostructures via two-photon lithography and thermal evaporation, enabling homogeneous adsorption of various probe molecules. Individual Al nanostructures exhibit strong local electromagnetic field enhancements, in turn leading to intense SERS activity. The average SERS enhancement factor (EF) for individual nanostructures exceeds 10^4 for non-resonant probe molecules in the visible. Approaches induce homogeneous adsorption of probe molecules on the Al nano-structures are straightforward and feasible through sublimation of probe molecules or immersion of Al nanostructures in solution. These Al nanostructures also retain more than 70 % of their original SERS intensities after one-month storage, displaying superb stability under ambient conditions. Moreover, the Al nanostructures possess angular orientation-dependent SERS response which further allows us to generate micron-sized readable hybrid quick-response (QR) code with two-tier of

security arrangement. The polarization-dependent SERS response of the Al nanostructures, molecular SERS fingerprints and structural design flexibility offered by two-photon lithography grant Al-based structures the applicability in low-cost modern chemical nano-analytics and forgery protection.

2.2 Introduction

Aluminum (Al) emerges as a promising substitute for gold (Au) and silver (Ag) in plasmonics research, primarily due to its relatively low cost and higher abundance on Earth's crust.^[1-3] More importantly, Al nanostructures can support tunable localized surface plasmon resonances (LSPRs) across the UV and visible, arising from the large negative real component and small positive imaginary component of Al's dielectric constant throughout this spectral region.^[3, 4] In addition, the SERS enhancement for molecules located at the tip of Al particles is theoretically predicted ranging from 10^3 to 10^4 in the visible region, which is yet verified experimentally.^[5] Moreover, the native ultrathin (~ 3 nm) passivating oxide layer (Al_2O_3) protects underneath Al against further corrosion, unlike bulk oxidation of Ag under ambient conditions.^[6-8] This passivating oxide layer can also interact with a wide range of chemical functional groups which facilitates surface modification.^[9-11] Consequently, Al nanostructures have given rise to applications in plasmonic colors,^[12-14] photocatalysis,^[15, 16] surface-enhanced Raman scattering (SERS)^[11, 17-19] and metal-enhanced fluorescence.^[20]

However, despite these numerous advantages, there are still various unresolved challenges in developing Al-based nanostructures as feasible platforms for practical SERS applications in the visible range. Currently, reported aggregates of Al nanocrystals for detecting traces of DNAs in the visible range have inferior homogeneity in SERS signals due to inconsistent electromagnetic field distribution on the aggregates with irregular shape and size.^[11] SERS-based Al substrates composed of aluminum/anodized aluminum oxide (AAO) films,^[21] annealed Al films,^[18] as well as shape- and size-controlled Al-based nanostructures (e.g. nanodisks, nanoholes, nanospheres and nanorods) suffer from non-

uniform SERS signals caused by inhomogeneous distribution of probe molecules.^[22-25] Moreover, the conventional positioning of Al-based nanostructures on substrates are stereotyped to random,^[4, 7, 11, 17, 18, 21, 26-28] square^[15, 19, 29-34] or hexagonal^[35, 36] orientations. Consequently, the fabrication of complex sub-micrometer periodic Al nanostructures over large areas for SERS applications has not been explored to date. Thus their potential applications in fingerprint anti-counterfeiting labels or microchannels for integrated sensors are restricted.

Here, we demonstrate the on-demand micropatterning of Al nanostructure arrays to generate homogeneous SERS signals in the visible range and highlight the capability of our platform as promising optical security labels. Our strategy utilizes two-photon lithography and thermal evaporation of Al to modulate the morphology of Al nanostructures as well as to organize them into reproducible pre-designed graphic patterns. Comparison between theoretical simulations and experimental scattering spectra verifies the existence of surface plasmon modes at the Al nanostructures in the visible range, which can be achieved by controlling diameters of the Al nanostructures in the range of 160 to 220 nm. The presence of an ultrathin native oxide passivating layer on our Al nanostructures enables the homogeneous adsorption various chemical functionalities to serve as visible-range SERS probe molecules. The average SERS enhancement factors (EF) for our non-resonant probe molecules on single Al nanodots exceed 10^4 . The passivating oxide layer also aids in retaining more than 70 % SERS intensity after one-month storage. We further demonstrate the polarization-dependent SERS response of our array using elongated Al nanostructures. To develop a proof-of-concept Al-based security label, we design an array which contains two-tier encoded information. The first tier is a micron-sized machine-readable SERS quick-response (QR) code, and the second tier is a set of

ciphertexts. Our work establishes the potential of AI-based platforms as visible-range security labels.

2.3 Results and Discussion

We employ two-photon lithography in conjunction with thermal evaporation to fabricate arrays of structurally tailored SERS-active Al nanostructures. A femtosecond pulsed laser raster-scans through an acrylate-based photoresist (IP-L 780) to generate uniform polymeric nanodots via photo-induced polymerization (Figure 2.1A). These polymeric nanostructures are subsequently coated with (115 ± 8) -nm thick Al films via thermal evaporation (Figure 2.1A(ii) and Figure 2.1B). The fabricated Al nanodots are highly uniform in dimensions with diameters of (196 ± 20) nm and average heights of (300 ± 25) nm (Figure 2.1C).

Figure 2.1. (A) Fabricating Al nanostructure using (i) two-photon lithography, followed by (ii) Al thermal evaporation. (B) (i) TEM micrographs of Al layers deposited on silver nanocubes (edge-to-edge length = (120 ± 10) nm). The TEM micrograph displays depositing morphology of the Al layers on nanostructures with similar width as the silver nanocubes, facilitating model design for FDTD simulations on the Al-deposited IP-L 780 polymeric nanostructures. (ii) SEM cross-sectional view of Al layer coated on a silicon wafer. Average thickness of the Al layer is (115 ± 8) nm. (iii) Model of Al-coated IP-L 780 nanostructure used for FDTD simulations. (C) (i) Tilted SEM micrographs of Al nanodots array and their close-up views (inset). (ii) AFM height profile of the Al nanodots.

Next, we use angle-resolved X-ray photoelectron spectroscopy (XPS) to quantify the thickness of aluminum oxide (Al_2O_3) on our Al nanostructures. Our Al nanostructures are typically exposed to ambient conditions (25 °C, 65% relative humidity) for 24 hours. Angle-resolved XPS measurements show the formation of a very thin oxide layer at the surface of the Al nanostructures (Figure 2.2A). Decreasing tilt angles increases the surface sensitivity of the XPS measurements, in turn giving rise to higher oxide content (Figure 2.2A).

On the other hand, thickness of aluminum oxide layer is calculated using Equation 2.1:^[37]

$$d_{\text{Oxide}} = \lambda_{\text{Oxide}} \sin \theta \ln \left(\frac{\rho_{\text{Al}} \lambda_{\text{Al}} A_{\text{Oxide}}}{\rho_{\text{Oxide}} \lambda_{\text{Oxide}} A_{\text{Al}}} + 1 \right) \quad (2.1)$$

ρ_{Al}	= Volume density of aluminum = 0.1 mol/cm ³
ρ_{Oxide}	= Volume density of aluminum oxide = 0.0777 mol/cm ³
λ_{Al}	= Inelastic mean free path of electrons in aluminum = 2.8 nm
λ_{Oxide}	= Inelastic mean free path of electrons in aluminum oxide = 2.6 nm
A_{Al} and A_{Oxide}	= Area under the curve of fit curves to the experimental results
θ	= Tilt angle of the substrate with respect to the XPS detector. This angle corresponds to the take-off angle of photoelectrons from the substrate's surface.

From the results obtained from the angle-resolved XPS, we estimate the thickness of the native oxide layer to be ~ 3 nm (Figure 2 for XPS spectra and calculations from Equation 2.1) which is in good agreement with reported thicknesses of native oxide layers on Al surfaces.^[37-40]

Figure 2.2. Angle-resolved XPS measurements to determine Al_2O_3 thickness on Al-coated substrates (A) 1 day after Al deposition, (B) 1 day after Al deposition and 4 hour after immersion in triethoxyphenylsilane (TEPS) ethanolic solution, and (C) 1 month after Al deposition and 4 hour after immersion in TEPS ethanolic solution. (i) Dependence of XPS spectra on tilt angle for 100-nm thin Al film which was evaporated under the same conditions as in fabrication of Al nanostructures. (Inset) Schematic depiction on tilt angle, defined as the angle between the plane of the sample surface and the entrance to the analyzer. The low energy (~ 72.9 eV) and high energy (~ 74.4 eV) shoulders correspond to the Al 2p line from Al and Al_2O_3 , respectively. (ii) Calculated elemental distribution of Al and Al_2O_3 as a function of tilt angle based on angle dependence of ratio between the area under the curves of the Al metal peak and Al 2p oxide peak. (iii) Calculated thickness of Al_2O_3 layer against tilt angles.

In addition, darkfield optical characterization of the Al nanodots show that these nanodots appear yellowish-green, suggesting that the plasmon bands of these Al nanodots are in the visible region (Figure 2.3A). Indeed, the darkfield scattering spectrum of the individual Al nanodots exhibit two plasmon resonance bands at 450 nm and 583 nm, respectively (Figure 2.3B, blue line). The dominant scattering band at 583 nm corresponds

well with the observed colors of these Al nanodots. We further utilize finite-difference time-domain (FDTD) simulations to gain insights on the optical properties of the Al nanodots. The simulated spectrum corroborates well with the experimentally measured spectrum (Figure 2.3B, pink line). However, we note a slight peak shift in the higher energy scattering resonance. The experimentally measured resonance centers at 450 nm while the simulated resonance is centered at 398 nm. We attribute this difference to the reduced quantum yield, and hence photon collection efficiency, of our CCD camera in this spectral region. The quantum yield of our CCD camera drops significantly from 52 % to 32 % when the scattered wavelength decreases from 450 nm to 398 nm.^[42] With a lower photon collection efficiency and hence lower sensitivity for measurements below 450 nm, we observe an apparent cut-off peak at around 450 nm.

Figure 2.3. (A) Darkfield image of Al nanodots. (B) Simulated (pink curve) and experimental (blue curve) scattering spectra of individual Al nanodots. Local electric field distribution of an Al nanodot at (C) 398 nm, (D) 583 nm, and (E) 532 nm.

We also perform electric field calculations to elucidate the nature of plasmon resonance modes on the Al nanodots (Figure 2.3C and 2.3D). The high-energy resonance at 398 nm generates electric field enhancement at the top and bottom of the Al nanodots, and we ascribe this resonance to an electric quadrupolar plasmon mode (Figure 2.3C). The low-energy resonance at 583 nm produces electric field enhancement only at the bottom of the Al nanostructure, and can be indexed to an electric dipolar resonance (Figure 2.3D). In addition, we observe a similar field distribution pattern at our laser excitation wavelength of 532 nm as the field distribution at 583 nm (Figure 2.3E). Such similarity indicates that the dipolar resonance is excited in our subsequent SERS experiments, providing the basis for field enhancements for these measurements. At these three wavelengths, plasmonic hotspots and surface electric field enhancements are observed at the localized interface between the Al coating, the polymeric structures, and the Al₂O₃ shells (Figure 2.3C – 2.3E). These enhancements correspond to charge concentrations induced by the polarization of the incident field.^[42] Consequently, our results suggest that Al nanostructures are practical and promising for the design and implementation of visible frequency plasmonics.

Prior to conducting SERS measurements, we demonstrate that the native oxide layer on our Al nanostructures also provides a versatile platform to introduce probe molecules with various chemical functionalities onto the Al surfaces. As a proof-of-concept, we graft naphthalene (NAP) and triethoxyphenylsilane (TEPS) onto the Al nanostructures (Figure 2.4A and 2.4B). We demonstrate functionalization of Al surfaces can be achieved by physisorption (for NAP) and chemisorption (for TEPS). We physically adsorb NAP onto the Al nanostructures via sublimation under vacuum of 0.010 mbar (Figure 2.4A). We covalently bind TEPS to the surface of Al nanodots via a condensation reaction between the silanol groups on hydrolyzed TEPS molecules and the surface Al₂O₃ (Figure 2.4B).^[43]

The water contact angles of Al substrates adsorbed with TEPS and NAP are $(65 \pm 1)^\circ$ and $(97 \pm 3)^\circ$, respectively (Figure 2.5B and 2.5C).

Figure 2.4. Schematic illustrations of grafting (A) naphthalene (NAP) and (B) triethoxyphenylsilane (TEPS) molecules to Al-coated surfaces.

As a comparison, the contact angle of the bare oxide layer is $(50 \pm 2)^\circ$ (Figure 2.5A). The increase in contact angle post-surface treatment arises from the introduction of hydrophobic benzene rings onto the Al nanostructures, thus reflecting successful adsorption of NAP and TEPS onto the Al substrates (Figure 2.5). NAP molecules are organized such that the plane of the benzene rings is parallel to the Al_2O_3 surfaces (Figure 2.4A),^[44, 45] while TEPS molecules remain nearly upright as their silane groups bind with the Al_2O_3 surfaces (Figure 2.4B).^[43, 46] The presence of residual ethoxy silane groups renders our TEPS-coated Al substrate slightly more hydrophilic than the NAP-functionalized one.^[46] Such versatility in functionalizing Al nanodots implies that we can expand beyond the typical amine- or thiol-terminated molecules which are used in Au- or Ag-based systems, which potentially increases our selection of probe molecules for subsequent anti-counterfeiting applications.

Figure 2.5. Water contact angle measurements for (A) Al-coated glass substrate, (B) TEPS- and (C) NAP-adsorbed Al-coated substrate.

We then proceed to investigate the SERS capabilities of our Al nanostructures in the visible range, using NAP and TEPS as the non-resonant Raman probes at the laser excitation wavelength of 532 nm. It is confirmed that there is negligible SERS background from the substrate (Figure 2.6), indicating the obtained SERS signals are coming from the probe molecules.

The SERS spectra of NAP and TEPS show typical fingerprints at 1090 cm^{-1} , 1330 cm^{-1} and 1590 cm^{-1} (NAP) / 1700 cm^{-1} (TEPS) (Figure 2.7). We use density functional theory (DFT) simulations to elucidate types of vibration for adsorbed NAP and TEPS molecules on Al surfaces (Figure 2.7 and Figure 2.8). The vibrations near 1090 cm^{-1} arises from CCC symmetric phenyl ring stretching, while the band at 1330 cm^{-1} represents the in-plane phenyl ring asymmetric stretching (Figure 2.8).^[47] The narrow band of NAP centered at 1590 cm^{-1} represents the in-plane phenyl ring asymmetric stretching mode (Figure 2.8A), while the broad fingerprint of TEPS at 1700 cm^{-1} comprises multiple in-plane and out-of-plane phenyl-ring stretching modes (Figure 2.8B).

Figure 2.6. Comparison of SERS intensity of (A) NAP adsorbed Al nanodots, (B) NAP adsorbed flat Al-coated glass substrate, (C) Al nanodots without probe molecules, (D) IP-L 780 polymeric nanodots and (E) blank cover slip only. As observed, there is no interference from background signals on SERS intensity of NAP adsorbed Al nanodots.

Figure 2.7. (A) SERS spectrum of NAP molecules adsorbed on Al-coated IP-L 780 nanodots (orange), normal Raman spectrum of 1 M NAP in ethyl acetate (blue), DFT simulated graph for NAP (pink) and normal Raman spectrum of ethyl acetate (violet). (B) SERS spectrum of TEPS molecules adsorbed on Al-coated IP-L 780 nanodots (orange), normal Raman spectrum of 1 M TEPS in ethyl acetate (blue), DFT simulated graph for TEPS (pink) and normal Raman spectrum of ethanol (violet). Intensity at $\sim 1330\text{ cm}^{-1}$ Raman band from both molecules is used for calculating SERS EF.

Figure 2.8. Schematic representatives for DFT-simulated vibrational modes of the (A) NAP and (B) molecules adsorbed on Al surfaces at Raman shift (i) 1090 cm^{-1} , (ii) 1330 cm^{-1} and (iii) 1590 cm^{-1} for NAP or 1700 cm^{-1} for TEPS.

We observe that the experimental Raman peaks of NAP at 1090 cm^{-1} , 1330 cm^{-1} and 1590 cm^{-1} are respectively shifted to 1055 cm^{-1} , 1411 cm^{-1} and 1624 cm^{-1} in the simulated results (Figure 2.7A). On the other hand, Raman peaks of TEPS at 1090 cm^{-1} , 1330 cm^{-1} and 1700 cm^{-1} are shifted to 1050 cm^{-1} , 1310 cm^{-1} and 1642 cm^{-1} respectively in the simulated results (Figure 2.7B). The main reason for the blue/red shift in the Raman peaks is the difference in structure between the compact passivating Al_2O_3 layer and the simulated Al_2O_3 -molecules model. Moreover, the DFT simulation with single molecule neglects the neighbouring effects of close-packed molecules grafted on surface which deviate the experimental SERS bands.^[48, 49] Nevertheless, the presence of aforementioned Raman characteristic peaks provides strong evidence on the adsorption of NAP and TEPS molecules onto Al surfaces.

Further, analyses of the vibrational mode at 1330 cm^{-1} over 50 Al nanodots show strong SERS signals of (7656 ± 467) counts and (7163 ± 319) counts for NAP and TEPS respectively (Figure 2.7 and Figure 2.9). Vibrational mode at 1330 cm^{-1} is chosen since there is no overlap with any other potential interference. Moreover, the measured SERS intensities remain highly consistent with a low relative standard deviation (RSD) of $< 10\%$. Consistent SERS intensity with low RSD also indicates homogeneous adsorption of the Raman probe molecules on the Al nanodots (Figure 2.9C – 2.9F). Notably, our Al nanodots remain stable and provide consistent SERS performance after keeping under nitrogen environment for a month. The thickness of native oxide layer remains at $\sim 3\text{ nm}$ on the as-fabricated Al nanodots (Figure 2.2C). More importantly, the SERS intensity of the 1330 cm^{-1} mode for NAP-adsorbed Al nanodots decreases by less than 30% after 1-month storage in a desiccator (Figure 2.9B). Such stability implies that the native oxide layer is sufficiently dense to prevent oxygen diffusion towards the Al core, in turn dramatically slowing down the corrosion of the Al nanodots and preserving the SERS-activity of our platform.^[35, 50] Our work here is the first report to showcase practical performance of individual Al-based nanostructures in the visible range.

We further estimate the average SERS enhancement factors (EF) of the two probe molecules on the Al nanodots based on the 1330 cm^{-1} mode. SERS EF are calculated using the formula $EF = (I_{SERS} / N_{SERS}) / (I_{Normal} / N_{Normal})$ (Equation 2.2), where I_{SERS} is the intensity of the 1330 cm^{-1} Raman band from NAP adsorbed on Al-coated IP-L 780 microstructures, I_{Normal} is the intensity of the 1330 cm^{-1} Raman band from 1 M NAP – ethyl acetate solution, while N_{SERS} and N_{Normal} represent the number of NAP molecules contributing to I_{SERS} and I_{Normal} , respectively. Both SERS and normal Raman measurements are conducted using point mode with laser power of 40 mW and exposure time of 20 s. To determine volume of laser spot, the lateral spot size of the focus beam is measured from the displayed laser spot

that gives the highest sample signal. The axial size of the laser is determined by adjusting the stage position along the z-axis (optical path) to get the z-distance from maximum signal intensity to zero intensity. The lateral and axial spot sizes are used to get optical resolutions of the 532 nm laser, which are: $x = (914 \pm 110)$ nm; $y = (850 \pm 55)$ nm; and $z = (4880 \pm 247)$ nm (Figure 2.10A). Volume of the laser spot is approximately equal to 2.978×10^{-12} cm³.

Figure 2.9. (A) SERS spectra of NAP and TEPS adsorbed on Al nanodots. (B) Stability of NAP intensity over 1 month. 2D SERS images of Al nanodots functionalized with (C) NAP and (D) TEPS generated using the 1330 cm⁻¹ fingerprint. Consistent SERS signals measured across a row of (E) NAP- and (F) TEPS-coated Al nanodots (yellow lines in (C) and (D)).

To facilitate the EF calculation, we use DFT to estimate molecular occupying area of the probe molecules on Al surfaces. The simulated distance between H atoms for phenyl rings and O atoms on the probe molecules are listed in Figure 2.10B and 2.10C. From calculations, the theoretical molecular occupying area of a NAP molecule on Al is 0.348 nm² (Figure 2.10B(ii)). On the other hand, the estimated molecular area of a TEPS molecule based on O atoms on silane is 0.062 nm² (Figure 2.10C(iii)) and the area becomes 0.181 nm² when phenyl group (Figure 2.10C(iv)) is considered. Our calculated molecular areas of NAP and TEPS molecule are much smaller than the reported molecular areas of NAP and benzene, which are 0.65 nm², and 0.30 to 0.43 nm², respectively.^[51-53] Therefore, our calculated molecular areas assume the closest packing of probe molecules on the Al surfaces. The actual SERS EFs are thus underestimated. Consequently, for NAP, I_{SERS} = 7656 counts, N_{SERS} = 173402 molecules, I_{Normal} = 1134 counts, and N_{Normal} = 1.794×10^9 molecules. On the other hand, for TEPS, I_{SERS} = 7163 counts, N_{SERS} = 973290 molecules, I_{Normal} = 779.65 counts, and N_{Normal} = 1.794×10^9 molecules. By using Equation 2.2, the SERS EFs are estimated to be 7.0×10^4 and 1.7×10^4 for NAP and TEPS, respectively.

The high SERS EFs of our Al nanodots obtained in the visible range indicate SERS-based Al nanostructures with high sensitivity are not limited to deep-UV/UV laser excitation to achieve SERS EFs of 10^3 to 10^6 .^[23, 24, 31, 54] We confirm presence of SPRs on the Al nanodots at 532 nm excitation by calculating electric field enhancement ($(|E|/|E_o|)^2$) (Figure 2.3, Figure 2.11). 532-nm irradiation induces dipole resonance on the Al nanodot which generates a relatively strong $(|E|/|E_o|)^2$ of approximately 72.4 (Figure 2.11), illustrating the Al nanodot is proficient in enabling efficient scattering of SERS signals. Although recently reported near-field visible-range simulated electric enhancement at the 2-nm gap of two Al nanocrystals is around 10^5 to 10^6 ,^[12] literatures have yet reported average experimental SERS EF of a single Al nanostructure. Our results affirm

contribution of each Al-based nanostructure as an alternative high-performance non-resonant SERS sensor in the visible electromagnetic range.

Figure 2.10. (A) Optical resolutions of 532 nm laser passing through Nikon TU Plan ELWD 100x objective lens (NA 0.8) for (i) x-, (ii) y- and (iii) z-axis, respectively. (B)(i) top view of DFT-simulated structural configuration of NAP molecules, and (ii) geometry calculation on theoretical molecular occupying area of NAP molecules on Al. (C)(i) Side view and (ii) top view of simulated structural configuration of TEPS molecules, respectively. Geometry calculation on theoretical molecular occupying area of TEPS molecules on Al based on (iii) distance between O atoms and (iv) size of phenyl ring on TEPS, respectively.

Figure 2.11. Electric field intensity $(|E|/|E_0|)^2$ of an Al nanodot simulated at excitation wavelength of 532 nm.

Aside from nanodots, we demonstrate that polarization-dependent SERS responses can be achieved using Al nanowires. Our model system is a concentric ring made up of continuous circular Al nanowires which are functionalized with NAP (Figure 2.12). The width and the periodicity of the Al nanowires are intentionally set at (170 ± 20) nm and 2 μm to minimize plasmonic coupling between the rings of nanowires (Figure 2.12).^[29, 55]

Figure 2.12. SEM micrographs of concentric ring structure consisting of continuous Al nanowires at different magnifications.

The concentric-ring setup allows us to continuously measure the polarization-dependent SERS response between 0° to 360° , where the angle between the polarized laser excitation and nanowire orientation (θ) is defined with respect to the vertical y-axis (Figure 2.13A). At x-polarization, the SERS signal changes from bright to dim as θ changes from 0° to 90° (Figure 2.13B). A reverse gradient of SERS intensities is observed from the x-y SERS image at y-polarization (Figure 2.13C). With an increase in θ , the LSPR's strength that is coupled to the incident light decreases correspondingly at x-polarization, therefore reducing the SERS intensity of NAP at 1330 cm^{-1} from 0° to 90° (Figure 2.13D). By normalizing the SERS intensity at various θ against the measured I_{max} at $\theta = 0^\circ$, we obtain a gradient of discrete SERS values ranging from 1.00, 0.90, 0.69, 0.40, 0.19, 0.02 to 0.00 when the orientation of the Al nanowires is aligned at 0° , 15° , 30° , 45° , 60° , 75° , and 90° with respect to the vertical y-axis, respectively (Figure 2.13E). A plot of normalized SERS intensity against θ further demonstrates a good fit to a $\cos^2\theta$ function (Figure 2.13E). In addition, a regular “dumbbell”-shaped SERS intensity distribution can be observed in the θ -dependent SERS-intensity plot (Figure 2.13F). A continuous polarization-dependent variation in experimental SERS intensity across the concentric ring is thus achieved, with the strongest SERS intensities observed at 0° and 180° at x-polarization.

Figure 2.13. (A) SEM micrograph of Al concentric rings. (B) X- and (C) y-polarized 2D SERS images of the Al concentric rings generated using the 1330 cm^{-1} fingerprint of NAP. (D) X-polarized SERS spectra of the Al concentric circles collected at different angles ($\theta = 0 - 90^\circ$). (E) Plot of normalized SERS intensity against θ at x-polarization. (F) “Dumbbell”-shaped distribution of simulated (dotted purple line) and normalized experimental (red circles) SERS intensity against θ at x-polarization for (B).

The polarization-dependent tunability of SERS signals arises from the directional coupling of Al nanowires with incident light.^[55] The anisotropic morphology of nanowires allows these structures to support longitudinal and transverse localized surface plasmon resonances (LSPR). At x-polarization, the transverse LSPR mode of the nanowires at $\theta = 0^\circ$ couples efficiently to the incident light. This coupling results in strong electromagnetic fields along the nanowires and generates the strongest SERS intensities. Increasing θ reduces the coupling strength and thus leads to progressively weaker SERS intensity following a $\cos^2\theta$ relationship. At $\theta = 90^\circ$, momentum mismatch between the incident photon and the propagating plasmons significantly reduces the coupling between incident light and the longitudinal mode of the Al nanowires. Consequently, the weakest SERS

intensity is observed at $\theta = 90^\circ$ (Figure 2.13B and Figure 2.13D). Furthermore, the $\cos^2(\theta)$ -dependence of SERS intensity obeys the fundamental theory of SERS. SERS intensity can be described as $I_{SERS} \propto |E(\omega) / E_o(\omega)|^2 |E(\omega_s) / E_o(\omega_s)|^2$, where $E(\omega)$ and $E(\omega_s)$ are the enhanced local fields at the excitation and scattering frequencies respectively, and E_o refers to the incident field. $E(\omega)$ can be more accurately described as $E(\omega, \theta) = E(\omega) \cos(\theta)$ for x-polarization in our system.^[55] For example, the theoretical SERS intensity at $\theta = 0^\circ$ is $I_{\theta=0^\circ} = I_x \cos^2(0^\circ) = I_x$, corresponding to the maximum SERS intensity at x-polarization; at $\theta = 60^\circ$, $I_{\theta=60^\circ} = I_x \cos^2(60^\circ) = 0.25 I_x$. Compared to current works which focus on using polarization to study the fundamental optical response of Al nanostructures^[3, 56-59] or for use in color display,^[12, 13, 30, 34] our work extends the application of Al nanostructures in creating a dynamic range of gray-scale polarization-dependent SERS intensities. Such consistent polarization-dependent SERS signals suggest that Al nanostructures can be applied to multi-digit data storage and anti-counterfeiting platforms.

To design a proof-of-concept Al-based security label, we proceed to develop a steganograph comprising two tiers of covert information (Figure 2.14A). A steganograph hides a secret message within an ordinary image.^[60] The first tier of security feature is a microscale SERS quick-response code (QR code, Version 1, 21×21 modules), which conventionally is a square two-dimensional barcode with black dots strategically arranged on a white background (Figure 2.14B). As compared to linear barcodes, QR codes possess larger data storage capacity and error correction features for faster authentication in omnidirectional reading. The second tier of security feature consists of two ciphertexts “ntu” and “cbc”, which are embedded at the top and bottom position markers of the QR code, respectively (Figure 2.14C).

Figure 2.14. (A) SEM micrograph of our two-tier security label comprising a QR code overlaid with ciphertexts. (B) QR code for the official website of the Singapore government. (C) Embedded ciphertext within (B). (D) x- and (E) y-polarized 2D SERS images depicting the encrypted QR code and ciphertexts, respectively. Images are generated using the 1330 cm^{-1} fingerprint of NAP. (F) SERS spectrum of NAP extracted from the square region in (E). (G) (i) Reading the QR code in (D) using a smartphone directs the reader to the (ii) Singapore government's official website.

Our SERS QR code comprises an array of vertically-oriented Al nanorods and nanodots which can be directed to the Singapore government's website (www.gov.sg) (Figure 2.14D). The ciphertexts are fabricated using Al nanodots in conjunction with horizontally-oriented Al nanorods (Figure 2.14E). The designed steganograph can only be fully decoded using the unique polarization-dependent SERS response of our Al nanostructures. Physical characterization using scanning electron microscopy reveals a complex pattern which does not show the ciphertexts, and scanning this pattern using a QR code reader also does not load the coded website (Figure 2.14A). In contrast, the ciphertexts comprising "ntu" and "cbc" can be discerned in the y-polarized SERS Raman imaging (Figure 2.14E). We can also measure the covert SERS molecular fingerprints of adsorbed NAP directly from the Raman images which further increase the security level in authentication (Figure 2.14F). More importantly, the QR code shows up in the x-polarized SERS image generated can be immediately directed to the coded website by a QR code reader (Figure 2.14G). There is no signal cross-talk between the two polarizations, where the QR code reader is unable to detect a valid QR code in the y-polarized SERS image and the ciphertexts are invisible in the x-polarized SERS image. Our microscale steganograph is therefore an ideal candidate for providing advanced security, possessing images which are readable and trackable using commercial imaging scanners and multiple security features for protection against forgery. Even though visible-range plasmonic security labels^[55, 61-63] have been reported, the reported geometrical patterns have no source tracking function. In addition, we are also the first to overlay ciphertexts with QR codes to achieve the highly sophisticated two-tier security features. This achievement has thus far remained challenging to attain via optofluidic maskless lithography.^[64] Our approach is also more practical as compared to fluorescence based double-layered QR codes fabricated within single silver zeolite microcarrier via two-photon activation.^[65]

2.4 Conclusion

In summary, we demonstrate Al nanostructures have significant SERS activity in the visible electromagnetic range. The visible-range SERS-active Al nanostructures with high structural consistency and with homogeneous adsorption of probe molecules (NAP or TEPS) can be fabricated via two-photon lithography and thermal evaporation. Our plasmonic Al nanostructures possess distinct physical morphology and chemical Raman information with narrow spatial and spectral resolution. Average SERS enhancement factor of the Al nanodots can exceed 10^4 . After at least a month, SERS performance of the Al nanostructures remains stable. By manipulating SERS intensity through controlling orientation of the Al nanorod structures and the polarization of incident light, we successfully fabricate micron-sized QR code with two-tier security arrangement. These two enhanced security features are readable 2D QR code and encrypted alphabets. The feasibility of our approach to generate highly complex plasmonic authentication codes can contribute to the urge in continuous development of new anti-counterfeiting labels for staying ahead of the curve. Patterns made up of Al nanostructures also provide opportunities for cost-effective multiplex data storage and disposable label-free plasmonic nanosensors.

2.5 Experimental Section

2.5.1 Materials. IP-L 780 photoresist was purchased from Nanoscribe Inc, Germany. Triethoxyphenylsilane (TEPS, > 98 %), naphthalene (NAP, 99 %), propylene glycol monomethyl ether acetate (PGMEA, > 99.5 %), acetic acid (≥ 99.7 %), isopropyl alcohol and ethanol were purchased from Sigma-Aldrich. Aluminum (Al) target (99.99 %) was purchased from Zhongnuo Advanced Material (Beijing) Technology Co. Ltd, China. All chemicals were used without further purification, unless otherwise stated. Milli-Q water ($> 18.0 \text{ M}\Omega\cdot\text{cm}$) was purified with a Sartorius Arium® 611 UV ultrapure water system.

2.5.2 Fabrication of Polymeric Nanostructures. Polymeric nanostructures were fabricated using the Nanoscribe® Photonic Professional (Nanoscribe Inc., Germany). The system is equipped with an erbium-doped, femtosecond laser source with a centre wavelength of 780 nm, pulse repetition rate of 80 MHz and pulse length of 100 fs. IP-L 780 nanostructures were designed using computer-aided design (CAD) software, 3ds Max®. Dimensions of the structures were defined by the Nanoslicer and DeScribe softwares. All structures were written on square glass substrates (width = 22 mm and thickness ≈ 0.13 to 0.16 mm). The direct laser writing (DLW) processes were performed using an inverted microscope with an oil immersion lens (Zeiss Plan Apochromat, 100 $\times$, NA 1.4), and a computer controlled piezoelectric stage. The average laser power was around 10 mW and writing speed was 20 $\mu\text{m/s}$. After writing, sample substrates were developed in PGMEA, followed by isopropyl alcohol for 30 min each. The substrate was then blow-dried using nitrogen gas.

2.5.3 Generation of QR Code. To create Version 1 quick response (QR) code (21 × 21 modules) for the Singapore government's official website (www.gov.sg), we used a QR code generator software offered by QRStuff, QR Code Generator, Inc. Lines and dots of specific length and spacing were then arranged according to the QR code pattern using 3ds Max® software.

2.5.4 Metallization of The Polymeric Structures. Good-quality Al film was deposited on the substrates at a rate of 15 Å/s with a thermal evaporator system (Syskey, Taiwan) operating under high vacuum of 2.7×10^{-7} mbar.

2.5.5 Deposition of TEPS on Al-coated Nanostructures. The substrate with Al-coated nanostructures was submerged in 3 mL of ethanol for 30 minutes. TEPS solution (approx. pH 5) containing 300 µL TEPS, 50 µL acetic acid, 500 µL Milli-Q water and 7 mL ethanol was prepared and left for 5 minutes. The substrate was then added to the TEPS solution and immersed for 3 hrs at room temperature. The substrate was subsequently washed with copious of ethanol to remove excess silane compounds. Next, the substrates were dried under low vacuum at room temperature for 6 hrs to aid the removal of water and ethanol, as well as to promote the formation of siloxane bonds.^[43]

2.5.6 Deposition of NAP on Al-coated Nanostructures. 20 mg of NAP suspension (1mL water and 0.5 mL ethanol) was frozen and then deposited on the Al-coated polymeric structures via sublimation at – 82 °C and under vacuum of 0.010 mbar.

2.5.7 Surface-enhanced Raman Scattering (SERS). SERS measurements were conducted using a Ramantouch microspectrometer (Nanophoton, Osaka, Japan) and a uRaman microscopy module (uRaman-532-TEC-Ci, Technospex Pte. Ltd, Singapore). SERS spectra of NAP and TEPS adsorbed on the surface of Al nanodots were generated by using excitation wavelength of 532 nm, a 100 × objective lens (Nikon TU Plan ELWD, NA 0.8), excitation power of 40 mW and integration time of 20 s. For normal Raman evaluation of solution containing 1 M TEPS or 1 M NAP in ethyl acetate, Raman spectra were collected using the 100 × objective lens (Nikon TU Plan ELWD, NA 0.8) at laser power of 40 mW with 20 s exposure time. For polarized Raman measurements, polarizer and half-wave plate were used to adjust the laser travelling pathway emitted from the Ramantouch microspectrometer. Raman intensities and spectra were obtained by averaging at least 50 individual spectrum. Baseline subtract treatment on spectra were processed using OriginPro software.

2.5.8 Materials Characterization. To prepare samples for scanning electron microscopy (SEM) imaging, samples attached on glass substrates (with or without Al coating) were sputter-coated with platinum particles at 40 mV for 40 s using a JEOL JFC-1600 Auto Fine Coater. Coated surfaces were viewed using a JEOL 7600F SEM operating at accelerating voltage of 5.0 kV and beam current of 5 in SEI mode. AFM was used to determine actual width and height of the Al-coated polymeric structures using a Bruker Multimode 8 with NanoScope V controller from Bruker. ScanAsyst® mode (a PeakForce Tapping® based image optimization technique) image was acquired using a pyrex-nitride cantilever from Nanoworld (PNP-TR, force constant: 0.32 N/m and, resonance frequency: 67 kHz) in ambient condition. Data analysis was carried out using Bruker NanoScope Software 8.10. Static contact

angles were measured with a 4 μ L ultrapure water droplet using an Attension Theta Lite tensiometer equipped with a USB 2.0 digital camera. Average contact angle and standard deviation were calculated from five measurements at each of at least three locations across a substrate. Angle-resolved X-ray photoelectron spectroscopy (AR-XPS; SPECS Phoibos 100 spectrometer equipped with a monochromatic Mg X-ray radiation source) was employed to determine thickness of aluminum oxide layer formed on surfaces of the structures. Anode voltage and emission current used were 12.5 kV and 16.1 mA, respectively. For XPS calibration, 284.5 eV for C 1s binding energy peak was used. Presented results based on XPS measurements were average data taken from total 5 different regions of 3 substrates. 2D SERS image of the micron-sized plasmonic QR code was decoded using a QR code reader application (QR Scanner, Honestly App) on a smartphone (Xperia C3, Sony Mobile Communications Inc. or iPhone 6, Apple Inc.).

2.5.9 Dark-field Scattering Measurements. The scattering from the individual microstructures was measured on a dark field microscope (Nikon Eclipse Ti-U) that was integrated with a halogen lamp (12V, 100 W), a flat field imaging spectrograph (Andor Shamrock SR-303i-B), and a charge-coupled device camera (Andor iDus DU401A BR-DD). The camera was thermoelectrically cooled to -60 °C during measurement. A dark-field objective (Nikon LU Plan Fluor EPI P 100 $\times$, NA 0.9) was employed for exciting and measuring the scattering from the individual microstructures. Andor-Einst software was used to control the detector, spectrograph and motorized systems. The obtained scattering spectra were calibrated based on Equation 2.3. $Q(\lambda)$ represents the scattering spectrum, $I_{Mea}(\lambda)$ is the recorded spectrum of the microstructures, $I_{Bckgrd}(\lambda)$ is the scattering spectrum

collected from the blank region, $I_{Silica}(\lambda)$ is the spectrum collected from a glass substrate covered by a condensed film of silica nanoparticles, and $I_{Peak}(\lambda)$ is the peak value of the spectrum collected from the condensed silica film. The term, $I_{Mea}(\lambda) - I_{Bckgrd}(\lambda)$, eliminates the background scattering. The term, $1/(I_{Silica}(\lambda)/I_{Peak}(\lambda))$, removes nonuniformity of the source spectrum and the response of the entire optical system.

$$Q(\lambda) = \frac{I_{Mea}(\lambda) - I_{Bckgrd}(\lambda)}{I_{Silica}(\lambda)/I_{Peak}(\lambda)} \quad (2.3)$$

2.5.10 Electromagnetic Simulations. All electrodynamic simulations were performed using finite-difference time-domain (FDTD) Solutions v8.6 (Lumerical Solutions). During the simulations, an electromagnetic pulse from 300 to 1600 nm was launched into a box containing the target Al nanostructure. The Al nanostructure and its surrounding medium were divided into meshes of 0.5 nm in size. The model of the Al nanostructure was built according to the experimental parameters (Figure 2.1B(iii)). The simulated Al nanostructure consisted of a truncated cone with a hemisphere on top. The truncated cone was 37 nm in height, while its top and bottom bases had radii of 87 nm and 97 nm, respectively. Radius of the hemisphere was 87 nm. The surface of the Al nanostructure was covered with 3-nm thick Al_2O_3 layer. The Al nanostructure was set on a polymeric truncated cone (IP-L 780) with radii of 97 nm and 150 nm for its top and bottom bases, respectively. The refractive index of the polymer was fixed at 1.4.^[62] The surrounding medium was set to 1. The dielectric function of Al and Al_2O_3 was taken from previously measured values.^[66] The electric field intensity at wavelength of 398 nm, 532 nm and 583 nm was monitored at the surface of Al.

2.5.11 Density Functional Theory (DFT) Calculations. We used the Gaussian 09 computational chemistry package to determine theoretical molecular occupying area of NAP and TEPS molecules on Al. Geometric optimization of both NAP- and TEPS-adsorbed Al was carried out using the unrestricted B3LYP exchange-correlation functional. And the 6-311g (d p) basis set was used for all atoms.

2.5.12 Data Analysis. Measurements of dimensions of the as-fabricated Al-coated IP-L 780 nanostructures were conducted using ImageJ. All data for the physical characterization of Al-coated polymeric nanostructures were averaged using at least 10 measurements.

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Chapter 3

Quantitative Formulation of Protein Photoresists for Fabricating Dynamic Microstructures with High Aspect Ratios and Uniform Responsiveness

3.1 Abstract

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 that 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.

3.2 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 microactuators^[10] 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 substrates^[7] 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.

3.3 Results and Discussion

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 3.1). Fabrication parameters such as laser power and laser dwell time on the protein microstructure integrity are also investigated (Scheme 3.1).

Scheme 3.1. 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.

We first create a photoresist which is optically transparent at the laser excitation wavelength (Figure 3.1), 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 3.1).^[26, 27]

Figure 3.1. 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.

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 writing^[15] 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 3.2A). 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 3.2A).

Figure 3.2. 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.

The structural integrity of the cross-shaped BSA microstructures increases with increasing BSA concentration (Figure 3.2Ai-iii). At BSA concentration of 290 g/L, only the substrate-attached column can be formed (Figure 3.2Ai). Increasing the BSA concentration to 380 g/L produces highly distorted free-standing cross-shaped microstructures (Figure 3.2Aii). We only observe the distinct cross-shaped geometry without significant structural distortion at BSA concentrations of 460 g/L (Figure 3.2Aiii). Although dependence of protein matrix porosity on BSA concentration was reported,^[15] our experiments here further 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 3.2B). 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 3.2Aiv–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 3.3 and Figure 3.4).^[30, 34] As observed during experiments, 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 3.3 and Figure 3.4). Linear concentration dependence of absorbance on rose bengal concentration at 390 nm is also independent of BSA concentration and presence of PVP (Figures 3.3B and 3.3C). Our results are in consistent with findings which reported independence of rose bengal-sensitized photodynamic cross-linking of proteins on addition of acrylamide copolymers^[35, 36] or dye-labelled long-chain dextran.^[37]

Figure 3.3. (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 $R^2 = 0.9988$.

Figure 3.4. Optical microscopy images of microstructures taken at pH 5 to show effect of average laser power, BSA concentration and presence of PVP on photo-polymerizability of BSA photoresists using two-photon lithography. Microstructures are fabricated from different BSA photoresists over laser power ranging from 4 to 24 mW, with constant scan speed of 30 μ 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 3.2C.

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 3.2Av; Figure 3.2B). 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 3.2A, 3.2B).

By changing the BSA concentration, we are also able to tune the swelling capability of substrate-attached cylindrical microstructures (Figure 3.2C; Figure 3.4). The pH-responsiveness of the cylindrical microstructures is determined by their swelling percentage $((A_{pH11} - A_{pH5}) / A_{pH5}) \times 100 \%$ (Equation 3.1); where A_{pH5} and A_{pH11} are areas measured at pH 5 and pH 11, respectively). For microstructures fabricated from photoresists containing 290 g/L, 380 g/L, and 460 g/L of BSA, the swelling percentages are $(32 \pm 2) \%$, $(34 \pm 2) \%$, and $(36 \pm 1) \%$, respectively (Figure 3.2C, 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 $(30 \pm 1)\%$, $(29 \pm 2)\%$ and $(33 \pm 1)\%$ for microstructures fabricated from photoresists containing 290 g/L, 380 g/L and 460 g/L of BSA, respectively (Figure 3.2C, 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 3.2D,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 the fabricated BSA microstructures since the measured elastic modulus matches previous findings (0.03 to 3 MPa), which is on probing mechanical properties of structurally stable albumin hydrogels using AFM.^[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.

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. During two-photon excitation, rose bengal—an anionic xanthene compound—may undergo two pathways.^[38] In pathway 1, the excited rose bengal molecule reacts in its triplet excited state with a neighboring molecule by H atom or electron transfer. The reaction produces active oxygen species (ROS), for instance superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the $\text{OH}^\cdot$ radical. Molecules which are readily oxidized (e.g. phenols or amines) or reduced (e.g. quinines) will prefer this first reaction pathway.^[39] In the second reaction pathway, the excited triplet-state rose bengal molecule transfers its excited state energy to neighboring ground state molecular oxygen, and produces singlet-state oxygen ($^1\text{O}_2$). Singlet-state oxygen is highly reactive and oxidative. Therefore, in the second pathway, singlet oxygen reactions are more prevalent with compounds such as olefins, dienes, aromatics and heterocycles, as well as with electron-rich and substituted double-bonded functionalities.^[39] For photo-induced polymerization or crosslinking of proteins, it has been widely investigated that the generated ROS and singlet oxygen interact with amino acid residues such as histidine, cysteine, tryptophan and tyrosine in one protein molecule to generate reactive species. These reactive amino acids residues subsequently interact non-photochemically with surrounding similar amino acid residues or with free amino groups in other protein molecules to form a crosslink.^[36] Attributed to complexity in BSA protein molecular structure and conformation, there is no affirmation to conclude the actual photochemical pathway and the dominating amino acids involved in the photopolymerization and crosslinking.^[36, 40]

Concentration of rose bengal is proportional to the amount of free radicals generated upon laser irradiation (Figure 3.5), directly affecting the polymerization and crosslinking extent of BSA during fabrication. The equation used to calculate energy dose per unit area during laser irradiation is $E_d = (P \times t) / A$ (Equation 3.2).^[17] P is laser power (W), A is laser lateral spot size and t represents laser dwell time (s). It is observed that regions exposed to

energy dose per unit area $\geq 18.2 \text{ kJ/cm}^2$ experience internal burning and microstructure explosion (Figure 3.5 and Figure 3.6).

Figure 3.5. (A) Optical microscopy images of microstructures 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 unit area versus applied average laser power.

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 3.6). 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 3.5; Figure 3.6B). For successful microstructure fabrication, the threshold rose bengal concentration is 8 mM with a minimum laser power of 10 mW (Figure 3.5; Figure 3.6A). This condition enables us to achieve BSA microstructures with a maximum swelling of 40 %. At rose bengal concentrations $< 8 \text{ mM}$, 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 3.5; Figure 3.6A). In contrast, at rose bengal concentration $> 8 \text{ mM}$, 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 3.6A). However, no well-defined microstructures can be fabricated at rose

bengal concentrations ≤ 4 mM regardless of laser power (Figure 3.5; Figure 3.6). The damage threshold laser power in our system is 20 mW (energy dose per area (E_d) = 18.2 kJ/ cm²), whereby internal burning and microstructure explosion are observed at this laser power (Figure 3.4; Figure 3.5; Figure 3.6A,B).^[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 3.7).

Figure 3.6. 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 3.7. 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.

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 3.6C).^[5, 15, 41] We employ equation 3.3 to theoretically quantify the effect of rose bengal concentration and laser power on the number of initiating rose bengal molecules (N_{RB}) generated.

$$N_{RB} = \frac{1}{2} \frac{g_p N \phi \delta}{\tau f} \left(\frac{\lambda}{hc} \right)^2 \left(\frac{P}{A} \right)^2 t \quad (3.3)$$

where P is laser power, t is laser dwell time, g_p describes pulse shape of Fourier-transform-limited pulse (0.664), τ is pulse bandwidth (1×10^{-13} s), f is laser repetition rate (8×10^7 s⁻¹), λ is excitation wavelength (7.8×10^{-5} cm), 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 (10×10^{-50} cm⁴ s/photon for rose bengal).^[42]

Equation (3.3) 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.^[42] By correlating our experimental contour plot (Figure 3.6A) and calculated number of initiating molecules generated against rose bengal concentration and laser power (Figure 3.6C), 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 3.6D). We vary the laser dwell time from 10.0 ms to 1.4 ms by changing the laser scan speed from 10 μ m/s to 70 μ m/s during the laser writing process – 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.^[17] For consistency, point distance is fixed at 100 nm in all experiments.

With constant laser power of 12 mW, the microstructures are burnt at laser dwell time of 10 ms due to excessive laser exposure (Figure 3.8). At laser dwell time of 10 ms, the calculated energy dose per area is 33.0 kJ/cm² which is far beyond the damage threshold of our system (18.0 kJ/cm², Figure 3.8B). 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 3.6D). The fabricated microstructures are not well-constructed as laser dwell time decreases below 2.0 ms (Figure 3.8A). 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/cm² (Figure 3.8B). 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/cm³ at 10.0 ms to 5.1×10^{21} radicals/cm³ at 1.4 ms (Figure 3.8B). 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/cm³) for effective photo-polymerization and BSA cross-linking. Therefore, the optimal laser scan speed is determined to be 30 μ m/s for our system based on a laser power of 12 mW, corresponding to a dwell time of 3.3 ms.

Figure 3.8. 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 μ 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.

Effects of laser power and laser dwell time during two-photon lithography on porosity,^[15] surface roughness^[12] and swelling rate induced shape change^[10, 14, 16] of individual protein matrices have previously been investigated. Here, swelling properties of protein microstructures plays a supportive role in identifying and explaining influences of the properties of BSA photoresist and operating parameters on structural behaviors of the as-fabricated BSA microstructures, as well as provides information on property consistency of the BSA photoresists and the fabricated microstructures which will be discussed in the next context. 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 3.9A). 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 3.9B). All 160 microstructures display identical swelling percentages of $(33 \pm 2) \%$ (Figure 3.9C,D). Stable physical and chemical properties of the photoresist formulation indicate its competence for fabrication of microstructures with arbitrary shapes beyond the stated number of the cylindrical structures, area and processing time.

Figure 3.9. (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.

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 3.10). 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.

Figure 3.10. (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.

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-mm² ordered array of 80 free-standing cross-shaped (“+”) BSA microstructures (Figure 3.11). All fabricated free-standing cross-shaped microstructures are well-fabricated (Figure 3.11Ai,B). Each microstructure exhibits (35 $\pm$ 1) % swelling in area at pH 11 (Figure 3.12). 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 3.11B; Figure 3.12). The distinct edges of the microstructures further highlight the structural integrity of suspending arms against stage

motion induced drift in photoresist (Figure 3.12). It therefore verifies that analysis for the structural integrity based on the observation of the geometry of the cross-sections of the cross structures under optical microscope (Figure 3.2A) is in good correlation with SEM results. 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]

Figure 3.11. (A) Optical microscopy images of an array of 80 free-standing cross-shaped BSA microstructures immersed in (i) pH 5 and (ii) pH 11 solution. (B) SEM micrograph of the same array.

Figure 3.12. 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.

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 μm , and the height is varied from 2 to 24 μm to generate micropillars with aspect ratio ranging from 1.4 to 17.0 (Figure 3.13A). 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 3.13B). Raman intensity profiles in pH 5 solution (Figure 3.13B).

Figure 3.13. 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 3.13B). 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 μm ; aspect ratio = 11.3) obtained in pH 5 solution and after freeze-drying, respectively.

SEM characterization shows the successful fabrication of BSA micropillars with height of 16 μm and aspect ratio of 11.3 (Figure 3.13C). Notably, the free-standing BSA micropillars are mechanically robust and sufficiently stiff to maintain their structural integrity even after freeze-drying (Figure 3.13A, Inset: bottom). The measured FWHM height of the micropillars from the Raman intensity profiles show micropillars with heights of 16 μm (Figure 3.13B).

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 3.14A). The micropillars begin to bend or topple since they are not stiff enough to withstand weight of structures as pillar height is further increased to 20 μm and 24 μm (Figure 3.14), giving rise to smaller FWHM heights measured from the Raman intensity profiles (Figure 3.13). We observe a similar trend when comparing FWHM heights of micropillars fabricated from photoresist containing 460 g/L BSA (Figure 3.15). 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.

Acrylate- and epoxy-based photoresists have been extensively utilized for construction of high-aspect-ratio microstructures (aspect ratio > 5).^[20, 21, 43-45] On the other hand, most reported hydrogel-based photoresists are used to produce either vertical

microstructures with aspect ratios below 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.

Figure 3.14. 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 3.15. 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 3.15Bi – 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.

3.4 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.

3.5. Experimental Section

3.5.1 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 MΩ.cm) was purified with a Sartorius arium® 611 UV ultrapure water system.

3.5.2 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.

3.5.3 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 μm $\times$ 20 μm $\times$ 10 μm , ($l \times w \times h$) were fabricated from different BSA photoresists containing various BSA concentrations and PVP (Figure 3.2A). 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 μm and 3 μm in height. To construct an array of 80 free-standing cross-shaped microstructures (individual structure had dimension of 20 μm $\times$ 20 μm $\times$ 8 μm , $l \times w \times h$), photoresist containing BSA (460 g/L) and PVP (10 g/L) was used (Figure 3.11 A, B). 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 μm and 3 μm in height. Dimensions of micropillars are shown in Figure 3.13. 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 $\mu\text{m/s}$.

3.5.4 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.

3.5.5 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.

3.5.6 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.

3.5.7 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 $[(A_{pH11} - A_{pH5}) / A_{pH5}] \times 100 \%$ as A_{pH5} and A_{pH11} 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]

3.6 References

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Chapter 4

Transformative Two-Dimensional Array Configurations by Geometrical Shape-Shifting Protein Microstructures

4.1 Abstract

Two-dimensional (2D) geometrical shape-shifting is prevalent in nature, but remains challenging in man-made “smart” materials, which are typically limited to single-direction responses. Here, we fabricate geometrical shape-shifting of bovine serum albumin (BSA) microstructures, achieving polygon-to-circle shape-shifting behaviors. In addition, transformative 2D microstructure arrays are demonstrated by the ensemble of these responsive microstructures to confer structure-to-function properties. The design strategy of our geometrical shape-shifting microstructures focuses on embedding precisely-positioned rigid skeletal frames within responsive BSA matrices to direct their anisotropic swelling under pH stimulus using two-photon lithography. The layer-by-layer two-photon direct laser writing technique renders spatial resolution in the sub-micrometer length scale. By controlling the shape, orientation and number of the embedded skeletal frames, we have demonstrated the well-defined corner-to-arc transformation, which is essential for dynamic polygon-to-circle shape-shifting. By fabricating our shape-shifting microstructures in periodic arrays, we experimentally construct the first transformative 2D patterned arrays. Such versatile array configuration transformation give rise to structure-to-physical properties, including array porosity and pore shape, which are crucial for the

development of on-demand multi-functional “smart” materials, especially in the field of photonics and microfluidics.

4.2 Introduction

Shape-shifting is a prevailing phenomenon in nature where organisms perform shape-to-function activities in response to external environment, including movement, structural recognition for targeted adhesion and regulation of biochemical processes.^[1-4] For instance, *Amoeba proteus* undergoes multi-directional shape-shifting to form pseudopod (“false foot”) for navigation and rapid path alteration.^[5] Similarly, the recent advent of synthetic, micro/nano-sized shape-shifting “smart” materials are also defined by their precise, dynamic and well-defined two-dimensional or three-dimensional structural change in respond to stimuli.^[6-13] They are applied as autonomous grippers for cargo delivery,^[14-16] microactuators,^[17] and microsensors.^[18] These shape-shifting materials are generally limited to simple single-directional responsiveness, such as twisting,^[19] bending^[20] and swelling.^[21] It remains a challenge to perform multiple-directional stimuli-responsiveness, which is vital for dynamic and sophisticated geometrical transformation. Such tunable and versatile geometrical features is essential for both regulation of shape-dependent properties (such as surface area-to-volume ratio and flow characteristics) and the application in structural recognition for modulation of chemical reactivity and directing multi-channel microfluidic flow.^[22, 23]

Furthermore, computational simulations have demonstrated that the ensemble ordered array of these dynamic, geometrical shape-shifting micro/nano-sized building blocks is promising for reversible topographical change in structural configurations.^[24, 25] This is analogous to the self-assembly of atoms in highly symmetrical lattices,^[26] where the molecular arrangement of atoms directly affect the chemical, physical and/or optical properties, such as in the case of graphite and diamond.^[27] To date, 2D array with transformative configurations has not been realized experimentally at the sub-micrometer length scale. Hence, we hypothesize that both geometrical shape-shifting microstructures

and their ensemble as transformative 2D patterned arrays with stimuli-responsiveness are essential for the scientific advancement of autonomous, multi-functional “smart” materials, especially for the field of photonics,^[28] sensors^[29] and microfluidics.^[23]

Here, we demonstrate the dynamic geometrical shape-shifting of pH-responsive bovine serum albumin (BSA) microstructures from well-defined polygons to simple circles using two-photon lithography. Moreover, two-dimensional (2D) BSA microstructure arrays with dynamic and transformative array configurations *via* precise geometrical shape-shifting are reported. Our strategy involves layer-by-layer photo-induced polymerization of BSA to precisely modulate cross-linking density within a single protein microstructure entity at sub-micrometer spatial resolution. This is achieved using two-photon lithography to create region of skeletal framework (high cross-linking density) to direct swelling of low cross-linking density regions for geometrical transformation. The shape, orientation and number of the embedded skeletal frames are precisely modulated to effect corner-to-arc transformation for geometrical shape-shifting. Efficacy of shape-shifting is defined by area, radius of curvature and tangent length at edge of final transformed shapes. We then demonstrate on-demand transformation of array configurations, where protein microstructures fabricated in 2D arrays with open-structured hexagonal configuration undergoes reversible geometrical shape transformation to form hexagonal and percolating hexagonal configurations, respectively, under pH stimuli. The transformation of array configurations confers tunable structure-to-physical properties, such as array porosity and pore shape, which is crucial for future development of 2D/3D multi-functional “smart” materials.

4.3 Results and Discussion

We achieve geometrical shape-shifting of microstructures through the spatial control of anisotropic swelling at sub-micrometer precision. The design involves embedding a less-responsive and highly cross-linked bovine serum albumin (BSA) segment within a responsive BSA matrix of lower cross-linking density (Scheme 4.1). The highly cross-linked BSA segments function as skeletal frames to induce mechanical constraints and to direct the structural anisotropic swelling of low cross-linked BSA matrix.^[30-32]

Scheme 4.1. (A) Schematic of dynamic geometrical shape-shifting BSA microstructure fabricated via (B) the sub-micrometer-scaled spatial modulation of cross-linking density distribution using two-photon lithography. Z-layer distance (Δd) refers to separation between each consecutive raster-scanned x-y plane during the layer-by-layer two-photon direct laser writing process.

The fabrication of segmented BSA microstructures with sub-micrometer scale spatial modulation of cross-linking density is performed using two-photon direct laser writing. Typically, laser is raster-scanned repeatedly at a pre-defined x-y plane to polymerize BSA structure, where the cross-linking density of the microstructure is manipulated by modulating the z-layer distance (Δd ; Scheme 4.1) during the layer-by-layer laser writing process at sub-micrometer precision. Since Δd is inversely proportional to the cross-linking density, it is expected that lower cross-linked structures exhibit higher swelling ability due to greater freedom of cross-linked network expansion, and vice versa.^[33] Highly cross-linked BSA segments are written at $\Delta d = 100 \text{ nm}$ and exhibit a less-responsive swelling

ratio of 1.1 when surrounding pH is changed from 5 to 11 (Figure 4.1A). In contrast, low cross-linked segments are written at $\Delta d = 400$ nm which allow them to swell more pronouncedly under stimulus, with a larger swelling ratio of 1.5 (Figure 4.1B). With an isoelectric point between pH 4.7 - 4.9, BSA is significantly more negatively charged at pH 11 than at pH 5.^[34] Therefore, the BSA microstructure is able to absorb more water through the formation of multiple ion-dipole interactions upon increase in pH, which causes its swelling.^[35] Hence, the cross-linking density is inversely proportional to Δd , which agrees well with literature.^[33] This observation clearly demonstrates the ability of two-photon lithography to fabricate well-defined microstructures and also to precisely control the spatial distribution of cross-linking density within a microstructure.^[36-38] Hereafter, BSA segments fabricated at $\Delta d = 100$ nm and 400 nm are termed as “skeletal frames” and “responsive BSA matrix”, respectively.

pH 5	pH 11	Swelling Ratio ($A_{\text{pH } 11}/A_{\text{pH } 5}$)
(A) $\Delta d = 100$		1.10 ± 0.03
(B) $\Delta d = 400$		1.52 ± 0.03

Figure 4.1. Optical microscopic images illustrating the area swelling ratio of square BSA microstructures fabricated using a z-layer distance (Δd) of (A) 100 nm and (B) 400 nm, respectively. Area swelling ratio is calculated by dividing area at pH 11 ($A_{\text{pH } 11}$) with the area at pH 5 ($A_{\text{pH } 5}$).

For polygon-to-circle shape-shifting of BSA microstructures, the key criterion is the transformation of corner/edge-to-arc, which is critical for the generation of well-defined arcs of a circle from the initial corners/edges of a polygon (Scheme 4.2). Our approach lies at the induction of directed anisotropic swelling of responsive segmented BSA microstructures. We start with setting benchmarks to evaluate the corner/edge-to-arc transformation, and also the effectiveness of geometrical polygon-to-circle shape-shifting of our BSA microstructure using two critical parameters, namely (1) area of occupancy, and (2) protrusion index.

Scheme 4.2. Schematic illustrating the edge-to-arc conversions of responsive BSA matrices upon anisotropic swelling at pH 11.

Firstly, area of occupancy is calculated by dividing the area of protrusions generated by experimentally shape-shifted microstructures ($A_{\text{structure}}$) over area of ideal circular shape that is unoccupied by the isotropically-swelled polygon microstructure (A_{ideal}) (Figure 4.2A). The larger the area of occupancy (area of occupancy = $A_{\text{structure}}/A_{\text{ideal}} \times 100\%$), the better a microstructure conforms to its ideal shape and has more well-defined geometrical transition. Secondly, protrusion index can be calculated using ($r/R_c \times 100\%$; Figure 4.2B), where r is the radii of curvature of generated protrusions in experimentally transformed

BSA microstructure and R_c is a reference radius of curvature of an isotropically swelled circular microstructure. A protrusion with protrusion index $> 50\%$ is classified as an “arc” (Figure 4.2B). That is, a protrusion approximates to an arc when it has at least 50% larger radius of curvature as compared to that of an isotropically swelled ideal circular microstructure (Figure 4.2B). Therefore, we define a polygon-to-circle shape-shifting event successful only when area of occupancy and protrusion index is $> 50\%$ (Figure 4.2, Figure 4.3).

Figure 4.2. (A) Schematics illustrating the area of occupancy of BSA microstructures (outline in blue) in an ideal shape (outline in black) when it undergo anisotropic swelling to cover (1) 0%, (2) 25%, (3) 50%, and (4) 75% of the initial empty area, for polygon-to-circle shape-shifting. Area of occupancy (area of occupancy = $A_{\text{structure}}/A_{\text{ideal}} \times 100\%$) is obtained by comparing area occupied by shape-shifted BSA microstructure at pH 11 ($A_{\text{structure}}$, shaded in blue) with initial empty area of ideal shape (A_{ideal} ; Figure 4.2A(1)). (B) Schematic representations of shape-shifted triangular BSA microstructures with protrusion index (protrusion index = $r/R_c \times 100\%$) of (2) 75%, (3) 50% and (4) 25%, respectively. “ r ” is the radius of circles (outline in blue) that can be fitted into the protrusions and (1) R_c is the reference radius of curvature at pH 11 of an isotropically swelled circular structure in the absence of skeletal frames (refer to Figure 4.8E; $R_c = 12.2 \mu\text{m}$; $\Delta d = 400 \text{ nm}$; swelling ratio ~ 1.5).

By controlling both shape and orientation of embedded skeletal frame within the BSA microstructure, we can induce directed anisotropic swelling of the responsive segmented BSA microstructures. For square-to-circle shape-shifting, we embed four spike-shaped skeletal frames in “×” arrangement in a square BSA microstructure (Figure 4.3C). These skeletal frames are aligned along the diagonal axes of the square microstructure (oriented at 45°, 135°, 225° and 315°), with each spike bound to one of the squares’ corner. This creates four regions of responsive BSA matrices, each sandwiched between two adjacent skeletal frames. As pH 5 increases to pH 11, the straight edges of the square microstructure swell anisotropically to produce arcs (Figure 4.3C(2)). These corner/edge-to-arc transformations drive the square-to-circle shape-shifting of BSA microstructure in < 2 s with area of occupancy and protrusion index of $(74 \pm 8) \%$ and $(89 \pm 1) \%$, respectively, which is also reversible on returning to pH 5 (Figure 4.4). The anisotropic swelling of responsive BSA matrix is due to pinning of skeletal frames to the four corners of the square, which restrict swelling at/near the corners. The embedded skeletal frames therefore create a broad arc-shaped swelling gradient between two adjacent corners of the square (Figure 4.3C), characterized by restricted swelling near the corners and a higher swelling ability at midpoints of the square’s edges. These individual arcs generated upon swelling at pH 11 are joined at the initial corners’ positions, forming the continuous arc of shape-shifted circular BSA microstructures.

Figure 4.3. Schematics depicting (A) area of occupancy and (B) protrusion index to describe polygon-to-circle shape-shifting. A successful polygon-to-circle shape-shifting event occurs when area of occupancy and protrusion index are $> 50\%$. Square-to-circle shape-shifting of BSA microstructures embedded with four spike-shaped skeletal frames aligned along the (C) diagonal axes and (D) edge axes of the square. (E) Control square BSA microstructure in the absence of skeletal frame. (1, 2) Optical microscopic images of as-fabricated BSA microstructure at pH 5, and their transformed shape at pH 11, respectively. (3) Schematic illustrating the anisotropic swelling profile of respective BSA microstructures.

Figure 4.4. Representative movie frames demonstrating the dynamic and rapid polygon-to-circle transformation when pH 5 increases to pH 11 (top), and also the reversed circle-to-polygon shape-shifting (bottom) when pH 11 returns to the initial pH 5. (1) Pentagon-to-circle and circle-to-pentagon transformations. (2) Hexagon-to-circle and circle-to-hexagon transformations.

The orientation of spike-shaped skeletal frames is crucial in producing well-defined polygon-to-circle transformation. We construct a similar square BSA microstructure containing spike-shaped skeletal frames but aligned along the edge's axes (oriented at 0° , 90° , 180° and 270°) instead (Figure 4.3D(1)). At pH 11, regions of responsive BSA matrix do not generate arcs from edges (Figure 4.3D(2)). Instead, it swells to yield a ~ 1.5 -fold larger pinched square, which does not demonstrate geometrical shape-shifting. This observation highlights the importance of orientation-dependent anisotropic swelling of the

responsive BSA matrix. When skeletal frames are aligned along the square edge's axes, a swelling gradient is created between midpoints of two edges (Figure 4.3D(3)). In this case, midpoints of the square edges experience the lowest swelling ability due to mechanical constraints imposed by the skeletal frame, whereas the four corners of the square exhibit highest swelling ability. As a result, the square's corners are preserved upon swelling at pH 11, leading to the formation of a larger, pinched square. Furthermore, we observe that a square BSA microstructure without skeletal frames exhibits isotropic swelling and not geometrical shape-shifting (Figure 4.3E). Both controls therefore clearly depict the importance of the embedded skeletal frame and its precise orientation to direct anisotropic swelling for polygon-to-circle transformation in BSA microstructures.

We also determine that sharp-tipped, spike-shaped skeletal frame is essential to bind and restrict swelling near the corners effectively for generation of well-defined polygon-to-circle transformation. To compare the effect of frame shape on the distinctness of polygon-to-circle shape-shifting, we embed sharp-tipped spike-shaped skeletal frames (Figure 4.5A) and blunt-tipped stick-shaped skeletal frames (Figure 4.5B) into a low cross-linked pentagon microstructure.

Skeletal frame	(A) Spike	(B) Stick
Pentagon pH 5 ⚡
pH 11 Circle
Area of occupancy (%)	74 ± 8	223 ± 7
Protrusion index (%)	89 ± 1	50 ± 8

Figure 4.5. Polygon-to-circle shape-shifting of BSA microstructures embedded with (A) spike- and (B) stick-shaped skeletal frames. Optical images (left) and schematic diagrams (right) illustrating the gradient swelling profile of each BSA microstructure. Ideal circular shapes are outlined in blue over respective transformed BSA microstructures at pH 11.

Pentagon microstructure embedded with spike-shaped skeletal frames transforms into near-perfect circle at pH 11 (area of occupancy = (74 ± 8) %, protrusion index = (89 ± 1) %; Figure 4.5A). On the other hand, the microstructure embedded with stick-shaped skeletal frames swell to form a contorted circle with area of occupancy and protrusion index of (223 ± 7) % and (50 ± 8) % (Figure 4.5B), respectively. We would like to highlight that stick-shaped skeletal frames lead to an area of occupancy which is > 2 -fold larger than A_{ideal} , denoting the presence of large deviation between the swelled microstructure and the ideal circle which therefore cannot be considered a well-defined polygon-to-circle transformation. The well-defined polygon-to-circle transformation contributed by spike-shaped skeletal frames is due to the precise attachment of their sharp tips to the corners of pentagon microstructure (Figure 4.5A). At pH 11, the pentagon microstructure with spike-shaped skeletal frames embedded swells anisotropically to produce a circle. This defined anisotropic swelling is due to the pinning of the skeletal frames to the five corners of the pentagon which restrict swelling at/near the corners. As a

result, a broad arch-shaped swelling profile occurs along all the edges of the pentagon to form arcs (protrusion index = $(89 \pm 1) \%$) which leads to the pentagon-to-circle transformation.

However, the blunt tips of the stick-shaped skeletal frames are not well attached to the corners of the pentagon microstructure. At pH 11, the pentagon microstructure also undergoes anisotropic swelling, but to form a contorted circle instead. The contorted circle exhibits much sharper protrusions (protrusion index = $(50 \pm 8) \%$) and has area extended beyond its ideal circular shape (area of occupancy = $(223 \pm 7) \%$; Figure 4.5B). These observations clearly demonstrate that the sharp, spike-shaped skeletal frame is essential to bind and restrict swelling near the corners effectively for the generation of well-defined arcs (protrusion index = $(89 \pm 1) \%$) in polygon-to-circle transformation. This is superior to blunt, stick-shaped skeletal frames, which are poorly attached to the polygon's corners and hence do not promote distinct polygon-to-circle shape-shifting.

Based on our design strategy above, we fabricate a library of well-defined polygon-to-circle shape-shifting BSA microstructures are fabricated simply by embedding spike-shaped skeletal frames within responsive polygon matrices, with sharp tips of skeletal frame bound to corners of the polygons (Figure 4.6). Using three, four, five and six spike-shaped skeletal frames, triangle, square, pentagon and hexagon BSA microstructures undergo rapid shape-shifting into circles as a result of well-defined corner/edge-to-arc conversion at pH 11, with both area of occupancy and protrusion index $> 50 \%$ (Figures 4.6 to 4.8). It is clear that specific well-defined circle-to-polygon or polygon-to-circle transformations can be programmed through defining the number of skeletal frames embedded in the BSA microstructures.

Figure 4.6. (A) Schematic depicting the polygon-to-circle shape-shifting of BSA microstructure on stimulation at pH 11. Optical microscopic images of dynamic shape-shifting of BSA microstructures from (B) triangle, (C) square, (D) pentagon, and (E) hexagon to circles. (1, 2) Optical microscopic images of as-fabricated BSA microstructure at pH 5, and their transformed shape at pH 11, respectively. Polygon-to-circle shape-shifting efficacy is expressed in terms of (F) area of occupancy and (G) protrusion index.

<p>(A) pH 5 Polygon</p> <p>(E) pH 11 Circle</p>		Area unoccupied by polygon in ideal circle at pH 11, $A_{ideal} (\mu m^2)$	Area occupied by shape-shifted microstructure in ideal circle, $A_{structure} (\mu m^2)$	Area of occupancy, $A_{structure}/A_{ideal} (%)$
(A)	(E)	304 ± 4	231 ± 6	76 ± 3
(B)	(F)	235 ± 2	173 ± 5	74 ± 3
(C)	(G)	100 ± 2	74 ± 7	74 ± 8
(D)	(H)	71 ± 1	50 ± 6	70 ± 8

Figure 4.7. Schematic representations (top) and optical microscopic images (bottom) of dynamic geometrical shape-shifting BSA microstructures from (A) triangle, (B) square, (C) pentagon, and (D) hexagon at pH 5 to circular shapes (E - H) at pH 11. Ideal shapes of circle (E - H) are outlined in blue over respective transformed BSA microstructures at pH 11. A successful shape-shifting event occurs only when the area of occupancy ($A_{structure}/A_{ideal} \times 100\%$) of shape-shifted BSA microstructure is >50% upon pH stimulation.

Figure 4.8. Schematic representations (top) and optical microscopic images (bottom) of dynamic geometrical shape-shifting BSA microstructures from (A) triangle, (B) square, (C) pentagon, and (D) hexagon at pH 5 to circular shapes (F - I) at pH 11. Ideal circles (cyan) are fitted into corners of the respective transformed BSA microstructures to determine their radius of curvature (r). (E, J) Optical microscopic images of circular BSA microstructure (z-layer distance, $\Delta d = 400$ nm) without skeletal frame at pH 5 and pH 11, respectively. The control circular BSA microstructure undergoes isotropic swelling (swelling ratio ~ 1.5) at pH 11 and has a radius of curvature (R_c , red) of $12.2 \mu\text{m}$.

Collectively, we propose general rules-of-thumb for the fabrication and programming of pre-defined polygon-to-circle shape-shifting into BSA microstructures. Firstly, polygon-to-circle shape-shifting requires corner/edge-to-arc transformation, which is attained by aligning the sharp tips of spike-shaped skeletal frames to polygon corners. Secondly, the number of embedded skeletal frames determines the number of regions of responsive BSA matrices available for corner/edge-to-arc transition. These general rules-of-thumb therefore highlight the versatility and simplicity of our strategy to fabricate a wide variety of programmable geometrical shape-shifting BSA microstructures. Such rapid, dynamic, well-defined and on-demand geometrical shape-shifting microstructures are essential for the future construction and study of reconfigurable “smart” materials.^[25] Furthermore, our

precisely engineered BSA microstructures are the first demonstration of geometrical shape-shifting, which is in contrast to conventional responsive microstructures that are typically limited to simple single-directional swelling. Such geometrical shape-shifting is essential in nature. For instance, pollen grains possess a stiff outer layer, akin to our skeletal frames, which enables their geometrical shape-shifting *via* different self-folding mechanisms to protect against dehydration.^[39]

Next, we fabricate transformative 2D patterned arrays with open-structured hexagonal array configurations using geometrical shape-shifting BSA microstructures and study the changes to physical properties as the array configuration transforms in response to pH stimulus. To start, we construct a periodic hexagonal array comprising of triangular BSA microstructures oriented inwards with edge-to-edge alignment analogous to open-structured hexagonal configuration (Figure 4.9A). The edges of the triangles approach each other as the individual BSA microstructures undergo triangle-to-circle shape-shifting (Figure 4.9B(2)) at pH 11, hence this open-structured hexagonal array configuration transitions to a percolating hexagonal configuration upon swelling.

As triangular microstructures swell into circular shape from pH 5 to pH 11, edge-to-edge distance between microstructures decreases (58 ± 4 %) from $(7.8 \pm 0.1) \mu\text{m}$ to $(3.3 \pm 0.3) \mu\text{m}$ as swollen edges approaching each other (Figure 4.10) resulting topological transition. The percolating hexagonal array also experiences a reduction in array porosity from 69 % to 38 % as pH increases from pH 5 to pH 12 (Figure 4.9C) while pore shape is observed to change from asterisk-shape to six-pointed star (Figure 4.9A, B). With triangular BSA microstructures arranged in open-structured hexagonal array configuration, change in topology, pore shape and pore size are observed together with topographical transition from open-structured hexagonal configuration to percolating hexagonal configuration in response to pH stimulus.

Figure 4.9. Raman images illustrating transformation of open-structured hexagonal-to-percolating hexagonal array configurations. (A) Raman image uses a unit cell of six triangle-to-circle shape-shifting microstructures arranged in a hexagon manner. Optical microscopic images depicting topographical change in array configurations during (B) open-structured hexagonal-to-percolating hexagonal transformations. (1, 2) Arrays of geometrical shape-shifting BSA microstructures at pH 5 and pH 11, respectively. (C) Change in porosity of the two-dimensional arrays during open-structured hexagonal-to-percolating hexagonal transformations at different pH. All data were averaged using 10 measurements of individual unit cells and the error bars were calculated as the standard deviation from the mean.

Figure 4.10. Graphs illustrating change in edge-to-edge distance between adjacent triangular BSA microstructures constructing an open-structured hexagonal array after transforming into a percolating hexagonal array at pH 11. Percentage of reduction in edge-to-edge distance is calculated by dividing difference in edge-to-edge distance before and after triangular-to-circle shape-shifting over edge-to-edge distance measured at pH 5. An array is considered to undergo topological transition due to change in physical connectivity when edge-to-edge distance decreases $>50\%$. Transformation of open-structured hexagonal array configuration to percolating hexagonal array configuration is hence accompanied by topological transition as BSA microstructures have reduced edge-to-edge distance $>50\%$.

Overall, the example of array configuration transformation clearly exemplifies the importance of our design and strategy on geometrical shape-shifting microstructures for the first experimental demonstration of dynamic, on-demand transformative 2D arrays. The precise modulation of physical properties, such as connectivity and array porosity, in geometrical shape-shifting microstructure arrays marks the advent of multi-functional, tunable “smart” materials for photonic or microfluidic devices. Our work can also be extended to future studies on reconfigurable 3D crystal.

4.4 Conclusions

We have demonstrated the fabrication of a large library of well-defined polygon-to-circle shape-shifting bovine serum albumin (BSA) microstructures by adopting three general rules-of-thumb: (1) polygon-to-circle shape-shifting requires the transformation of corner/edge to arc which is attained by orienting spike-shaped skeletal frames toward the polygon's corners, and (2) number of corners/arcs created is controlled by the quantity of skeletal frames embedded. We apply these shape-shifting microstructures to construct the first transformative 2D array capable of undergoing reversible open-structured hexagonal to percolating hexagonal configuration transformations. Such versatile transformations in array configurations confer immense potential in the tuning of array physical properties, such as connectivity and array porosity, necessary for controlled interaction with light and fluids. This enables us to potentially tune electromagnetic wave in near-infrared region, modify fluid flow behavior and also identify and direct different phases of fluids (pH variation in this case) crucial in the field of photonics and microfluidics, respectively.^[28, 40-42] Our work also provides scientific insights potentially applicable for the future construction of 3D reconfigurable crystals.

4.5 Experimental Section

4.5.1 Materials. Lyophilized powdered bovine serum albumin (BSA) (96+ %), HEPES (99.5+ %), rose bengal (95 %), powdered sodium chloride (NaCl; 99+ %) and phosphate buffered saline tablets (PBS) were 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.

4.5.2 Preparation of BSA Protogel Precursor Solution. 2.9 g of BSA powder and 1.94 mL of DMSO were added to an aqueous solution of 8.5 mM rose bengal and 100 mM NaCl in 20 mM HEPES buffer to make up a total volume of 10 mL. The mixture was filtered through a hydrophilic PVDF syringe filter (pore size = 0.22 μ m) to remove impurities before use.

4.5.3 Fabrication of BSA Microstructures. Fabrication of the protogel structures were performed using the Nanoscribe® Photonic Professional. The system is equipped with a femtosecond laser source with a center wavelength of 780 nm. Structures of the protogels were designed using a CAD software, 3ds Max®. Parameters of the structures were defined by the Nanoslicer and DeScribe softwares. 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 0.16 mm) with 13 mW laser power, 0.2 μ m line (x-y) distance, scan speed of 30 μ m/s and various layer distance along z-axis (Δd). Resist containing BSA and rose bengal was

deposited on a glass substrate. Photopolymerization started at the interface between resist and glass substrate, allowing fabricated structures to be firmly attached to the glass substrate. Resist within the confocal volume of the laser underwent polymerization and cross-linking on glass substrate. After fabrication, the substrate was soaked in 20 mM PBS buffer to remove excess unpolymerized resist, and subsequently washed with water and stored in nitrogen box. The fabricated microstructures remained fixed on glass substrates after development.

All individual circle-to-polygon and polygon-to-circle shape-shifting microstructures were fabricated with dimensions of $20\text{ }\mu\text{m} \times 20\text{ }\mu\text{m} \times 5\text{ }\mu\text{m}$, $l \times w \times h$. Microstructures constituting the 2D patterned arrays with different configurations were fabricated with dimensions of $12\text{ }\mu\text{m} \times 12\text{ }\mu\text{m} \times 5\text{ }\mu\text{m}$, $l \times w \times h$.

4.5.4 Dynamic Geometrical Shape-shifting of BSA Microstructures. All as-fabricated BSA microstructures were equilibrated in pH 5 solution for 3 minutes and subsequently transferred to a pH 11 solution. The BSA microstructures were equilibrated for another 3 minutes. Both the BSA microstructures at pH 5 and pH 11 were monitored using optical microscope and Raman microscopy. For the *in-situ* monitoring of shape-shifting process using optical microscope, the optical images were captured continuously as the pH of the solution was gradually increased from pH 5 to pH 11.

4.5.5 Materials Characterization. Bright field optical microscopy was performed using an Olympus BX51BD microscope. Raman imaging was obtained using the Ramantouch microspectrometer (Nanophoton Inc, Osaka, Japan) equipped with an Apochromat NIR water immersion objective from Nikon (60× magnification, NA 1.0). The wavelength and power of excitation laser were set at 532 nm and 0.10 mW, respectively, and Raman

acquisition was performed with an exposure time of 0.8 s/line. All data for the physical characterization of BSA microstructures were averaged using at least 10 measurements.

4.5.6 Data Analysis. Measurements of swelling ratio and pH-induced changes in porosity of the transformative 2D arrays were conducted using ImageJ.

4.6 References

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Chapter 5

Customizable Dynamic Thermoresponsive Poly(N-isopropylacrylamide) Microstructures via Two-photon Lithography

5.1 Abstract

Microfabrication of three-dimensional (3D) microstructures with stimuli-responsiveness is essential for fabricating functional microstructures to achieve biomimicry which can help designing products in sustainable ways. Yet, availability of commercially available water-soluble photoinitiators is scarce which limit the development of aqueous-based photoresists containing vinyl monomers with stimuli-responsive functional groups. Here, we develop an aqueous-based photoresist consisting of N-isopropylacrylamide (NIPAAm) and rose bengal (RB) for constructing hydrogel microstructures with high susceptibility to temperature via two-photon lithography. We quantitatively optimize processing parameters for the construction of poly(N-isopropylacrylamide) (PNIPAAm) microstructures with high structural integrity. The controlled thermal responsiveness is achieved by precise spatial manipulation of the laser scanning layer distance along the z-axis at the nanoscale to tune crosslinking density of PNIPAAm. Therefore, by nanoscale modulation of the laser scanning layer distance, we are able to control and quantify shrinkage percent of the PNIPAAm microstructures with high accuracy. Our findings are beneficial to the development of other aqueous-based

stimuli-responsive hydrogel photoresists for the construction of intricate 3D geometries with multiple on-demand functionalities.

5.2 Introduction

Adaptive hydrogels with stimuli-responsiveness towards environmental cues (such as solution pH, surrounding temperature, humidity, and etc) are frequently used for designing miniature structures. The aim is to produce sustainable solutions for solving complicated issues on materials design which are pending due to limitations of conventional technologies, for instance 3D structures fabricated using UV lithography suffer from poor resolution. Two-photon lithography, a layer-by-layer three-dimensional (3D) femtosecond laser-assisted printing technique with precise spatiotemporal control, has been used in the fabrication of dynamic microactuators,^[1-3] microlenses and photonic crystals with reversible transition of refractive indices,^[4-7] as well as geometrical shape-shifting microstructures.^[8] Reported precursors used for the above-mentioned fabrications are limited to pH-responsive protein molecules,^[1, 4, 5, 8, 9] water- and salt-tuneable acrylamides,^[3] organic-solvent susceptible polydimethylsiloxane (PDMS),^[6] as well as self-synthesized oligo(aniline)-based molecules.^[7] However, thermoresponsive materials exhibiting reversible heat-induced structural variations which are made using two-photon polymerization— important materials for biomedical applications,^[10, 11] programmable micro-robots,^[12, 13] and anti-counterfeiting^[14, 15]— remain unreported.

One prominent temperature sensitive material is poly(N-isopropylacrylamide) (PNIPAAm). The non-ionic PNIPAAm chains undergo reversible phase transition in aqueous solution above its lower critical solution temperature (LCST) of approximately 32 °C.^[16, 17] Photo-induced polymerization of these biocompatible thermo-responsive molecules is conducted via conventional UV irradiation or UV lithography.^[18-20] UV irradiation produces bulk hydrogel while UV lithography—a planar lithography technique — allows fabrication of 3D structures by stacking written 2D structures with resolution > 300 nm.^[21, 22] Photopolymerization of NIPAAm using conventional UV irradiation is

normally initiated by thermally-unstable redox initiators (for instance N,N,N',N'-tetramethylethylenediamine and ammonium persulfate) which require long irradiation time to reach gelation.^[18, 19] On the other hand, UV lithography employs sparingly water-soluble photoinitiator and methanol as co-solvent in the photoresist formulation, and this impairs stability of the photoresist due to rapid methanol evaporation.^[20, 23] It is reported that multiphoton excitation of xanthene dyes has high efficiency in initiating polymerization of acrylamide and triacrylate in aqueous solution for the fabrication of submicron 3D structures.^[24, 25] Yet, only simple geometrical microstructures with no stimuli-responsiveness are reported. The capability of water-soluble xanthene dyes on initiating two-photon induced polymerization thus broadens the variety of commercially accessible photoinitiators for two-photon lithography.

Here, we demonstrate a quantitative study to understand the effects of fabrication parameters on our capability to achieve structurally well-defined PNIPAAm microstructures with controlled thermal responsiveness. To achieve well-defined microstructures with reproducible dynamic thermal responsiveness, we develop a set of sweep tests to optimize fabrication parameters such as laser power and laser dwell time. We achieve precise modulation over the extent of structural shrinkage from 27 % to 44 % via spatially manipulating laser scanning layer distance along the z-axis with accuracy at nanometer scale. The PNIPAAm microstructures remain sturdy and can withstand reversible swelling and shrinking over 4 heating/cooling cycles. Since most of the hydrogel precursors are water soluble and with aim to achieve green chemistry, our research on establishing aqueous-based photoresists containing thermo-responsive monomers is potentially useful for the development of other two-photon polymerizable aqueous-based stimuli-responsive hydrogel photoresists.

5.3 Results and Discussion

We construct poly(N-isopropylacrylamide) (PNIPAAm) hydrogel cylindrical microstructures in a layer-by-layer fashion via two-photon induced polymerization of the N-isopropylacrylamide (NIPAAm) photoresist (Scheme 5.1). The layer-by-layer three-dimensional (3D) microfabrication is achieved through raster-scanning the x-y plane coordinates along different predetermined z-planes repeatedly.^[26, 27] The separation between two layers is called the “layer distance” (Δd), and it can be accurately manipulated using the NanoScribe software. Moreover, spatial resolution of two-photon lithography is within the nanometer scale.^[26, 27]

Scheme 5.1. (A) Schematic illustration of PNIPAAm fabrication using two-photon lithography. (B) 3D PNIPAAm cylinder is fabricated using a layer-by-layer laser writing process. The separation between two neighbouring layers is called the layer distance (Δd).

The aqueous-based NIPAAm photoresist comprises NIPAAm as the monomer, N,N'-methylenebisacrylamide (bis-AAm) as the crosslinker, rose bengal (RB) acting as the photoinitiator, and triethanolamine (TEOA) as the reducing agent (Figure 5.1). The NIPAAm precursor renders the hydrogel microstructures dynamically heat-responsive.^[16, 18, 28] At the laser focal point, excitation of RB in the presence of TEOA triggers the polymerization reaction.^[24, 25]

Figure 5.1. Molecular structures of the (A) monomer—N-isopropylacrylamide (NIPAAm), (B) crosslinker—N,N'-methylenebisacrylamide (bis-AAm), (C) photoinitiator—rose bengal (RB) and reducing agent—triethanolamine (TEOA).

To fabricate microstructures with optimized thermal-responsiveness, we investigate the influence of laser power and laser dwell time on structural properties of the PNIPAAm micro-cylinders (diameter = 5 μm , height = 3 μm , and $\Delta d = 200$ nm) (Figure 5.2). The laser power ranges between 6 – 30 mW and the laser dwell time is tuned between 2.5 – 10.0 ms. The laser dwell time governs the duration of interaction between the laser beam and NIPAAm photoresist. A sweep test on dependence of structural integrity on laser power and laser dwell time is conducted (Figure 5.2). From the results obtained, three regions can be classified as Region (I) which has no microstructures be written out, Region (II) has well-defined cylindrical microstructures, and Region (III) displays deformed microstructures due to localized explosion (Figure 5.2). A minimum laser power of 10 mW

is required for microfabrication at laser dwell time of 10 ms to successfully fabricate microstructures with high structural fidelity (Figure 5.2). At higher laser power (≥ 20 mW, Region (III)), significantly shorter laser dwell times are needed to avoid internal burning and explosion of structures (Figure 5.2). Moreover, the calculated energy dose per area (E_d) experienced by the laser irradiated zone increases with laser power and laser dwell time. Calculation for E_d is demonstrated below by using $P = 1.2 \times 10^{-2}$ W and $t = 0.0033$ s,

$$\begin{aligned}
 E_d &= (P \times t) / A \\
 &= (1.2 \times 10^{-2} \times 0.0033) / 3.629 \times 10^{-9} / 1000 \\
 &= 11.0 \text{ kJ/cm}^2
 \end{aligned}
 \tag{5.1}$$

Figure 5.2. SEM micrographs of PNIPAAm microstructures illustrate two-photon polymerization processing window of the NIPAAm photoresists and showcase the effect of average laser power and laser dwell time on two photo-polymerizability.

Figure 5.3. Calculated energy dose per area versus applied average laser power and laser dwell time. The region within the two dotted lines represents optimal conditions for microfabrication using NIPAAm photoresists. Region (I), (II) and (III) are corresponding to those labeled regions in Figure 5.2.

The optimum range of E_d is from 12.8 to 30.3 kJ/cm² (Figure 5.2, Figure 5.3, Region (II)). Notably, no well-defined microstructures can be fabricated at $E_d \leq 12.8$ kJ/cm² (Region (I)). Furthermore, microstructures fabricated beyond the optimum range of E_d experience internal burning, followed by explosion are observed at $E_d > 30.3$ kJ/cm² (Figure 5.2, Figure 5.3, Region (III)).^[9, 29] However, microstructures written at laser dwell time of 10.0 ms with laser power range from 38.6 kJ/cm² to 49.6 kJ/cm² (14 – 18 mW) are exceptional cases, where other factors such as amount of initiating RB molecules at the focus point need to be considered.

We also determine how the number of initiating RB molecules (N_{RB}) in the focal volume affects fabrication of PNIPAAm microstructures. Equation (5.2) is used to theoretically evaluate the roles of laser power and laser dwell time on N_{RB} generated.

$$N_{RB} = \frac{1}{2} \frac{g_p N \phi \delta}{\tau_f} \left(\frac{\lambda}{hc} \right)^2 \left(\frac{P}{A} \right)^2 t \quad (5.2)$$

where P is laser power, t is laser dwell time, g_p relates to 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 is 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.^[9, 30] Equation (5.2) assumes, after experiencing absorption events within the laser focal volume, all RB molecules are activated and converted to photochemical reaction initiators. Moreover, the laser lateral spot size (A) is also supposed to remain unchanged with respect to the photoresist's properties and processing parameters.^[30] Calculation for number of initiating rose bengal (RB) molecules (N_{RB}) generated within the laser focal volume is demonstrated below:

$$g_p = 0.664$$

$$\tau = 1 \times 10^{-13} \text{ s}$$

$$f = 8 \times 10^7 \text{ 1/s}$$

$$\lambda = 7.8 \times 10^{-5} \text{ cm}$$

$$h = 6.626 \times 10^{-34} \text{ J s}$$

$$c = 3 \times 10^{10} \text{ cm/s}$$

$$\begin{aligned} A &= \pi \times [(0.61 \times \lambda)/(\text{Numerical aperture of objective lens})]^2 \\ &= \pi \times [(0.61 \times 7.8 \times 10^{-5})/1.4]^2 \\ &= 3.629 \times 10^{-9} \text{ cm}^2 \end{aligned}$$

$$\phi = 1 \quad (\text{RB molecules have quantum efficiency} > 98 \%)$$

$$\delta = 10 \times 10^{-50} \text{ cm}^4 \text{ s/photon}^{[24]}$$

As a demonstration by using $P = 1.2 \times 10^{-2}$ W, $t = 0.0033$ s and $N = 1.3448 \times 10^{19}$ molecules/cm³:

$$\begin{aligned}
 N_{RB} &= 0.5 \times [(g_p N \phi \delta) / (\tau f)] \times [\lambda / (h c)]^2 \times (P / A)^2 \times t \\
 &= 0.5 \times [(0.664 \times 1.3448 \times 10^{19} \times 1 \times 10 \times 10^{-50}) / (1 \times 10^{-13} \times 8 \times 10^7)] \times [7.8 \times 10^{-5} / (6.626 \times 10^{-34} \times 3 \times 10^{10})]^2 \times (1.2 \times 10^{-2} / 3.629 \times 10^{-9})^2 \times 0.0033 \\
 &= 3.1 \times 10^{22} \text{ radicals/cm}^3
 \end{aligned}$$

Figure 5.4. Calculated number of initiating RB molecules against average laser power and laser dwell time. The region within the two dotted lines represents optimal conditions for microfabrication. Region (I), (II) and (III) are corresponding to those labelled regions in Figure 5.2.

Stronger laser power and longer laser dwell time enable the generation of more initiating RB molecules for polymerization and crosslinking of NIPAAm molecules within the exposure zones (Figure 5.4). When stronger laser powers are employed, shorter laser dwell times are needed to trigger the requisite number of initiating RB molecules for promoting polymerization and crosslinking of NIPAAm monomers (Figure 5.4). However, no well-defined microstructures can be fabricated at $N_{RB} \leq 3.3 \times 10^{22}$ radicals/cm³ (Region

(I)) regardless of laser power and/or laser dwell time. This is attributed to the poor photo-induced polymerization and crosslinking events during laser irradiation (Figure 5.2; Figure 5.4). Hence, 3.3×10^{22} radicals/cm³ of N_{RB} is the minimum requirement for successful microfabrication. However, when N_{RB} exceeds 1.6×10^{23} radicals/cm³, internal burning and microstructure explosion are observed (Figure 5.2, Figure 5.4, Region (III)). Moreover, calculated data shown in Figure 5.4 are in good agreement with experimental results illustrated in Figure 5.2. Consequently, the efficiency for the two-photon polymerization process can be reflected more accurately by the number of initiating molecules (N_{RB}) generated than by the calculated energy dose per area (E_d). Based on the sweep test results, the very broad processing window for two-photon induced polymerization and crosslinking of our aqueous-based environmental-friendly photoresist endows robustness to our PNIPAAm photoresist.

The impact of layer distance (Δd) on structural integrity of the PNIPAAm cylindrical microstructures (diameter = 5 μm and height = 3 μm) is also investigated (Figure 5.5). It is to figure out what is the minimum Δd for well-defined microstructures. We vary the Δd from 100 nm to 1000 nm by maneuvering movement of the piezoelectric stage in the z-direction during two-photon lithography via executing commands in the NanoScribe software.^[1, 8] Laser power and laser dwell time remain constant with value of 20 mW and 5 ms, respectively, throughout the experiments. The SEM micrographs illustrate the structural integrity of PNIPAAm cylindrical microstructures constructed using layer distance range of 100 – 800 nm are considerably well-structured (Figure 5.5A). However, microstructure fabricated with $\Delta d \geq 800$ nm becomes slanted and has a crater on its top surface (Figure 5.5A). The results verify that the upper limit of Δd is 800 nm in order to create structurally stable PNIPAAm microstructures.

Figure 5.5. (A) SEM micrographs of 3D PNIPAAm cylindrical microstructures constructed using a layer-by-layer laser writing process. The distance between each neighbouring layer is termed the layer distance (Δd). (B, C) Measured dry-state top area and height of the PNIPAAm cylindrical microstructures fabricated with different layer distances, respectively.

The top surface and height of the PNIPAAm microstructures shrinks upon drying. Shrinkage degree of the top area and height increases with Δd (Figure 5.5B and 5.5C). Top area of the PNIPAAm cylindrical microstructures decreases from $(20 \pm 1) \mu\text{m}^2$ at $\Delta d = 100$ nm to $(13 \pm 2) \mu\text{m}^2$ at $\Delta d = 1000$ nm—equals to 65 % of reduction in area of the top surface. On the other hand, the PNIPAAm cylindrical microstructures also experience a 67 % reduction in height from $(4.2 \pm 0.8) \mu\text{m}$ to $(2.8 \pm 0.5) \mu\text{m}$ when Δd increases from 100 nm to 1000 nm, respectively. The decrease in top surface area and height of the PNIPAAm microstructures as Δd increases are due to the limited amount of photo-induced polymerization and crosslinking.^[1, 8] When PNIPAAm microstructures are fabricated at $\Delta d = 100$ nm, the microstructures interact longer with laser pulses, and hence expose to higher

energy dose.^[31] It is expected that microstructures with $\Delta d = 100$ nm possess the highest degree of polymerization and crosslinking density. As a result, microstructures fabricated at $\Delta d = 100$ nm are sturdier and have insignificant shrinkage even after drying (top surface area measured in water at 20 °C and at dry state are $(19 \pm 2) \mu\text{m}^2$ and $(20 \pm 1) \mu\text{m}^2$ (Figure 5.5B), respectively). On the other hand, when Δd increases, fabricated PNIPAAm microstructures become softer due to reduction in events of polymerization and crosslinking. These shrinkage results determined at dry state provide a clue on the maximum shrinkage the PNIPAAm cylindrical microstructures experience at the top surface area and in height. The extends of shrinkage of the microstructures at dry state (in terms of top surface area and height) are treated as benchmarks, since they are the smallest achievable shrinkage, for studying dynamic swelling-shrinking behaviours of the PNIPAAm microstructures in water at different temperatures.

We investigate the dynamic thermal responsiveness of the PNIPAAm microstructures through various thermal-induced shrinking experiments. The shrinking abilities of the PNIPAAm microstructures above the lowest critical solution temperature (LCST) of PNIPAAm are quantified by the percentage area shrinkage $[(A_{20} - A_{Temp}) / A_{20}] \times 100 \%$, where A_{20} and A_{Temp} represent top surface areas measured at 20 °C and at any other temperatures, respectively. A general trend for the PNIPAAm microstructures is observed when top surface area of the microstructures decreases with increasing water temperature, with the microstructures possessing the smallest area at 40 °C (Figure 5.6A). This is because 40 °C is above the LCST of PNIPAAm— which is 32 °C.^[16, 17, 28] At 40 °C, chains and/or networks of PNIPAAm are expected to shrink and collapse due to unfavourable entropy of mixing which in turn increase the chain/network hydrophobicity and induce water exclusion.^[16, 17] On the contrary, PNIPAAm chains/networks become completely hydrated and the microstructures swell at 20 °C due to the presence of excessive intra- and

inter-molecular hydrogen bonding formed between the hydrophilic amide functional groups and water molecules (Figure 5.6).^[16, 17] Moreover, the degree of this reversible shrinking capability can be effectively manipulated by changing the layer distance (Δd), and therefore the crosslinking density, during microfabrication.^[19, 28]

Figure 5.6. (A) Optical images of heat-dependent shrinking of PNIPAAm cylindrical microstructures constructed with different layer distances are obtained at (i) 20 °C and (ii) 40 °C, and (B) their top surface area shrinkage percent. The dotted circles outline the top surface for better visualization. Top surface area shrinkage percent is defined as $[(A_{20} - A_{Temp}) / A_{20}] \times 100 \%$ as A_{20} and A_{Temp} are top surface areas measured at 20 °C and at any other temperatures, respectively. (C) Reversible swelling of the PNIPAAm cylindrical microstructures of layer distances 200, 400, 600 and 800 nm over four swelling-shrinking cycles. Dotted orange outlines are areas used for calculations.

The PNIPAAm microstructures fabricated with a larger Δd shrink more extensively in water at 40 °C (Figure 5.6). The trend is in good agreement with our findings obtained from Figure 5.5. By increasing the Δd of the PNIPAAm microstructures from 200 nm to 800 nm, the top surface area shrinkage percent of the microstructures increases from $(27 \pm 3) \%$ to $(44 \pm 2) \%$ (Figure 5.6B). The PNIPAAm layers enable the hydrogels to undergo reversible shrinking transition in response to temperature changes in the immersing water.^[16-18] PNIPAAm microstructures with larger laser scanning layer distance are less compact. Therefore, at larger laser writing layer distance, PNIPAAm chains have higher flexibility and have more residing water molecules to be expelled at temperature above the LCST, resulting in greater thermo-sensitivity and higher top surface area shrinkage percent of the PNIPAAm hydrogels.^[19] Consequently, we are able to precisely control the degree of polymerization, crosslinking density and thus area shrinkage percent of the PNIPAAm microstructures by carefully manipulating Δd during two-photon lithography.

In terms of the results obtained from determining dynamic swelling-shrinking capability of our PNIPAAm microstructures, the area shrinkage percent of the 3D hydrogels at 40 °C/ 20 °C with respect to the Δd are also greatly reproducible (Figure 5.6C). This is verified through four consecutive swelling/de-swelling cycles using 8 cylindrical microstructures with dimensions same as those used in experiments mentioned in previous sections. Furthermore, Δd used are 200 nm, 400 nm, 600 nm and 800 nm. The PNIPAAm microstructures exhibit excellent consistency in area shrinkage percent over 4 swelling/shrinking cycles (Figure 5.6C), with the average top area shrinkage percent ranging from 27 % to 44 % at 40 °C as layer distance of the PNIPAAm microstructures increases from 200 nm to 800 nm.

The presence of crosslinks within the PNIPAAm hydrogels enable the microstructures to retain their structural integrity during the swelling and de-swelling cycles, even the microstructures which are fabricated with $\Delta d = 800$ nm. These crosslinks further contribute to the highly reproducible dynamic swelling/shrinking performance of the PNIPAAm microstructures (Figure 5.6C). The slight deviation from original top surface area after first swelling/deswelling cycle can possibly be due to insufficient equilibrium cooling time at 20 °C for the PNIPAAm chains/networks to relax back to their initial extended state.

The precise spatiotemporal control of the two-photon induced polymerization and crosslinking grants the fabricated PNIPAAm microstructures with well-defined structures and excellent programmable swelling/shrinking behaviors. By establishing knowledge on the swelling/shrinking properties of a PNIPAAm hydrogel, we can further extend its programmable shape-change advantages for achieving anti-counterfeiting, molecular sieves and 3D shape-shifting microstructures.

5.4 Conclusion

In summary, we have successfully fabricated poly(N-isopropylacrylamide) (PNIPAAm) microstructures with excellent dynamic thermo-responsive behaviors using our formulated NIPAAm photoresist via two-photon lithography. It is the first report utilizing a xanthene dye — rose bengal — as photoinitiator for triggering two-photon induced polymerization of NIPAAm during fabrication of dynamic microstructures. We adopt the reported general rules during the formulation of stable aqueous-based hydrogel photoresists. The rules are the prepared photoresists (1) must contain suitable photoinitiator(s) to be two-photon polymerizable, (2) have to be optically transparent in the near-infrared wavelength to prevent one-photon absorption and generate excessive heat, (3) can be cured rapidly within the confined laser focal spot, and (4) can produce mechanically stable structures with resistance to withstand subsequent structure development.^[32] Our innovative exploration opens up new opportunities for 3D microfabrication of functional microstructures with water-soluble photoresists. Moreover, our NIPAAm photoresist has very broad processing window which offers great versatility in selecting appropriate writing conditions for architectural designs to achieve functional properties such as anti-counterfeiting, molecular sieves and 3D shape-shifting microstructures. The shrinkage variance in the PNIPAAm hydrogel microstructures also showcases good precision in spatiotemporal control of the hydrogel crosslinking density and the resultant shrinkage percent. Therefore, our NIPAAm photoresist is a promising candidate which holds great potential applications in tissue engineering, construction of molecular sieves for ultrafiltration and detection, as well as in fabrication of shape-change 3D hydrogels with customized microstructures. Additionally, our NIPAAm photoresist formula is also suitable for two-photon induced polymerization of other olefinic compounds containing

stimuli-responsive functional groups, for instance acrylic acid, sulfonic acid, allylamine, vinyl imidazole, *etc.*

5.5 Experimental Section

5.5.1 Materials. N-Isopropylacrylamide (NIPAAm) (> 97 %), N, N'-methylenebis(acrylamide) (bis-AAm) (99 %), rose bengal (RB, 95 %), triethanolamine (TEOA, $\geq$ 99 %), (3-aminopropyl)triethoxysilane (APTES, 99 %), dimethyl sulfoxide (DMSO; AR grade) and ethanol (AR grade) were purchased from Sigma Aldrich. 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.

5.5.2 Preparation of NIPAAm Precursor Solution. NIPAAm (30.0 mg), bis-AAm (1.25 mg), RB (1.25 mg) were dissolved in solution containing 8.5 μ L of nitrogen-purged DMSO and 49.0 μ L of nitrogen-purged water. 4.0 mg of TEOA was then added to the as-prepared NIPAAm solution. The solution was brought for sonication and was used immediately for microfabrication once solids were dissolved.

5.5.3 Surface Treatment of Glass Substrates. Both sides of glass coverslips (22 mm $\times$ 22 mm, with thicknesses of 0.13–0.16 mm) were cleaned with oxygen plasma for 5 mins at 100 W. The coverslips were then submerged in 2 % (v/v) APTES in ethanol for 5 mins, followed by rinsing with a 50 % (v/v) water/ethanol mixture. Subsequently, the substrates were blown dry with nitrogen gas and dried in a 65 °C oven.

5.5.4 Fabrication of PNIPAAm Microstructures. Poly(N-Isopropylacrylamide) (PNIPAAm) microstructures were generated using the Nanoscribe® Photonic Professional. Details of the Nanoscribe® Photonic Professional system can be found in the Experimental Section of Chapter 3. Photoresist of NIPAAm was deposited on the APTES-treated glass

coverslips and all microstructures were written on the glass substrate. Photo-induced polymerization and crosslinking of NIPAAm and bis-AAm began from the interface between photoresist and glass coverslip. The fabricated hydrogel microstructures were therefore securely anchored to the surface of the glass coverslips even after development. At the end of the lithography, the coverslips were gently rinsed with water to get rid of unreacted precursors.

All individual circular microstructures fabricated for systematic studies on operating conditions have a diameter of 5 μm and 3 μm in height. Laser power, laser scan speed and slicing distance along z-axis were varied during tuning experimental conditions to obtain two-photon lithography operating windows.

5.5.5 Scanning Electron Microscopy (SEM) Imaging. Samples were sputter-coated with platinum particles at 20 mV for 30 s using a JEOL JFC-1600 AUTO FINE COATER. Platinum-coated samples were characterized with a JEOL 7600F SEM, where accelerating voltage of 5 kV and beam current of 6 in LEI mode were used.

5.5.6 Materials Characterization. Bright-field optical images of the PNIPAAm microstructures obtained at different temperatures were conducted using an Olympus BX51BD microscope and a 60 $\times$ water-immersion objective lens (Nikon NIR APO, DIC N2, WD 2.8 mm). Temperature of the glass substrate and water was controlled using a Linkam T95-PE System Controller (Linkam Scientific Instruments Ltd). The increment (reduction) in temperature when heating (cooling) the sample and water was set to 5 $^{\circ}\text{C}/\text{min}$ with equilibrium time fixed at 5 mins for every 10 $^{\circ}\text{C}$ interval.

5.5.7 Data Analysis. Determination of area shrinkage percent of the as-fabricated PNIPAAm microstructures were conducted using ImageJ. Area shrinkage percent was obtained from equation $[(A_{20} - A_{Temp}) / A_{20}] \times 100 \%$ as A_{20} and A_{Temp} are areas measured at 20 °C and at any other temperatures, respectively. Energy dose per area (E_d) was calculated using equation $(P \times t) / A$, where P represents laser power, t as laser dwell time and A is laser lateral spot size.^[29]

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Chapter 6

Summary and Outlook

6.1 Summary

Two-photon lithography is a versatile technique for creating well-defined targeted three-dimensional (3D) nano- and microstructures, by virtue of its proficiency in precise spatiotemporal control in the 3D space with nanometer-scaled accuracy. Furthermore, to achieve miniature multi-functional biomimetic architecture, novel self-formulated two-photon polymerizable photoresists containing molecules with stimuli-responsive moieties are extensively investigated among this scientific community. Although it is a thrilling exploration, inconsistent properties of the reported self-formulated photoresists hamper the ability to achieve large-area fabrication of intricate biomimetic microstructures. In this thesis, besides generating micropatterns using commercially available acrylate-based photoresist, we also systematically resolve stability issues of the self-prepared stimuli-responsive hydrogel photoresists using simple yet effective approaches.

In Chapter 2, fabrication of anticounterfeiting security labels is conducted via two-photon lithography using acrylate-based commercially available photoresist (IP-L 780). The micropatterns are subsequently coated with aluminum (Al) through thermal evaporation for surface-enhanced Raman spectroscopy (SERS) study. We demonstrated that SERS activity of the Al nanostructures can be extended from the conventional deep-UV/UV to the visible range, with average SERS enhancement factors exceeding 10^4 . The Al nanostructures also retain $> 70\%$ of their original SERS intensities after one-month storage. Notably, the observed angular orientation-dependent SERS response of the Al nanostructures further contribute to the construction of micron-sized readable hybrid

quick-response (QR) code with two-tier of security features. The versatility of my established approach in generating Al-based plasmonic patterns with high complexity highlights their potential usage for low-cost multiplex data storage, disposable label-free plasmonic nanosensors and anti-counterfeiting labels.

In Chapter 3, we presented the first quantitative analysis on aqueous-based bovine serum albumin (BSA) photoresists for two-photon lithographical fabrication of microstructures with high aspect ratios (> 10) and uniform pH-responsive swelling over areas $> 10^4 \mu\text{m}^2$. We illustrated the use of dynamic viscosity to quantitatively determine the relationship between BSA photoresist properties and the structural integrity of the fabricated microstructures. By systematically investigating the complex process-structure-property relationships in the BSA system, we elucidated the role of various chemical components (such as concentrations of protein and photoinitiator) in the photoresist affecting the viscosity of the photoresist. For the first time, we demonstrated the addition of an inert high molecular weight polymer to enhance the photoresists' viscosity for the successful fabrication of responsive microstructures with excellent mechanical properties over large areas. The optimized photoresist can be potentially used for high throughput fabrication of microstructures without posing any limitations on physical dimensions and its stimuli-responsiveness.

In Chapter 4, we employed the formulated BSA photoresist to fabricate pH-induced geometrical shape-shifting BSA microstructures. To achieve multiple-directional geometrical shape-shifting microstructures, we derived three governing rules to generate corner-to-arc transformation during shape-shifting. These rules involve specific shape, orientation and quantity of the embedded rigid skeletal frames to guide the responsive BSA matrices undergoing anisotropic swelling into specific geometric shapes. It is a simple and conducive approach to create a library of microstructures with programmable dynamic

geometrical shape-shifting behavior. We also constructed reconfigurable 2D lattices capable of undergoing pH-triggered reversible lattice transformations by positioning our microstructures into periodic arrays. These reconfigurable lattices confer tunable structure-to-physical properties of the arrays by systematic modulation of unit cell structures and area, as well as pore size. Thus, our reconfigurable lattices are promising especially in developing of on-demand smart materials as biomimetic and microfluidic devices.

In addition to BSA photoresist, we are also actively involved in exploring promising formulations for other precursors containing stimuli-responsive moieties which are suitable for two-photon lithography. In Chapter 5, an aqueous-based photoresist containing N-isopropylacrylamide (NIPAAm) precursors is successfully prepared for microfabrication of 3D thermo-responsive poly(N-isopropylacrylamide) (PNIPAAm) microstructures. Rose bengal (RB) was employed as photoinitiator to enhance efficiency of two-photon induced polymerization of NIPAAm, with the assistance of triethanolamine. We quantitatively optimized laser power and laser dwell time for the construction of PNIPAAm microstructures with high structural integrity. By manipulating the laser scanning layer distance along the z-axis, crosslinking density of the PNIPAAm microstructures were precisely controlled and hence the thermal responsiveness of the written PNIPAAm microstructures. Importantly, this two-photon polymerizable NIPAAm formulation can potentially be extended to other olefinic molecules with stimuli-responsive functional groups in order to realize multi-functional engineered biomimetic microstructures.

6.2 Outlook

Microfabrication is a process to achieve miniaturization of physical structures with micrometer to submicrometer scales for enhancing performance or overcoming limitations of existing products fabricated via conventional methods.^[1-4] Conventional microfabricating techniques are either limited to 2D structures or cannot provide submicrometer scale resolution.^[1, 2] The aforementioned issues render two-photon lithography as an appealing technique to generate structurally sophisticated 3D microstructures with precise details. To move forward with the aim to broaden selection of photoresists, two-photon lithography processable photoresists have been widely researched and improved on. Photoresists containing stimuli-responsive components are currently capturing the attention of the research communities for the microfabrication of functional microstructures.^[5-7]

Although it is encouraging to have aqueous-based N-isopropylacrylamide (NIPAAm) photoresist be developed for the construction of temperature-sensitive PNIPAAm hydrogel microstructures, the formulation reported in Chapter 5 is still unsatisfactory for fabricating suspending and high-aspect-ratio microstructures. It is essential to investigate and improve dynamic viscosity of the formulated photoresists, which measures resistance of a fluid to flow when shear force is applied. As reported, movement of the substrate during two-photon lithography induces drift in low-viscosity photoresists in directions the substrate set to motion leading to deformation of written structures.^[8] Therefore, to prevent structural distortion due to drift in liquid photoresists, viscosity of the photoresists can be improved by increasing concentration of polymerizable materials, using solvent(s) of high viscosity such as glycerol and ethylene glycol or introducing viscosity-enhancing agents such as polyvinylpyrrolidone, polyethylene oxide, cellulose or high-molecular weight PNIPAAm

chains.^[9, 10] With sufficient knowledge on the interrelationship between physical parameters of the photoresists containing monomers with stimuli-responsive moieties and chemical/physical properties of fabricated microstructures, we can expand currently available aqueous-based two-photon processable photoresists to realize microfabrication of advance engineered 3D architectures with multiple functionalities.

Network structures are commonly found in nature, for instance resurrection ferns, wheat awns, pine cones and animal dermis.^[11-13] They compose of hierarchically arranged rigid fibres reinforced with elastic networks to form sturdy yet flexible materials, allowing these structures to respond steadily to environmental stimuli. Besides changing swelling properties through modulation of laser scanning layer distance along z-axis, we hypothesize that by introducing highly-crosslinked wired meshes of different architectural orientations to soft stimuli-responsive matrices, we can extend our capability in generating microstructures with on-demand out-of-plane shape-shifting Origami microstructures.^[4, 14] Microstructures fabricated based on as-mentioned bio-inspired design concepts are expected to demonstrate considerable actuating efficiency and instantaneous shape change.^[11, 15, 16]

For future progress in fabricating stimuli-responsive microstructures, I propose to construct single microstructures with dual stimuli responsiveness, for instance pH and temperature. This may act as a foundation to render microstructures with multi-functionalities. In addition, I also elucidate shape-directed self-assembly of shape-shifting microstructures into long-range planar superlattices at air-liquid or liquid-liquid interface. It is inspired by the “Cheerios effect” on the “self-assembly” of light-weight substances at air-liquid interface which can be observed in our daily life, for instance clustering of breakfast cereal, chili flakes or paper clips at water surface.^[17] Non-shape-shifting binary anisotropic microstructures of same materials with complementary size and shape will be

firstly investigated to systematically achieve the desired assembly conditions. Our understanding on the rational structural design and involved mechanisms will be crucial towards exploring the potentials of this shape-specific assembly approach. The findings will then be extended to generate long-range reconfigurable superlattices with predictable and designable arrangements of same materials by using stimuli-responsive shape-shifting building blocks, which is currently not exploited.^[18] Thereafter, we are going to study shape complementary guided self-assemblies of anisotropic structures made of different materials. We can then gain insights on influences of external stimuli on interactions between the anisotropic structures of different materials, and hence phase transitions and change in properties of the assembled superlattices.^[19]

Followed by the successful development of various aqueous-based stimuli-responsive hydrogel photoresists and printing approaches, the ultimate goal of the shape-shifting project is to realize intelligent maneuverable micro-robots with efficient signal transduction networks and optimum structural designs and possess chemotaxis response similar to *E. coli* bacteria.^[20] Micro-robot chemosensation and embedded signal transduction pathway will be investigated to understand how the as-designed wireless responsive micro-robots perceive environmental changes. Furthermore, combined effects of stimuli-induced changes to packing interactions between numerous individual receptor subunits that may act to control behavioral responses and adaption mechanisms of the micro-robots will be identified. The factors on the chemical sensing abilities, structure mobility and proposed solutions on navigation will be tested and understood to provide a guide in materials selection and formulations, signal transduction pathway modulation and model design. These valuable insights will facilitate the exploration and invention of new generation of miniature mobile intelligent structures.

6.3 References

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List of Publications

1. Yih Hong Lee, **Chee Leng Lay**, Wenxiong Shi, Hiang Kwee Lee, Yijie Yang, Shuzhou Li, Xing Yi Ling; Creating Two Self-Assembly Micro-environments to Achieve Supercrystals with Dual Structures using Polyhedral Nanoparticles; Nature Communications, ASAP.
2. **Chee Leng Lay**, Charlynn Sher Lin Koh, Jing Wang, Yih Hong Lee, Ruibin Jiang, Yijie Yang, Zhe Yang, In Yee Phang, Xing Yi Ling; Aluminum Nanostructures with Strong Visible-range SERS Activity for Versatile Micropatterning of Molecular Security Labels; Nanoscale, 2018, 10, 575-581.
3. Mian Rong Lee, Hiang Kwee Lee, Yijie Yang, Charlynn Sher Lin Koh, **Chee Leng Lay**, Yih Hong Lee, In Yee Phang, Xing Yi Ling; Direct Metal Writing and Precise Positioning of Gold Nanoparticles within Microfluidic Channels for SERS Sensing of Gaseous Analytes; ACS Applied Materials & Interfaces, 2017, 9, 45, 39584-39593.
4. Yijie Yang, Yih Hong Lee, **Chee Leng Lay**, Xing Yi Ling; Tuning Molecular-Level Polymer Conformations Enables Dynamic Control over Both the Interfacial Behaviors of Ag Nanocubes and Their Assembled Metacrystals; Chemistry of Materials, 2017, 29, pp 6137–6144.
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6. **Chee Leng Lay**, Mian Rong Lee, Hiang Kwee Lee, In Yee Phang, Xing Yi Ling; Transformative Two-Dimensional Array Configurations by Geometrical Shape-Shifting Protein Microstructures; ACS Nano, 2015, 9, 9708-9717.