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Fabrication and Applications of Metallic and Polymeric Smart Materials using Two-photon Lithography

Lee Mian Rong

**Supervisor: Associate Professor Ling Xing Yi
Division of Chemistry and Biological Chemistry
School of Physical and Mathematical Sciences**

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Abstract

This thesis details my PhD research from the past four years focused on the fabrication and applications of metallic and polymeric shape-shifting “smart” materials using the technique of two-photon lithography. I begin the thesis with a brief introduction into the principles of two-photon lithography, followed by applications of two-photon lithography in the fabrication of polymeric shape-shifting materials, and the direct laser writing of metallic structures.

From the fabrication of polymeric shape-shifting materials, the main challenges I identify are that currently, (1) most of the polymeric shape-shifting materials are macroscale, and (2) usually exhibit only directional shape change such as bending or twisting. I aim to resolve these issues by demonstrating the fabrication of 10 - 30 μm sized shape-shifting protein microstructures that are not only able to undergo quantifiable directional shape-shifting (chapter 2), but also geometrical shape-shifting from circles to polygons such as triangles, square, pentagons and hexagons (chapter 3).

I also discuss some of the challenges faced in direct metal writing; for instance, although direct metal writing has been explored extensively for the past decade, most fabricated structures comprise sparse, or dense but irregularly shaped nanoparticles. I aim to fabricate monodisperse, dense gold nanoparticle microstructures where the physical and optical properties can be tuned by varying surfactant concentration, and fabricate these microstructures within a microfluidic channel for SERS sensing of small gaseous molecules (chapter 4). I also recognise that the precision and morphology of fabricated metallic structures are largely dependent on the fabrication parameters and composition of the photoresist used, however, an investigation into the effects of varying fabrication parameters on the resulting structure is lacking. Hence, I create a robust Au precursor solution and optimized the writing parameters to fabricate highly precise, well-defined Au microstructures even after long hours of lithography (~4 hours) (chapter 5). Finally, I conclude the thesis with a short summary and outlook to discuss future work in the field of two-photon fabrication (chapter 6).

1 Introduction

ABSTRACT. Advancements in lithography have enabled researchers to create two-dimensional (2D) and three-dimensional (3D) structures from various materials as sensors, integrated circuits, anti-counterfeit labels, shape-shifting structures for drug delivery and surgery. Of which, multi-photon lithography emerges as a promising technique to fabricate high resolution 2D and 3D, micro- to millimeter sized structures with nanometer-scale resolution, well beyond the diffraction limit of light. In the introduction, I will explore briefly different types of lithographic techniques, followed by a discussion of the principles and mechanism of two-photon lithography applications of two-photon lithography to the two-photon polymerization of functional, stimuli-responsive polymeric structures, and the two-photon photoreduction of metallic salts to form metallic microstructures and their applications.

1.1 HISTORY OF LITHOGRAPHY

Lithography, meaning “to write in stone”, is a technique of printing that was invented in Bavaria by celebrated German actor and author Johann Alois Senefelder in 1796.¹ An oil-based pattern was typically pressed onto a smooth limestone slab and subsequently treated with acid, causing areas of the stone that were not protected by the oil-based pattern to etch away. Following which, an oil-based ink was then applied to the wet, etched stone slab, which adsorbs only onto the pattern and not on the etched areas which retain water. The inked pattern can then be transferred onto a paper to produce a printed page.² This concept of separating the desired pattern template from the printing surface gave rise to the birth of modern lithographic techniques such as UV lithography, electron beam lithography, x-ray lithography and multiphoton lithography, which enable us to print not only ink, but also 2D and 3D micro- to centi-meter sized structures from various materials such as ceramics, metals and most commonly, organic monomers and polymers.

1.2 TYPES OF LITHOGRAPHY

One of the oldest and most widely utilized modern lithographic techniques is photolithography. Photolithography is a masked lithography technique that utilizes ultraviolet wavelengths to produce a pattern from a polymeric photoresist (light sensitive material).³⁻⁵ Depending on user preference, a negative or positive tone photoresist can be used.⁶ In a negative tone photoresist, areas that are exposed to the light source will be cured and become insoluble in the developer post-lithography, whereas when a positive tone resist is used, exposed areas become soluble in the developer post-lithography and can be easily removed (Figure 1.1).⁷⁻⁸

Figure 1.1. Illustration of differences between positive and negative tone resists. Reproduced with permission from ref. 8. Copyright 1999 American Chemical Society.

In UV lithography, the pattern or shape to be formed is controlled by employing a mask. The mask is essentially a thin transparent wafer that is coated with a material opaque to UV light, such as a deposited metallic layer, to prevent light transmission. If a positive tone photoresist is used, the mask must possess the same pattern as the desired pattern to be printed, conversely, if a negative tone resist is used, the mask must possess the reverse pattern from the one to be printed. Using this technique, Kumacheva et al fabricated centimetre-scale temperature- and pH-responsive shape-shifting gel composite sheets that can tune their shape in accordance to temperature or pH changes, for purposes in sensing and robotics (Figure 1.2).⁹

Figure 1.2. Patterning of temperature and pH responsive shape-shifting macrostructures. Reproduced with permission from ref. 9. Copyright 1999 American Chemical Society.

The key advantage of photolithography lies in the ability to generate the desired pattern quickly as the entire substrate can be exposed to the light simultaneously, however, the limits to the resolution of photolithography is low due to the diffraction limit of light, which is defined using Abbe's equation:¹⁰⁻¹¹

$$d = \frac{\lambda}{2 N.A.}$$

where d is the diffraction limit, λ is the incident wavelength and $N.A.$ is the numerical aperture of the focusing objective lens. Recent advancements in nanotechnology has spurred researchers to work towards fabricating smaller functional devices for applications in nanomedicine, nanoelectronics and nano-optics. Hence, while photolithography is a robust, versatile and inexpensive method to fabricate desired structures quickly, seeking other higher resolution fabrication techniques is still necessary.

Some techniques that have been proposed to achieve higher resolution are X-ray, extreme-UV, and electron beam lithography. X-ray lithography was first demonstrated in 1975 at Bell Laboratories, where Maldonado et al utilized x-rays of 4-6 Å to fabricate 2D zig-zag lines from an organic resist.¹² The resolution of x-ray lithography is ~ 30 nm,¹³ however, as the radiation wavelength is short, the distance between the x-ray source and the lithography mask, as well as between the mask and resist both affect the fabrication resolution.¹⁴ In extreme-UV (EUV) lithography, a CO₂ laser is used to stimulate the emission of 13.5 nm radiation wavelengths from a tin droplet to achieve sub-10 nm resolution.¹⁵ Gonsalves et al were able to fabricate 2D patterns with 25 nm lines from a hybrid organometallic polymer resist.¹⁶ Ekinici et al further reduced structure sizes to 22 nm by decreasing the incidental radiation to ~ 6 nm in beyond-EUV (BEUV) lithography,¹⁷ however, reducing the source radiation even further to ~ 1 nm has met with multiple technical challenges and hence efforts to develop this technique have dwindled.¹⁸ Electron beam lithography offers the highest resolution (~ 1 nm) compared to other lithography methods as electrons are used for fabrication instead of light, hence the resolution of the fabricated structures is limited only by the wavelength of the electrons and not

the diffraction limit. As a result, intricate nanostructures with sub-10 nm feature sizes can be achieved easily.¹⁹⁻²¹ The high resolution that electron beam lithography offers places it at a strong advantage to fabricate complex integrated electrical circuits and microelectromechanical systems.²² However, both the set-up and the vacuum required to generate high resolution electron beams are expensive to upkeep, and current technology also only allows for the fabrication of 2D nanostructures, which limits the variety and functions of structures that can be obtained.

1.3 MULTIPHOTON LITHOGRAPHY (TWO PHOTON LITHOGRAPHY)

Multi-photon lithography is a promising method to create high resolution 2D and 3D, micro- to milli-meter sized structures of nanoscale resolution, well beyond the diffraction limit of light. Currently, this unique capability is unparalleled; while conventional photolithography is able to fabricate 3D structures via a layer-by-layer method, the resolution of these structures is poor (1 – 3 μm), and although electron beam lithography offers significantly better resolution than multi-photon lithography, the complexity of fabricated structures is limited to only 2D. In multi-photon lithography, the photoresist absorbs more than one photon to become excited. To date, only two-photon and three-photon absorption have been achieved. In this thesis, I will focus my discussion on two-photon absorption only.

Two-photon absorption was first described by Maria Goeppert-Mayer (Figure 1.3) in her dissertation in 1930, where she calculated the probability that an electron relaxing from a higher energy orbital to a lower energy orbital could emit 2 photons instead of 1.²³

Figure 1.3. Portrait of Maria Geoppert-Mayer. Reproduced with permission from ref. [34](#). Copyright 2009 Wiley-VCH Verlag GmbH & Co KGaA, Weinheim.

Three decades later, Kaiser and Garrett observed this phenomenon experimentally when a sample of $\text{CaF}_2\text{Eu}^{2+}$ crystal emitted blue fluorescence when it was irradiated with red light. They found the relationship of the intensity of fluorescence observed to be proportional to the intensity of light squared, which is a strong indication of a two photon absorption process.²⁴

1.3.1 MECHANISM OF TWO-PHOTON ABSORPTION

As the name suggests, in two-photon absorption, two photons that are of identical or different energies are absorbed by the photoresist to an excited state. Where the photons are identical, each photon has an energy of $(h\nu/2)$, approximately half the energy gap of the photoresist $(h\nu)$. The absorption process can also be step-wise or simultaneous; in a step-wise absorption process, one photon is first absorbed to a real and stable intermediate state before the second photon, whereas in a simultaneous absorption process, there is no real intermediate state. Instead, one photon is absorbed to a transient, virtual intermediate state, and a second photon will only be absorbed if it appears within the lifetime of the virtual intermediate state (~ 5 fs),²⁵ hence, high intensity lasers are required for two-photon absorption processes. In this thesis, we focus on simultaneous two-photon absorption, and from herein, the term “two-photon absorption” refers to the simultaneous two-photon absorption process.

The high resolution of two-photon absorption arises from the unique interaction between the photoresist and the laser. In single-photon absorption, the material is excited at the particular wavelength of $(h\nu)$, hence absorption follows the Beer-Lambert law and begins on the surface of the photoresist even when it is not the focal plane of the laser (Figure 1.4). In two-photon absorption, however, the material is not excited at the applied wavelength of $(h\nu/2)$. As a result, due to the high laser intensity required to initiate the excitation of the photoresist via a transient intermediate state, excitation only occurs at the focal plane of the laser.²⁶ Hence, 3D structures can be fabricated within the resist, and the resolution of the fabricated structures is mainly determined by the focal volume of the laser spot, which can be tuned by modulating the laser power and exposure time based on the type of photoresist used.²⁷

Figure 1.4. Single-photon absorption as compared to two-photon absorption of fluorescein. Adapted from ref. 26 with permission from Macmillan Publishers Ltd, copyright 2003.

Two photon absorption is defined as a non-linear, “third order” process, and can be illustrated by the following equation, which illustrates the non-linear polarization of an optical material under intense illumination:²⁸

$$P = \epsilon_0 \chi E + \epsilon_0 \chi^2 E^2 + \epsilon_0 \chi^3 E^3 \quad \dots \text{.(Eq. 1)}$$

Where P is the degree of polarization, χ , χ^2 , χ^3 represent the 1st, 2nd, and 3rd order non-linear susceptibilities respectively which also denote the 1st, 2nd, and 3rd order processes – examples of which are linear absorption, second harmonic generation, and two-photon absorption respectively (Figure

1.5),²⁹ E is the magnitude of the applied electromagnetic field and ϵ_0 denotes the permittivity of free space.

Figure 1.5. Scheme illustrating the 1st, 2nd and 3rd order processes

Because there is no contribution by even-numbered order susceptibilities in resonant processes such as two-photon absorption, the equation can be re-represented as:³⁰

$$P = \epsilon_0 \chi E + \epsilon_0 \chi^3 E^3 \quad \dots \quad (\text{Eq. 2})$$

As with any other electronic transition, there are conditions that must be fulfilled for two-photon absorption to occur. First, in an allowed transition, the angular momentum must be conserved, that is, because photons possess their own angular momentum, the absorbing atom or molecule must experience a change in angular momentum state in exchange for the gain in photon angular momentum.³¹

Second, for centrosymmetric molecules (molecules possessing a symmetry of inversion), parity must be conserved for two-photon absorption. While non-centrosymmetric molecules generally enjoy less restrictive selection rules, for centrosymmetric molecules, a special condition termed the Laporte selection rule applies. This selection rule states that in a *single-photon* electronic transition, there must be a change in parity for the transition to be “allowed”.³² The concept of parity is abstract and can be thought of as a form of symmetry; if the inversion of spatial coordinates does not change the sign of the angular momentum wavefunction, then the parity is said to be even, or *gerade* (*g*).³³ On the other hand, if the sign of the angular momentum wavefunction changes, the parity is odd, or *ungerade* (*u*). Hence in *single-photon* absorption, an “allowed” electronic transition for centrosymmetric molecules would be from $g \rightarrow u$ or $u \rightarrow g$, whereas $g \rightarrow g$ and $u \rightarrow u$ transitions are

“forbidden”. However, in *two-photon* absorption, the Laporte selection rule is relaxed. This is because upon the absorption of the first photon, the transient, virtual intermediate state that is generated assumes a parity opposite to that of the initial state.³⁴ The presence of this transient opposite parity then enables an incoming second photon to induce an electronic transition to the final state, and hence in two-photon absorption, $g \rightarrow g$ and $u \rightarrow u$ transitions are “allowed” (Figure 1.6).

Figure 1.6. Two-photon absorption in centrosymmetric molecules compared to non-centrosymmetric molecules. Reproduced with permission from ref. [34](#). Copyright 2009 Wiley-VCH Verlag GmbH & Co KGaA, Weinheim.

1.4 APPLICATIONS OF TWO-PHOTON LITHOGRAPHY

1.4.1 TWO-PHOTON POLYMERIZATION

In recent years, the concept of two-photon absorption has been most popularly applied to polymerization processes for the lithography of precise structures. Two-photon polymerization is usually initiated by an ultrafast, near-infrared laser, and the precision of the structures written is a result of the significantly smaller linewidth that two-photon absorption provides as compared to single-photon absorption. The first demonstration of two-photon polymerization was in 1997 when, faced with the difficulties of using photolithography to synthesize precise 3D-structures for use in microfabrication, Maruo et al proposed the use of two-photon-absorbed polymerization to achieve higher spatial resolution through better focused lasers in the lithography of defined, 3D-structures.

The team fabricated a partially free-standing microhelix with potential applications in optical studies.³⁵ Subsequently, Kawata et al fabricated a highly detailed dancing ballerina and microbull ~ 10 μm long, similar to the size of an erythrocyte.³⁶ This precision in fabrication further highlights the potential of two-photon lithography as a 3D microfabrication technique to construct polymeric scaffolds,³⁷⁻³⁹ and structures for shape-shifting and recognition,⁴⁰⁻⁴² data storage,⁴³⁻⁴⁵ anti-counterfeiting⁴⁶⁻⁴⁸ and surface-enhanced Raman scattering (SERS) sensing applications.⁴⁹ In this thesis, we focus on shape-shifting as an application and in particular, we will like to promote two-photon polymerization for the fabrication of shape-shifting microstructures.

1.4.1.1 APPLICATIONS OF TWO-PHOTON POLYMERIZATION: SHAPE-SHIFTING

1.4.1.1.1 INTRODUCTION TO SHAPE-SHIFTING

Shape-shifting is a phenomenon often seen in nature, where plants and animals, such as the venus flytrap, the pufferfish and the mimosa, undergo a geometrical or unidirectional change in shape in response to a stimulus by bending or twisting.⁵⁰⁻⁵² In synthetic polymers, this ability to shape-shift is mimicked using a special group of polymers known as “smart materials”, or “stimuli-responsive materials”, which demonstrate the capability to sense and “respond” reversibly to an environmental stimulus by changing either physical properties, chemical properties, or generating an electromagnetic response.⁵³ These materials can be further classified by the type of stimulus that the material responds to, such as temperature,⁵⁴ pH,⁵⁵⁻⁵⁶ light,⁵⁷⁻⁶⁰ humidity,⁶¹⁻⁶³ magnetism,⁶⁴ chemical stimuli,⁶⁵⁻⁶⁶ and can be used to create smart structures that can perform sensing, changes in color or shape-shifting and actuation (Figure 1.7).

Figure 1.7. Laser-induced folding of microstructures. The microgrippers close immediately upon irradiation. Scale bars are 500 μm in (b) and 1 mm in (c). Reprinted from ref. [60](#), with the permission of AIP Publishing.

Shape-shifting polymeric structures are commonly fabricated via photolithography, where structures range from millimetre to centimetre sizes. In most cases, such polymeric structures are hydrophilic and absorb water, hence they are termed “hydrogels”. Stimuli-responsive shape-shifting in hydrogels can hence be defined as the non-uniform swelling of localized areas within a material. This can be attained by using a mixture of materials⁶⁷⁻⁶⁹ or varying the cross-linking density or responsiveness within different areas of a single-phase material to generate a gradient osmotic potential across the hydrogel.⁷⁰⁻⁷¹ As a result, the hydrogel undergoes anisotropic swelling upon water uptake and hence a change in shape.

1.4.1.1.2 TECHNIQUES TO INDUCE SHAPE-SHIFTING: MIXTURE OF MATERIALS

Inducing shape-shifting by incorporating a mixture of materials within the same structure can be achieved firstly through coupling a stimuli-responsive polymer with a non-responsive polymer, where the non-responsive polymer either functions as a reinforcement structure to restrict movement

(swelling) in the stimuli-responsive polymer, or undergoes a shape change due to the swelling of the stimuli-responsive polymer. Gracias et al fabricated segmented, origami-like microstructures using a thermoresponsive polymer, poly(*N*-isopropylacrylamide-*co*-acrylic acid) as the main structure body and a non-responsive polymer, polypropylene furamate as stiff “hinges” along the bending joints of the structures. The “hinges” function as an extrinsic stress to impose swelling restrictions on specific areas of the polymer such that upon swelling, the polymer bends along the restrictive “hinges”.⁷² Ionov et al fabricated a bi-layer hydrogel structure where one layer is thermoresponsive gelatin and the other layer is a non-responsive polymer. The non-responsive polymer restricts swelling on one side of the bi-layer structure allowing the structure to fold and unfold irreversibly when temperature is increased from 22 °C to 37 °C.⁷³ Zhao et al fabricated self-healing, shape changing bi-layer hydrogels by incorporating a non-responsive hydrogel with a polyacrylamide based hydrogel containing carboxylic acids, to form a bi-layer structure which responds to both humidity and pH changes (Figure 1.8). The mechanism behind the shape change is due to the presence of the non-responsive hydrogel that imposes a swelling restriction on one side of the responsive hydrogel.⁵⁵ By selectively incorporating the non-responsive polymer onto specific areas of the structure, an inhomogeneous osmotic potential is created across the structure, causing the structure to swell anisotropically to effect a shape change.

Figure 1.8. Stimuli-responsive shape-shifting of bi-layer structure. (a) the reversible bidirectional bending of a bi-layer hydrogel and (b) the shape changing behavior of a self-healed gel. Reproduced with permission from ref. 55. Copyright 2014 Wiley-VCH Verlag GmbH & Co KGaA, Weinheim.

Two or more polymers which are responsive to different types of stimulus can also be incorporated into a single system to enable the system to respond to various stimuli, or to impart multiple shape change ability. For instance, Aizenberg et al created the Hydrogel-Actuated Integrated Responsive System (HAIRS), which involves curing a stimuli-responsive polymer over non-responsive, high aspect ratio flexible epoxy nanopillar array fabricated from a silicon master. The group extended this system to the incorporation of 2 types, pH-responsive and thermoresponsive polymers selectively onto specific areas of the epoxy nanopillar array via two-photon polymerization. By subjecting the system to a combination of conditions, such as high pH, low temperature, or high temperature, high pH etcetera, specific areas of hydrogel can be programmed to swell or shrink, resulting in multiple shape changes of the epoxy nanopillar array.^{40, 67}

1.4.1.1.3 TECHNIQUES TO INDUCE SHAPE-SHIFTING: SINGLE-PHASE MATERIALS

In a single-phase material hydrogel, the responsiveness of different parts of the same structure can be altered to induce shape change via anisotropic swelling of the hydrogel structure. A famous example is the “self-walking gel” created by Maeda et al. The group incorporated a cyclic reaction, the Belousov-Zhabotinsky (BZ) reaction⁷⁴ within a co-polymer hydrogel containing a covalently bonded Ru(II) catalyst, a thermoresponsive polymer N-isopropylacrylamide (NIPAAm) and an ion-responsive polymer 2-acryloylamido-2-methyl propane sulfonic acid (AMPS). The hydrogel is fabricated between a hydrophilic glass plate and a hydrophobic Teflon plate. As a result, the hydrophilic AMPS assemble mostly along the side of the glass plate while the hydrophobic ruthenium(II) catalyst assembles mostly along the side of the Teflon plate, creating different osmotic potentials on each side of the hydrogel. As the BZ reaction proceeds, the catalyst cycles between an oxidised Ru(III) state and a reduced Ru(II) state, which possess different Lower Critical Solution Temperature (LCST). The LCST is the specific temperature at which the polymer undergoes a hydrophilicity change. Hence at a constant environment temperature, the swelling ability of the

hydrogel changes with the cycling of catalyst oxidation states, enabling the hydrogel to undergo bending and stretching motions to “walk” across a surface (Figure 1.9).⁷⁰

Figure 1.9. Optical images showing the (a) actuation of the self-walking gel and (b) graph showing the displacement of the gel with time. Reproduced with permission from ref. 70. Copyright 2007 Wiley-VCH Verlag GmbH & Co KGaA, Weinheim.

Liu et al fabricated shape memory hydrogels using a Zn^{2+} responsive co-polymer made from acrylonitrile and 2-acryloamino-2-methyl-1-propanesulfonic acid (AMPS). The hydrogel contains multiple cyano groups that can form dipole-dipole interactions with one another. The shape changing ability is introduced via nanoimprinting of patterns into the hydrogel, such that one face of the structure is patterned and the other face is smooth. Soaking the gel into a 50 % ZnCl_2 solution for a short duration allows for Zn^{2+} ions permeating the gel to function as a bridge between 2 cyano groups. As a result, the polymer network contracts and the gel is observed to shrink. The anisotropic shrinking of the patterned gel results in an overall shape change. Depending on the patterns that are imprinted into the hydrogel, the gel can be made to curl into a spiral or twist into a helix (Figure 1.10).⁷⁵

Figure 1.10. The shape-shifting of a single-phase patterned hydrogel in response to Zn^{2+} ions. Depending on the patterns that are imprinted into the gel, the gel can be made to (a – c) curl or (d – f) twist. Reproduced from ref. [75](#) with permission of The Royal Society of Chemistry.

Most polymer chains are able to form inter-chain bonds with structurally related polymers, also known as cross-linking. A high cross-linking density implies that many inter-chain bonds, or cross-links, are formed; the polymer network is hence more rigid and possesses lower swelling ability. On the other hand, a low cross-linking density implies that there are fewer cross-links, hence polymer networks are more flexible and has greater swelling ability. By varying the cross-linking density within a single material, a gradient osmotic potential can similarly be generated across the hydrogel to result in anisotropic swelling and thus shape change.

Varghese et al varied the cross-linking density within a polyelectrolyte gel, (poly(2-acryloylamido-2-methyl propane sulfonic acid) which contains the anion SO_3^-), to control the bending direction of the gel.⁷¹ The hydrogel is placed in a salt bath and an electric field is applied. The negative ions in the salt bath migrate to the anode and positive ions to the cathode, resulting in a build-up of negative ions at the anode. This creates a gradient osmotic potential across the hydrogel, where the anode side of the hydrogel is able to absorb more water and swell to a greater extent, causing the gel to curve towards the cathode, as observed for a highly cross-linked hydrogel. However, in a lightly cross-linked hydrogel, the gel first curves towards the cathode, where upon water absorption, the effective anionic concentration decreases at the anode and increases at the cathode, causing the gel to straighten, and then curve towards the anode instead (Figure 1.11).⁷¹

Figure 1.11. (A) Bending direction of PAMPS hydrogel. The PAMPS hydrogel (B, C) bends towards the cathode when highly cross-linked and (D, E) bends towards the anode when lightly cross-linked. Reproduced with permission from ref. [71](#). Copyright 2011 Wiley-VCH Verlag GmbH & Co KGaA, Weinheim.

1.4.1.2 TWO-PHOTON LITHOGRAPHY OF SHAPE-SHIFTING STRUCTURES: BOVINE SERUM ALBUMIN

Two-photon lithography is a promising technique to fabricate stimuli-responsive shape-shifting structures. The high resolution two-photon polymerization allows for the precise fabrication of intricate, nano- to micro-scale shape-shifting structures that can be potentially applied to intravenous drug delivery, self-assembly and multi-functional lab-on-a-chip substrates. However, there has been very few reports on fabricating shape-shifting microstructures via two-photon lithography.

Bovine Serum Albumin (BSA) is a large, globular protein molecule comprising 583 amino acids and is the most commonly reported polymer used for the two-photon fabrication of shape-shifting microstructures. In 2000, Pitts et al carried out the first two-photon photo-initiated

crosslinking of BSA and another protein, Fibrinogen directly from solution to construct 3D traps, demonstrating the two-photon fabrication of 3D protein structures for the first time.⁷⁶ Butler et al discovered in 2006 that fabricated protein structures, such as Bovine Serum Albumin hydrogels exhibit dynamic swelling properties at extreme pH values.⁷⁷ This finding opened up a plethora of areas for the possible application of a dynamic, responsive system as such, for instance drug delivery,⁷⁸ or the dynamic self-assembly of particles.

The pH responsiveness of proteins arises from the fact that individual amino acids possess a side chain group which can be acidic, alkali, hydrophilic or hydrophobic, where acidic and alkali side chains can be charged or neutral depending on the acid/base dissociation constant (pK_a or pK_b) of the side chain. The combination of amino acids will then determine physical properties, such as overall charge, melting point, solubility as well as isoelectric point of the protein (pI) of a protein, which is the pH at which there is no net charge on the protein (overall charge is zero),⁷⁹ and hence the protein will be the most insoluble in aqueous solutions at the particular pH value. The pI of BSA is ~ 4.7 ; at pH values above and below, the protein is charged and soluble in water.⁸⁰ Hence, fabricated BSA structures are able to absorb water through the formation of ion-dipole interactions to result in swelling.

The polymerization of BSA occurs via a radical process in the presence of a photoinitiator such as Rose Bengal. At the focus point of the laser, Rose Bengal is excited to an excited singlet state, followed by intersystem crossing to an excited triplet state. The excited Rose Bengal triplet state is able to abstract a hydrogen atom from an amino acid³⁰ on BSA to produce a BSA radical and kick-start the photopolymerization process.^{76, 81}

Shape-shifting BSA structures can be created likewise by modulating the cross-linking density in different areas of the same structure. Shear et al investigated varying scan speed and laser power during lithography on the swelling ability of BSA structures and discovered that faster scan speeds and lower laser powers produce structures with higher swelling abilities due to lower crosslinking degree (Figure 1.12).⁸²⁻⁸³

Figure 1.12. (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. ⁸³. Copyright 2008 National Academy of Sciences.

The protein concentration and amount of photoinitiator used were also shown to affect the amount of cross-linking and hence the swelling ability of fabricated structures.⁸⁴ In my thesis, I will utilize another parameter, the z-layer distance to modulate the cross-linking density to fabricate responsive shape-shifting BSA microstructures.

1.4.2 TWO-PHOTON PHOTOREDUCTION

In recent years, advances in metamaterials, nanophotonics and microelectronics have led to increasing demands for patterned, conductive metallic structures with sub-micrometer resolution. Although wet chemistry methods remain the most popular for the synthesis of metallic nanostructures

due to the ease of fabrication as well as the promise of nanoparticle stability,⁸⁵⁻⁸⁶ such nanostructures cannot be easily assembled into desired designs with high precision. High resolution metallic patterns can also be fabricated using laser lithography and electron beam lithography, where the location and pattern of the nanostructures can be precisely programmed. However, as previously mentioned, electron beam lithography techniques are both technically challenging as there are multiple complicated steps to undertake, and logistically exhausting as the high vacuum facilities are expensive to upkeep.

In contrast, laser lithography, in particular two-photon lithography is a prominent, high resolution technique for the easy fabrication of metallic sub-micrometer to nano-structures as the process can be performed at ambient condition without the need for a harsh vacuum environment or complex processes. By varying parameters such as scanning speed and laser power, sub-micrometer 2D and 3D metallic structures can be generated, giving rise to applications as electrical conductive micropatterns in microelectronics, patterned SERS structures as well as various metamaterials.⁸⁷⁻⁹⁰ Such programmable structure design and patterning also deems two-photon direct metal writing a promising technique to create sub-micrometer scale metallic patterns that can be selectively incorporated on specific areas of a multi-functional lab-on-a-chip micro-platforms due to the precise control over fabrication location.

Currently, there are a few approaches to fabricate metallic nanostructures via two-photon direct metal writing. The first, and most commonly used approach is a two-step approach where polymeric nanostructures are first fabricated via two-photon lithography before a metallic layer is deposited to metallize the structures. In this approach, although structures can be written with nanoscale resolution and high precision, deposition of the metallic layer generates a large amount of material wastage as the deposition location is difficult to control. As a result, this approach is unsuitable for the fabrication of photonic devices or nano-electrical patterns as conductivity is not limited to only the written patterns.

1.4.2.1 METAL-POLYMER COMPOSITES

A second approach involves the direct formation of metallic nanostructures via a simultaneous, laser-induced photoreduction and nucleation process. This can be achieved through the formation of a metal-polymer composite or the addition of a surfactant within the metallic precursor solution. A metal-polymer composite can be obtained by mixing a metallic precursor with an organic monomer, where the metallic precursor and organic monomer are simultaneously reduced and polymerized respectively upon laser irradiation to form a metal-polymer composite.⁸⁸ Using this technique, Prasad et al fabricated Au-polymer composites as plasmonic metamaterials.⁹¹ Shukla et al also demonstrated the formation of 3D Au-polymer composites, with structure widths as small as 390 nm (Figure 1.13).⁹²

Figure 1.13. Metal-polymer composite structures as fabricated by two-photon lithography. Reproduced from ref. ⁹² with permission of The Royal Society of Chemistry

A metal-polymer composite can also be formed by mixing a metallic precursor with a polymer matrix, where the metallic precursor is reduced upon laser irradiation to form well-defined but sparse metal nanoparticles within the dense polymer matrix. The polymer matrix then functions as a physical support to fix the position of fabricated nanoparticles such that 3D scaffolds or disconnected nanoparticle layers can be created within the polymer matrix.⁹³ The polymer also protects embedded nanostructures against mechanical stresses which allows for higher durability of written nanostructures, which has been proposed for use in studies of the optical properties of single nanoparticles.

However, the presence of an insulating polymer matrix results in both lowered metal loading capacity within fabricated metallic nanostructures as well as decreased conductivity,^{88, 94} which reduces the efficiency of such structures in both nanoelectronics and photonics. Furthermore, the

presence of a thick polymer matrix encapsulating the fabricated nanostructures could significantly decrease the optical resolution of embedded nanostructures, and in the case of patterned SERS substrates, SERS signals from the polymer matrix could interfere with those from the analytes.⁹⁵

1.4.2.2 SURFACTANT-BASED METALLIC STRUCTURES

One promising method to circumvent this issue is to add a surfactant into the metal precursor solution instead of a polymer. The surfactant acts as a stabilizer to facilitate the nucleation of reduced metal atoms to form metal nanoparticles. In the absence of a polymer matrix, measured conductivities are significantly higher,⁹⁰ SERS signals are more accurate and distinct,⁹⁶ and optical resolution is improved. Using n-decanoysarcosine sodium (NDSS) as a surfactant, Tanaka et al fabricated 2D and 3D silver nanostructures where the smallest attainable line width is 180 nm (Figure 1.14). The group observed that an increase in surfactant concentration generally results in structures which are taller and more well-defined, due to the higher capping efficiency by NDSS to prevent further metallic growth.⁹⁷

Figure 1.14. SEM images of a 3D silver nanopillar and self-standing silver nanopillars. Reproduced with permission from ref. 94. Copyright 2009 Wiley-VCH Verlag GmbH & Co KGaA, Weinheim.

Similarly, Duan et al fabricated 2D gold nanostructures via two-photon photoreduction using trimethylammonium-based ionic liquid as a surfactant. The trimethylammonium cations adsorb strongly to generated gold nanoparticles to stabilize them, enabling the formation of stable, continuous lines with a minimum linewidth of 228 nm. Using this technique, the group fabricated split-ring resonators that exhibit an electric resonance of ~ 63 THz, demonstrating that metamaterials have been successfully fabricated.⁸⁹

In some cases, a photoinitiator is also incorporated within the precursor solution to promote increased nanoparticle synthesis. The surfactant and photoinitiator can then be easily washed off the nanoparticles or replaced with other ligands of interest, making this technique promising for the fabrication of catalytic microreactors as well as patterned SERS substrates for analyte sensing and monitoring of reaction processes.⁹⁸⁻⁹⁹ Adam et al fabricated an aggregated, 3D gold patterned SERS substrate in the presence of surfactant hexadecyltrimethyl ammonium bromide (CTAB) and photoinitiator (2, 4, 6-trimethylbenzoyl)-phosphine oxide (TPO). The presence of the photoinitiator resulted in increased photo-reduction of gold ion to form structures with irregular shape and size distributions, which demonstrated strong SERS enhancement factors of up to 10^8 .⁹⁶

1.5 MOTIVATION AND OBJECTIVES

Lithography has had a long history in time and has evolved from printing only ink and inscriptions to creating 2D and 3D nano- to centi-meter sized functional structures from various materials such as polymers, composites and even metals. In this chapter, we discussed the different types of lithography and some of the structures that have been created from various methods. In particular, we highlight two-photon lithography as a promising technique due to the high resolution and efficacy in fabricating intricate 3D structures.

We focus on 2 key areas: two-photon polymerization and two-photon photoreduction and introduce shape-shifting as a key feature of stimuli-responsive, smart polymers. Currently, most shape-shifting structures are fabricated via photolithography and range from millimeter to centimeter size.

These large sizes could be challenging in applications such as plasmonic studies, lab-on-a-chip microdevices and intravenous drug delivery. Two-photon lithography thus enables nano- to micro-meter scale shape-shifting structures to be created so as to potentially achieve shape-shifting at the microscale. Previously, the shape-shifting of BSA microstructures have been demonstrated, but there is no systematic study into the extent of shape change and how shape change extent can be programmed. In this thesis, our first objective will be on controlling and quantifying the extent of shape change in the two-photon lithography of shape-shifting BSA microstructures. We also observe that thus far, shape-shifting has been limited to unidirectional shape change such as bending, and twisting. Multi-directional shape-shifting is essential for the future construction of self-assembled 3D macrostructures as photonic crystals, and 2D arrays which can be used to tune electromagnetic wave in near-infrared region, and modify fluid flow behavior. Our second objective will hence be to use the obtained knowledge on controlling shape change and shape change extent to fabricate BSA microstructures that can undergo such geometrical shape-shifting.

Two-photon photoreduction to form metallic nano- to micro-scale structures has been explored extensively in the past decade. While metal-polymer composites remain a popular choice in direct metal writing, the presence of the polymer matrix could pose potential problems. Metal-polymer composites exhibit unremarkable conductivities when used in microelectronics. As a SERS substrate, the polymer matrix could interfere with analyte SERS signals, resulting in inaccurate or poor detection limits. Conversely, surfactant-based fabrication of metallic structures allows for greater flexibility of application as the surfactant can be easily removed or replaced with other ligands post-fabrication. However, currently most fabricated structures comprise sparse, or dense and irregularly shaped nanoparticles. There is also a lack of characterization of fabricated metallic structures. Hence our third objective is to fabricate gold nanoparticle microstructures, and demonstrate that nanoparticle sizes, particle density distribution, surface roughness and optical properties can be tuned by varying surfactant concentration. We select gold metal as gold is more stable to oxidation than silver to ensure longevity of fabricated metallic microstructures for long-term applications. By fabricating gold microstructures within a microfluidic channel, we also demonstrate remote SERS gas sensing of small

molecules. Our final objective is to demonstrate the ability to tune gold microstructure surface morphology by varying the ratio and type of additives added into the precursor solution, and optimize fabrication parameters through the systematic investigation of varying laser power, scanning speeds and surfactant concentrations on the resulting metallic structure to create stable, robust metallic microstructures.

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2 Shape-shifting 3D Protein Microstructures with Programmable Directionality via Quantitative Nanoscale Stiffness Modulation**

ABSTRACT. The ability to shape-shift in response to stimulus increases an organism's survivability in Nature. Similarly, man-made dynamic and responsive "smart" microtechnology is crucial for the advancement of human technology. Here we fabricate 10-30 μm shape-changing 3D BSA protein hydrogel microstructures with dynamic quantitative, directional, and angle-resolved bending via two-photon photolithography. Our technique for achieving controlled directional responsiveness is to incorporate a stiff reinforcement layer within a single microstructure by spatially control the cross-linking density of BSA at nanometer lengthscale. Atomic force microscopy measurements of Young's moduli of structures indicate that with increasing the laser writing distance at the z-axis from 0.1 μm – 0.5 μm , the modulus of the structure is decreased. Hence, through nanoscale modulation of the laser writing z-layer distance at the nanoscale, we are able to control the cross-linking density, allowing for the swelling extent of the microstructures to be quantified and controlled with high precision. We apply this method of segmented moduli within a single microstructure to the design of shape-shifting microstructures that exhibit stimulus-inducible chirality, as well as to the fabrication of a free-standing 3D micro-trap which is able to open and close in response to pH change.

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2.1 INTRODUCTION

In nature, the ability to shape-shift in response to stimulus increases an organism's survivability.¹⁻³ For instance, the cores and scales of pine cones are built in with stiff reinforcement layers that allow them to close and open in response to humidity to protect the seeds within.⁴ The concept of shape-shifting lies in the non-uniform responsiveness of localized areas within a material,⁵ where the application of an external stimulus⁶⁻⁸ causes distortion due to uneven swelling. This concept has inspired the creation of man-made, programmable 3D “smart” materials⁹ capable of bending,¹⁰ twisting,¹¹⁻¹² or actuating across distances.¹³ The advent of such materials has facilitated the innovation of tools¹⁴⁻¹⁶ which greatly advance human technology.

Polymers are the most commonly used material in the fabrication of 3D shape-shifting structures due to their high degree of malleability, which allows greater ease of manipulation. Aizenberg et al. pioneered in using hydrogel polymer film to induce bending of originally non-responsive Si nanocolumns.¹⁷ However, for polymeric structures with shape-changing capability, they can be generally classified into two methods:⁵ the first is to fabricate a dual component polymeric bilayer where each component is responsive to a different stimulus.¹⁸⁻¹⁹ However, the need for two components is not only time consuming but also technically challenging in terms of post-fabrication treatment and processing. The second method is to gradient the osmotic potential or cross-linking density in a single component polymeric material. Currently, single component 3D polymeric structures with shape change ability are limited to hundreds of micrometer to centimeter length scales,¹⁹⁻²² which are inadequate for the advancement of microtechnology. The fabrication of (sub)micrometer-sized 3D shape-shifting structures remain a challenge due to the difficulties and lack of fabrication tools for the precise fabrication and positioning of structures. Photolithography emerges as a promising technique for the fabrication of defined, 3D polymeric microstructures.²³⁻³¹ Using a masked-directed multiphoton lithography, pH-responsive³² bovine serum albumin (BSA) protein microstructures with differing cross-linking densities that qualitatively curl to different extents in response to pH change have been fabricated.³²⁻³³ However, the written structures are 2D, the bending

angle was not resolved and control over the directionality of curving was also not demonstrated which limit their versatility in practical applications.

To date, the ability to accurately control and quantify the shape-shifting direction and extent of a microstructure remains largely unexplored. Controlling both the direction and the extent of shape-shifting is highly important because the chemical and/or physical properties of a material can be greatly altered based on the extent of shape change.³⁴⁻³⁵ While directional shape-shifting mechanisms are frequently observed in nature to ensure survival, such as in the Venus flytrap,³⁶ there has not been much report on artificial microstructures with equivalent dynamic and precise control over directional shape-switching, especially at sub-50 micrometer length scale, which are critical in the development of functional microsystems in lab-on-a-chip microtechnology.

Here, we demonstrate the fabrication of dynamic shape-shifting pH-responsive BSA sub-50 micrometer structures with precise, programmable directionality using two-photon lithography, for the creation of biomimetic, stimulus responsive chemo-mechanical microactuators. 3D BSA microstructures are fabricated using a layer-by-layer laser writing process, where laser writing is repeated at the same x-y plane coordinates along different predefined z-planes (layer distances) with nanoscale precision. AFM measurements of the moduli of structures fabricated at increasing z-layer distances demonstrate an inverse relationship between the fabrication layer distance and modulus, which is in turn directly proportional to cross-linking density, allowing for the swelling extent of the microstructures to be quantified and controlled with high precision through nanoscale modulation of the z-layer distance. Inspired by the reinforcement layer in the pine cone, we create defined segments of differing modulus through varying the z-layer distance within the same microstructure to achieve programmable, directional responsiveness with resolved bending angles. We apply this technique to the design of originally achiral microstructures with stimulus-inducible chirality. In addition, a 3D free-standing micro-trap, will also be demonstrated to open and close in response to changes in pH.

2.2 RESULTS AND DISCUSSION

Fabrication of BSA microstructures. Free-standing, micrometer-sized 3D BSA protein hydrogel (protogel) structures are fabricated in a layer-by-layer manner via two-photon polymerization process with Rose Bengal serving as the photoinitiator and BSA as the monomer to achieve dynamic pH responsiveness. A piezoelectric scanning stage is used to maneuver the resist in the x, y, and z axes relative to the focus position of the laser with a spatial resolution down to nanometer length scale. At the laser focal point, Rose Bengal is excited and the polymerization reaction is initiated. Predefined 3D microstructures are fabricated in a layer-by-layer fashion, by allowing the laser-writing process to occur repeatedly at the same x-y plane coordinates along different predefined z-planes. The nominal distance between each layer is termed “layer distance” (Δd), and it can be defined using Nanoslicer® program (Figure 2.1A).

Figure 2.1. (A) Scheme of protogel fabrication using two-photon lithography. 3D BSA protein is fabricated using a layer-by-layer laser writing process. The distance between each successive layer is termed the layer distance (Δd). (B) SEM images of rigid and free-form protogels. Rigid protogels are adhered to the substrate via their x, y planes, whereas free-form protogels are elevated away from the substrate.

We begin our study by fabricating 3D rigid and free-form “Y” shaped BSA microstructures, respectively. The dimensions of the “Y” shaped structures are 5.0 μm in height (h) and 10.0 μm in length (l) for each arm of the structures, fabricated with 25 layers, using a fixed nominal layer distance of 0.2 μm . The rigid structures are bound by their x-y planes to the substrate, leaving them with only two degrees of freedom for movement. The free-form structures of the same dimension are fabricated

using the same parameters, and are elevated by cylindrical pedestals which are $5.0\ \mu\text{m}$ in diameter and $2.5\ \mu\text{m}$ in height, fixed at a fabrication layer distance of $0.2\ \mu\text{m}$. The free-form structures have three degrees of freedom for movement. The scanning electron microscope (SEM) images (Figure 2.1B) of the “Y” shape protogel structures show them to be stable and well-defined in both free-form and rigid forms, indicating that two-photon lithography is very versatile in creating robust, precise 3D protogel microstructures. The high precision of two-photon lithography allows for rigid, 3D “Y” shape microstructures as small as $1.3\ \mu\text{m}$ in height and $2.5\ \mu\text{m}$ in length to be fabricated (Figure 2.2).

Figure 2.2. The layer by layer fabrication of 3D, rigid protogels of layer distance $0.2\ \mu\text{m}$ using two photon lithography allow for fabrication of protogels up to $5\ \mu\text{m}$ in length. The aspect ratio of protogels are kept constant. Scale bar is $10\ \mu\text{m}$.

Using the “Y” structures ($h = 5.0\ \mu\text{m}$, $l = 10.0\ \mu\text{m}$) as the role model, we systematically tune the nominal layer distance of the rigid and free-form BSA microstructures during the lithography between $0.1\text{--}1.0\ \mu\text{m}$ in order to identify the minimum layer distance required for fabrication. The SEM images of the BSA microstructures fabricated using layer distance range of $0.1 - 0.5\ \mu\text{m}$ (at $\sim 100\ \text{nm}$ intervals) are well structured with defined edges, whereas the structures written with layer distance range of $0.6\text{--}1.0\ \mu\text{m}$ have lost their conformational integrity with increasing layer distance (Figure 2.3). This indicates that a minimum $\Delta d = 0.5\ \mu\text{m}$ is required to obtain stable BSA microstructures. From here onwards, we select the layer distance range of $0.1 - 0.5\ \mu\text{m}$ for our studies.

Figure 2.3. Ten sets of rigid and free-form structures with layer distances 0.1 – 1.0 μm are fabricated. It is observed that structures with layer distance of 0.6 – 1.0 μm (seen in rectangle box) appear to have lost their conformational integrity, hence, we only employ layer distance 0.1 – 0.5 μm for our studies. Scale bars are 20 μm .

Stimuli-responsiveness of BSA microstructures. The dynamic responsiveness of the protogel microstructures are demonstrated via pH swelling experiments. The swelling abilities of the protogels are quantified by the area swelling ratios (A/A_0), where A and A_0 denote the areas of the protogel at pH 11 and pH 5, respectively. The general trend for both rigid and free-form structures is that the structures possess the smallest area at pH 5 (Figure 2.4). This is because pH 5 is close to the isoelectric point of BSA,³⁷ where a protein has no net charge, and is expected to absorb less water than at other pH values due to fewer ion-dipole interactions. As the pH deviates from the isoelectric point, the protein structures appear to absorb more water and swell, due to more ion-dipole interactions present.

Figure 2.4. Bright field optical images of rigid structures fabricated with layer distances of 0.1 – 0.5 μm are placed in solutions of different pH (1-13). It is observed that at pH 5, the structures appear the smallest.

We compare the area swelling ratios of the protogel structures at various pH to determine the optimum pH for swelling studies. It is observed that the pH 13/pH 5 area swelling ratio is the largest (Table 2.1). This is likely due to the fact that there are more ionized groups on BSA at pH 13 than at other pH values. However, at pH values above 12, BSA protein undergoes an irreversible unfolding process. As a result, to ensure dynamic, reversible swelling of the protogel microstructures, the pH 11/pH 5 area swelling ratios are measured instead.

Table 2.1. Area swelling ratio of the structures in Figure 2.4 at various pH measured against pH 5

pH	Swelling ratio at different layer distances				
	0.1 μm	0.2 μm	0.3 μm	0.4 μm	0.5 μm
1.0	1.0 $\pm$ 0.0	1.1 $\pm$ 0.1	1.2 $\pm$ 0.0	1.3 $\pm$ 0.0	1.3 $\pm$ 0.0
3.0	1.0 $\pm$ 0.0	1.1 $\pm$ 0.1	1.2 $\pm$ 0.1	1.3 $\pm$ 0.0	1.4 $\pm$ 0.0
5.0	1.0	1.0	1.0	1.0	1.0
7.0	1.0 $\pm$ 0.0	1.1 $\pm$ 0.0	1.1 $\pm$ 0.1	1.3 $\pm$ 0.1	1.4 $\pm$ 0.2
9.0	1.1 $\pm$ 0.0	1.2 $\pm$ 0.1	1.3 $\pm$ 0.1	1.4 $\pm$ 0.2	1.5 $\pm$ 0.1
11.0	1.1 $\pm$ 0.1	1.3 $\pm$ 0.1	1.4 $\pm$ 0.1	1.5 $\pm$ 0.0	1.7 $\pm$ 0.1
13.0	1.3 $\pm$ 0.1	1.4 $\pm$ 0.1	1.6 $\pm$ 0.1	1.7 $\pm$ 0.1	1.8 $\pm$ 0.1

Besides the pH of the surrounding solution, we also find that the swelling capability of written protogels can be effectively controlled by tuning the layer distance during the laser-writing process. Structures written with a larger layer distance are able to swell to a greater extent at pH 11 for both rigid and free-form structures. We fabricate similar Y-shaped rigid and free-form structures with increasing layer distance from 0.1 μm to 0.5 μm (Figure 2.5A). It is observed that at pH 11, both the rigid and free-form structures swell to a greater extent with increasing layer distance (Figure 2.5B, C). Protogel (and hydrogels in general) consist of three-dimensional network where water is able to enter, or exit between the polymerized protein layers to allow the protogel to swell, or revert to the original state. A larger laser writing layer distance results in lower cross-linking density which in turn increases the swelling ability of the protogel because the structure is less compact and more water can enter to bind to individual ionized side chains, resulting in increased swelling ratios of the protogels as fabrication layer distance increases. Hence, we can accurately modulate the degree of swelling (and cross-linking density) of BSA structures by tuning the layer distance (from 0.1 – 0.5 μm) during the laser-writing process.

Figure 2.5. (A) SEM images of both the rigid and free-form structures, written with layer distances of 0.1 – 0.5 μm . (B, C) pH-dependent swelling of rigid and free-form structures constructed with different layer distances. Optical images of both rigid and free-form structures are obtained at (i) pH 5 and (ii) pH 11, and (D) their area swelling ratios. Area swelling ratio is defined as A/A_0 , where A = area of protogel at pH 11 and A_0 = area of protogel at pH 5. (E) Reversible swelling of the rigid protogel structures of layer distances 0.1 – 0.5 μm over four swelling cycles. All scale bars are 10 μm .

We also note that the free-form structures swell more than the rigid ones at all the layer distances investigated (Figure 2.5D), with the free-form structures swelling 10% larger than the rigid protogels on average. Comparing the rigid and free-form protogels, the highest area swelling ratio is obtainable at a fabrication layer distance of 0.5 μm for both rigid ($A/A_0 = 1.7$) and free-form ($A/A_0 = 1.9$) protogels. This is due to the complete freedom of movement in the x-y-z plane for the free-form structures. While both the rigid and free-form structures can swell in 3D, the x-y planes of the rigid structures are inhibited from free movement. Hence, the rigid structures are unable to swell in the x-y plane as freely as the free-form structures, leading to a lower area swelling ratio observed. The essence of programmable shape change lies in the swelling ability of a protogel, where a greater extent of swelling leads to a more apparent shape change.

In addition to dynamic swelling, the pH 11/pH 5 swelling ratios of the 3D protogels in relation to its layer distance are also highly reproducible. This is demonstrated through four consecutive swelling/de-swelling cycles using five rigid, “Y” shaped structures, with similar dimensions as described previously and possessing layer distances of 0.1 – 0.5 μm . The structures display excellent consistency in swelling ratios over four swelling cycles (Figure 2.5E), with the average swelling ratios ranging from 1.1 to 1.7 as layer distance of the structures increases from 0.1 – 0.5 μm . The presence of cross-linking within the protogel enables the structure to retain its conformation and ensures that the protogel does not disintegrate during swelling. This allows the protogel to exhibit reproducible dynamic swelling properties.

Mechanical properties of BSA microstructures. The mechanical properties of “Y” shape structures, each with a different layer distance from 0.1 – 0.5 μm are probed using atomic force microscopy (AFM), and their force-penetration relationship with the cross-linking density are investigated. To do this, a predetermined force is applied to deform the sample during the measurement in a process known as AFM-based nanoindentation. The deformation in the range a few hundred nanometers is recorded as a force-displacement force. We convert the force-displacement data to

force-penetration following the procedure of Janshoff et. al.,³⁸ and fit the force-penetration curves using the Hertz model with spherical indenter of 50 nm.¹

The plots of penetration force against the penetration depth of each of the structures at their isoelectric point of pH 5 aqueous medium (Figure 2.6A) demonstrate that in general, the protogel structures experience an increase in penetration depth when the layer distance of the structures increases from 0.1 μm to 0.5 μm .

Figure 2.6. (A) Plot of force-penetration depth obtained from AFM nanoidentation measurements. (B) The Young's modulus of the structures using different layer distance during the laser writing process. A trend of decreasing modulus value with increasing layer distance of protogel is observed in their original state at pH 5.

To quantitatively evaluate the mechanical strength of our protogel structures, the Young's moduli of the protogels are obtained from the force-penetration curve using a simple Hertz model. The equation of Hertz model is as follows:

$$F = \frac{E}{1 - \nu^2} \left[\frac{a^2 + R_s^2}{2} \ln \frac{R_s + a}{R_s - a} - aR_s \right]$$

$$\delta = \frac{a}{2} \ln \frac{R_s + a}{R_s - a}$$

¹ JPK Data Processing Software Manual

Where E is Young's modulus, R_s is the tip radius, δ is the deformation/indentation, ν is Poisson's ratio, α denotes the radius of contact, and F is the force applied. The parameters used for the fitting are force applied (F), radius of sphere (R_s), indentation (δ) and Poisson's ratio (ν), and the values obtained from the Hertz fitting were the Young's modulus (E) and radius of contact (α).

The Young's modulus, a measure of the stiffness of a structure, is directly proportional to the cross-linking density of the protogel.³⁹⁻⁴⁰ When the layer distance of the protogel structures are tuned from 0.1 μm to 0.5 μm , the Young's modulus measured decreases from ~ 6.5 MPa to 2.0 MPa (Figure 2.6B), indicating a decrease in stiffness (Young's modulus) of the protogel when the laser writing intervals is increased at z-axis. Considering all the BSA structures are of the same volume, structures with smaller layer distance experience higher number of laser scanning per volume, contributing to higher protogel cross-linking density. Given that a higher cross-linking density corresponds to a higher Young's modulus, this leads to the fabrication of a more rigid BSA structure as compared to structures fabricated with a larger layer distance. As a result, the force needed to penetrate a unit length of the structure is greater for small layer distances.

We also note that the overall Young's moduli of our BSA structures are at least one order of magnitude higher than the reported Young's moduli of commonly used hydrogels.⁴¹⁻⁴³ We attribute the higher Young's modulus to the two-photon lithography fabrication method used in this report, where high energy and high power density of ultrafast laser is tightly focused at a sub-micrometer volume to polymerize/cross-link BSA monomer in a layer-by-layer manner into 3D protogel structures. The two-photon lithography process generally takes tens of minutes to complete a BSA microstructure, whereas other photolithographic fabricated hydrogel systems use diffuse (UV) light, with lower power density per surface area to polymerize the hydrogel structures in a matter of minutes. The higher Young's moduli and therefore higher cross-linking density thus explain the observed lower swelling ratio (< 2) of our BSA structures as compared to the common hydrogel systems.

Creating shape-changing BSA microstructures. Thus far, the BSA microstructures exhibit homogeneous swelling over the entire structures. To achieve programmable directional shape change

within a single-component structure, we fabricate “dual-pillar” BSA structures by connecting two protogel structures of different swelling ratio (layer distances) to each other, a design that mimic pine cone structures. Our core strategy is to make use of the difference in swelling ratio of the two connecting pillars to manipulate the direction of the swelling. At high pH, the more rigid pillar structure possessing lower swelling ratio act as a reinforcement layer, where the less rigid pillar structure swells to a greater extent, causing the dual-pillar structure to bend towards the direction of more rigid structures. The directional swelling in our single component BSA microstructures is also analogous to bimetallic strips that bend when heated due to the difference in heat expansion coefficient.⁴⁴

To optimize the curving degree of the dual-pillar structures, five free-form structures, with layer distances of $(0.1, 0.X) \mu\text{m}$, where $X = 1, 2, 3, 4$, and 5 , are fabricated (Figure 2.7). The structures ($h = 5.0 \mu\text{m}$, $w = 5.0 \mu\text{m}$, $l = 15.0 \mu\text{m}$) are elevated on $3.0 \mu\text{m}$ cylindrical supports to maximize freedom of movement. The dual-pillar structures with layer distance of $(0.1, 0.4) \mu\text{m}$ and $(0.1, 0.5) \mu\text{m}$ appear to be curved which is likely due to their higher pliability as a result of the lower crosslinking density they possess.

Figure 2.7. SEM images of dual-pillar structures of $0.1, 0.X \mu\text{m}$, ($X = 1, 2, 3, 4, 5$). Scale bars are $20 \mu\text{m}$.

The directional bending of the dual-pillar structures are examined at pH 5 and pH 11 under the bright field microscope. Using the supporting pedestal in the center of the structure as a pivot point,

the angle of curvature of each side (α and β) of the structures away from the y-axis is measured to obtain the total bending angle, θ° ($\theta = \alpha + \beta$) in order to quantify the bending extent (Figure 2.8A). At pH 5, all five dual-pillar structures appear to be straight columns (Figure 2.8B(i) – F(i)). Increasing the basicity of the solution to pH 11, all structures exhibit controllable directional bending towards the more rigid reinforcement layers written using 0.1 μm layer distance, where the “0.X μm ” sides of the dual-pillar structures with higher swelling ability curve to a greater extent (Figure 2.8B(ii) – F(ii)).

Figure 2.8. Dual-pillar BSA structures are designed, with each pillar fabricated using a different layer distance. (A) The structures are expected to exhibit curving when pH of the solution is changed from 5 to 11, akin to that of a bimetallic strip. (B – F) show the optical images of various dual-pillar structures of layer distances (0.1 μm , 0.X μm), where X=1, 2, 3, 4, 5, in (i) pH 5 and (ii) pH 11. (G) Bending angle (θ°) of the structures at pH 11 was measured, and the bending angle is depicted by $(\alpha+\beta)^\circ$ as seen in (A). Scale bars are 5 μm .

The bending angles, θ° ($\theta = \alpha + \beta$) increase from 0° to 77° as the “0.X μm ” values increase from 0.1 μm to 0.5 μm . The (0.1, 0.4) μm dual-pillar structure is observed to exhibit the highest bending angle at 77° , instead of the expected (0.1, 0.5) μm dual-pillar structure, which has a bending angle of 67° (Figure 2.8G). A possible reason for the observed higher bending angle of the dual-pillar structure with layer distance of (0.1, 0.4) μm than the expected (0.1, 0.5) μm structure could be that the pillar with layer distance of 0.4 μm possesses higher modulus than the pillar with layer distance of

0.5 μm , as evident in our results in AFM measurements (Figure 2.6B). As a result, the pillar of layer distance 0.4 μm is hence able to exert a greater force to bend the pillar with layer distance of 0.1 μm in a (0.1, 0.4) μm dual-pillar structure to a greater extent, resulting in a greater bending angle measured. Having acquired the precise control over the bending direction of our dual-pillar structures, the position of the reinforcement layer and therefore the bending direction of the dual-pillar structures can be easily reversed, which allows us to program the direction of shape change of the structures (Figure 2.9).

Figure 2.9. (A) Dual-pillar structures with their positions reversed: (0.4, 0.1), (0.1, 0.1) and (0.1, 0.5) were fabricated and observed under the bright field microscope at pH 5. (B) Changing the pH of the solution from 5 to 11 resulted in the curving of the dual-pillar structures with directional control.

Using BSA as the only component, we have successfully fabricated segmented structures with different stiffness. Our design mimics the directional responsive materials that are usually observed using two-component materials, such as bimetallic strip. The control of the response (bending) of the structure can be easily modulated by varying the stiffness of the component with nanometer scale precision. Our structure is distinguished from the conventional fabrication process by the ease of design and fabrication using two-photon lithograph with greater flexibility.

Equipped with the capability of dynamic directional bending, we demonstrate that originally achiral microstructures can be transformed into chiral microstructures under stimulation. We fabricate a free-form “Y” shaped dual-pillar structure with each arm fabricated with segmented layer distance of 0.1 μm and 0.4 μm , respectively (Figure 2.10A). The process of inducing chirality is monitored in-

situ as the pH changes from pH 5 to 11. At pH 5 ($t = 0$ s), the structure appears to be achiral; however with the gradual increase to pH 11, the structure undergoes a shape change into a chiral fan-blade like structure within 0.5 s (Figure 2.10A), as a result of controlled directional curving.

Figure 2.10. The concept of directional control of dual-pillar structure's bending direction is applied to the fabrication of structures with dynamic, inducible chirality. (A) A (0.1, 0.4) dual-pillar “Y” shaped structure which is originally achiral at pH 5 transforms to a chiral protogel within 0.5 s upon a change in pH from 5 to 11. (B) An array of “I” shape dual-pillar structures which are originally achiral are programmed to undergo a shape change to the chiral “S” and reverse “S” structures in 0.5 s as pH increases from 5 to 11. Increasing the acidity of pH back to pH 5 results in the chiral “S” structures shape-shifting back to achiral “I” shape structures within 0.5 s.

The high reversibility of the process of inducing chirality in microstructures is illustrated through fabrication of an array of achiral, “I” shaped dual-pillar structures exhibiting programmable shape-shifting behavior. Movie frames demonstrate the gradual shape-shifting process of the “I” shape structures into “S” shaped structures within 0.5 s as the basicity of the solution increases steadily to pH 11 (Figure 2.10B). Decreasing the basicity of the solution sees a reverse in the process as the chiral “S” shape structures gradually shift back to their original “I” shape achiral state. Being able to program an achiral structure to exhibit dynamic, reversible chirality under a specific external stimulus and precisely control the chiral form through defining the position of the reinforcement layer is extremely significant. Depending on the pH of the external environment, the chirality of the structure can be switched on or off, which has potential applications for inducing plasmonic chirality in nanomaterials *via* far-field electromagnetic coupling with localized plasmons of achiral nanostructures.⁴⁵

Combining the precise positioning in fabricating dynamic microstructures and the accurate directional shape-shifting, we demonstrate the formation of dynamic microstructures with individual components demonstrating independent and heterogeneous swelling extent and bending directions within a single structure. Two sets of cross-shaped structures are fabricated; Figure 2.11A shows the cross-shaped structure with each arm consists of a dual-pillar with $\Delta d = (0.1, 0.X) \mu\text{m}$, where $X = 0.1, 0.2, 0.3$ and $0.4 \mu\text{m}$, respectively; whereas the structure in Figure 2.11B are written with opposing arms of $(\Delta d = 0.1, 0.1 \mu\text{m})$ and $(\Delta d = 0.1, 0.4 \mu\text{m})$, respectively. At pH 5, both structures appear identical (Figure 2.11C, D), and it is difficult to deduce that the different arms of the structures are written with different $(0.X) \mu\text{m}$ layer distances. However, at pH 11, the individual arms of the structures swell asymmetrically. While the arms of $(\Delta d = 0.1, 0.1 \mu\text{m})$ swell homogeneously; the dual-pillar arms written with $0.X > 0.1 \mu\text{m}$ bend towards the direction of the reinforced $0.1 \mu\text{m}$ layer, resulting in anisotropic shape change of the structures (Figure 2.11E, F).

Figure 2.11. Two free-form $(0.1, 0.X)$ μm dual-pillar cross-shaped structures are written to demonstrate consistency in swelling ratio. (A) A cross-shaped structure with each arm consists of a dual-pillar with $\Delta d = (0.1, 0.X)$ μm , where $X = 0.1, 0.2, 0.3$ and 0.4 μm , respectively. (B) A cross-shaped structure written with opposing arms of $(\Delta d = 0.1, 0.1 \mu\text{m})$ and $(\Delta d = 0.1, 0.4 \mu\text{m})$, respectively. (C, D) The structures at pH 5, which appear identical. (E, F) Heterogeneous shape-shifting of the structures when immersed in pH 11 solutions. Scale bars are $5 \mu\text{m}$.

We color code the dual-pillar structures of $(0.1, 0.1)$, $(0.1, 0.2)$, $(0.1, 0.3)$, and $(0.1, 0.4)$ as white, yellow, orange, and blue for easy verification. The extent of curvature of each arm follows the pre-designed trend of white < yellow < orange < blue in order of increasing curvature. The ability to independently control the swelling ratio and bending direction of individual components within a single microstructure has enabled us to manipulate the shape change of the 3D protogel microstructures at the sub-micrometer precision.

In addition, we also fabricate a free-standing 3D micro-trap structure with controllable bending direction (Figure 2.12). Four free-standing vertical dual-pillar structures ($\Delta d = 0.1, 0.4 \mu\text{m}$) are fabricated on a cylindrical support. The pH-responsive 3D shape change of the micro-trap is observed under the bright field microscope to monitor the top view x-y shape change, and subsequently with the Raman imaging to visualize the x-y-z three-dimensional shape change. The presence of Raman-active Rose Bengal allows for Raman imaging to be conducted, with peaks at 1612 cm^{-1} corresponding to quinodal structure stretching and at 1326 cm^{-1} , which corresponds to C-O symmetric vibration.⁴⁶

Figure 2.12. 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 and “closed”. All scale bars are $10 \mu\text{m}$.

The four dual-pillars are observed to be originally upright at pH 5 and separate from one another (Figure 2.12A), hence the 3D Raman imaging of the trap show it to be “open” at pH 5 (Figure

2.12B). Upon a change from pH 5 to pH 11, the pillars undergo a shape change by bending inwards until all four tips of the dual-pillar structures are in contact (Figure 2.12C), allowing the trap to “close” (Figure 2.12D). The programmable, directional shape change of the trap has potential applications in dynamic template-assisted assembly, where an array of traps can act as micro-robotic arms to capture microparticles when pH of the solution is adjusted to pH 11, and subsequently release them when the pH of the solution is adjusted back to pH 5.

2.3 CONCLUSIONS

In this chapter, we demonstrate the design and fabrication of pH stimuli-responsive protogel microstructures with programmable shape changing ability from a single material BSA, through modulating the layer distance of different parts of the structure. Using two-photon lithography, we are able to independently control the Young’s modulus (and hence the swelling ratio) of individual components within a single microstructure at nanometer length scale precision, which allows direct manipulation of the heterogeneous of shape change of the 3D protogel microstructures. We have designed and demonstrated using the concept of “dual-pillar” structure to control the swelling extent and swelling direction of BSA protogels, resulting in the inducible chiral structures and free-standing trap-like structures. The ability to program an achiral structure to exhibit chirality under a specific external stimulus is extremely useful. Depending on the external environment, the chirality of the structure can be switched on or off, which has potential applications in controlled, environment-dependent enantiomer recognition and identification. On the other hand, the programmable, directional shape change of the trap has potential applications in dynamic template-assisted assembly of particles; an array of traps can be written and act as micro-robotic arms to capture micro-objects by tuning the pH. The creation of dynamic, shape-shifting pH responsive free-standing 3D microstructures proves to be a milestone in the field of smart materials research. Besides the practical importance of the study, the ability to program the directionality as well as the extent of shape-change in microstructures *via* the nanoscale modulation of cross-linking density in a single material also

allows for existing trap and channel designs to be refashioned in micro-scale for installment in future micro-devices, which can greatly impact and advance human technology.

2.4 MATERIALS AND METHODS

Materials. Albumin from bovine serum, lyophilized powder (96+ %), HEPES (99.5+ %), Rose Bengal, powder, (95 %), phosphate buffered saline, tablet, were purchased from Sigma Aldrich; dimethyl sulfoxide (AR grade), hydrochloric acid (37 %), sodium hydroxide, pellet, were purchased from Goodrich Chemicals. The chemicals were used without further purification. Ultrapure water (> 18.0 M Ω .cm) was obtained from a Sartorius arium® 611 UV ultrapure water system.

Preparation of BSA protogel precursor solution. 2.1 g of BSA powder and 2.31 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. Final BSA : DMSO = 21 % : 25 %. The mixture was filtered through a solid phase extraction filter to remove impurities before use.

Preparation of different pH solutions. pH solutions were prepared in volumes of 10 mL using 1 M hydrochloric acid and 1 M sodium hydroxide. Solution pH of 1, 3, 5, and 7 were prepared from serial dilution of 1 M hydrochloric acid and ultrapure water whereas solution pH of 9, 11, 13 were prepared by serial dilution from 1 M sodium hydroxide and ultrapure water.

Protein Lithography. Lithography of the protogel structures were carried out by the Nanoscribe® Photonic Professional. The system is equipped with an erbium doped, femto second laser source, with a center wavelength of 780 nm, and pulse length of 100 – 200 fs. Structures of the protogels were designed using a Computer-aided Design (CAD) software, 3ds Max®. Parameters of the structures were defined by the Nanoslicer and DeScribe softwares. All structures were written with 16 mW laser power, 0.2 μ m line distance and scan speed of 30 μ m/s, varying only the layer distance.

In the polymerization of BSA, Rose Bengal functions as a photoinitiator, which is excited via a two-photon absorption process to a triplet state, subsequently abstracting a hydrogen atom from an oxidisable side chain of an amino acid in BSA. This generates an electron deficient protein molecule

which could then proceed to scavenge electrons from neighboring molecules of BSA²⁵, leading to the cross-linking of the protein molecules. After fabrication, the substrate was soaked in 20 mM PBS buffer to remove excess, unpolymerized resist, then washed with water and stored in nitrogen box.

In defining the range of layer distance used for our studies, ten sets of rigid and free-form 3D “Y” shape microstructures were fabricated to ensure reproducibility. Free-form structures are elevated on ($w = 5.0\ \mu\text{m}$, $h = 2.5\ \mu\text{m}$) cylindrical pedestals, fixed at a nominal layer distance of $0.2\ \mu\text{m}$ and requiring 13 layers to construct.

All dual-pillar structures are written using nominal layer distance of $0.1\ \mu\text{m}$ and $0.4\ \mu\text{m}$. The dual pillar structures in Figure 4A are fabricated with the pillar of $\Delta d = 0.1\ \mu\text{m}$ has dimensions of $(15.0 \times 1.0 \times 5.0)\ \mu\text{m}^3$ ($l \times w \times h$) and the pillar with $\Delta d = 0.4\ \mu\text{m}$ has dimensions of $(15.0 \times 5.0 \times 5.0)\ \mu\text{m}^3$. The “I” shaped dual-pillar structures (Figure 4B) are fabricated with the pillar with $\Delta d = 0.1\ \mu\text{m}$ has dimensions of $(6.0 \times 1.5 \times 24.0)\ \mu\text{m}^3$ and the pillar with $\Delta d = 0.4\ \mu\text{m}$ has dimensions of $(6.0 \times 5.0 \times 24.0)\ \mu\text{m}^3$. The four free-standing vertical dual-pillar structures in Figure 6 are written using pillars of $(15.0 \times 1.0 \times 5.0)\ \mu\text{m}^3$ and $(15.0 \times 5.0 \times 5.0)\ \mu\text{m}^3$ for $\Delta d = 0.1\ \mu\text{m}$ and $0.4\ \mu\text{m}$, respectively. The pillars are attached to a single cylindrical support $20.0\ \mu\text{m}$ in diameter and $2.0\ \mu\text{m}$ in height, written with a layer distance of $0.2\ \mu\text{m}$.

Swelling Studies. Five sets of rigid and free-form “Y” shaped structures of layer distance $0.1 - 0.5\ \mu\text{m}$ were fabricated and their swelling was observed at two extreme pHs, i.e. pH 5 and pH 11 using optical microscope. Substrates containing fabricated protogels were equilibrated in pH 5 solution for 3 minutes before it was transferred to a pH 11 solution, and equilibrated for 3 minutes. Areas of the structures were measured using ImageJ.

Atomic force microscopy (AFM). AFM measurements were carried out using a JPK Nanowizard®3 Bioscience AFM equipped with JPK Vortis™ SPMControl controller with XYZ closed-loop feedback (JPK instrument, Berlin) on an inverted microscope. Silicon cantilevers from BudgetSensors (model: Multi-75-Al-G with 30 nm Aluminum reflected coating) were used for the quantitative imaging (QI) mode and force spectroscopy. The samples were first incubated in the pH5

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 curves were fitted and tabulated into the graph. TipCheck from BudgetSensors was employed to determine the tip shape and sharpness of the tip after the experiment. Typically, an image of size $1\ \mu\text{m} \times 1\ \mu\text{m}$ was measured and the height scale set to 100 nm, from which the tip radius was estimated to be 50 nm.

Materials Characterization. SEM was done with a JEOL-JSM-7600F microscope, and bright field spectroscopy was done with an Olympus BX51BD microscope. Raman imaging was obtained by the Ramantouch microspectrometer (Nanophoton Inc, Osaka, Japan). The wavelength and power of excitation laser was set at 532 nm and 0.031 mW, respectively. Besides, the excitation laser light was focused into a line on the sample through a cylindrical lens and an Apochromat NIR water immersion objective from Nikon, 60 $\times$ magnification, NA 1.0). For each line, the samples were measured with an exposure time of 0.05 s. The structures are first immersed in pH 5 solution and equilibrated for 5 minutes before the x-y-z Raman imaging of the structure is conducted using a water immersion objective, and subsequently repeated for the 3D Raman imaging at pH 11. The x-y-z SERS map is constructed by using an image processing software which combines multiple x-y SERS maps at intervals of 0.5 μm into a single image.

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3 Geometrical Shape-shifting Protein Microstructures**

ABSTRACT. Inspired by the shape change mechanisms in nature, the recent years has seen the advent of synthetic shape-changing “smart” materials. However, these smart materials are usually fabricated as single macrostructures with directional shape change, such as bending or twisting, which limit their versatility. Here, we demonstrate the circle-to-polygon, geometrical shape-shifting of BSA microstructures fabricated via two-photon lithography. Our strategy is based on the precise embedding of highly cross-linked rigid “skeletal frames” within low cross-linked responsive BSA matrices to afford the directed anisotropic swelling of the microstructure under pH stimulus. By controlling the shape, orientation and number of the embedded skeletal frames, we demonstrate the shape-shifting from circles to triangles, squares, pentagons and hexagons with well-defined arc-to-corner transformations.

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3.1 INTRODUCTION

In nature, organisms are able to change shape to self-regulate and adapt to changes in the environment.¹⁻⁴ Inspired by the shape change mechanisms in nature, synthetic stimulus responsive, shape-changing “smart” materials have been created in recent years, which have facilitated the innovation of revolutionary tools.⁵⁻⁷ Currently, these smart materials are fabricated as single macrostructures with single-directional shape change, which limit their versatility.⁸⁻¹² However, future smart materials should possess flexibility in shape change, whereby the same material can take on multiple configurations for greater adaptability and functionality. Such a material can be constructed *via* the self-assembly of individual stimuli-responsive shape-shifting microstructures each possessing well-defined morphologies. More specifically, these individual smart nanostructures must each possess the dexterity to reconfigure precisely and reversibly, from one geometric form to another on demand,¹³⁻¹⁴ to enable a macrostructure constructed from the self-assembly of these individual shape-shifting microstructures to perform multiple shape change spatially and temporally, as controlled by the self-assembly mechanics of individual shape-changing smart nanostructures.

However, it remains experimentally challenging to fabricate such diverse shape-controlled nanostructures that can undergo reversible predictive shape reconfiguration to higher order shape, such as well-defined polygons, with instantaneous response time (≤ 500 ms). Currently, most shape-change materials are constrained to spherical,¹⁵ cylinder,¹⁶ and/or rod-shaped¹⁷⁻¹⁸ polymeric particles. For instance, spheres and ellipsoids shaped polystyrene-*b*-poly(2-vinylpyridine) block copolymer particles demonstrate pH-responsive shape change from ellipsoids to “accordion-like” segmented structures.¹⁹ Similarly, compartmentalized poly(lactic-co-glycolic acid) microcylinders demonstrated isotropic and anisotropic shape shifting from cylinders to pill-shaped particles to spheres.²⁰ The lack in shape change examples to higher order shapes is mainly due to the technical limitations in fabrication methods. Previously, we incorporated a stiff reinforcement layer within BSA microstructure to control its shape changing direction, where achiral microstructure can be transformed to chiral one under pH stimulus.²¹ The changes in shape of the microstructures are reversible, well-defined and instantaneous (~ 500 ms). However, multiple polygonal shape reconfigurations of individual microstructures, which is highly

desirable for variation in shape of self-assembled future generation smart materials, have yet to be achieved.

Here we demonstrate the dynamic pH-induced shape change of well-defined BSA microstructures, i.e. from circle to polygons (including triangle, square, pentagon and hexagon) in order to potentially create dynamic, self-assembled, shape-shifting structures for greater functionality. We fabricate shape-shifting protein microstructures using only a single material, BSA, where processing and post-treatment is technically less challenging²² as compared to existing techniques. Our strategy is based on the precise embedding of highly cross-linked, rigid “skeletal frames” within low cross-linked responsive BSA matrices to afford the directed anisotropic swelling of the microstructure under pH stimulus. By controlling the shape, orientation and number of the embedded skeletal frames, we demonstrate the shape-shifting from circles to triangles, squares, pentagons and hexagons with both well-defined arc-to-corner and arc-to-edge transformations.

3.2 RESULTS AND DISCUSSION

Design of Geometrical Shape-shifting Microstructures. 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 3.1). 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.^{7, 23-24}

Scheme 3.1. Schematic of dynamic geometrical shape-shifting BSA microstructure fabricated via 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 1) during the layer-by-layer laser writing process at sub-micrometer precision.

We fabricate highly cross-linked BSA segments at $\Delta d = 0.1 \mu\text{m}$. These segments exhibit poorer swelling ability upon pH stimulation (Scheme 3.1). In contrast, lower cross-linked structures are written at $\Delta d = 0.4 \mu\text{m}$ and generally exhibit better swelling ability due to greater freedom of expansion

of the cross-linked network. We can precisely modulate the cross-linking density of BSA microstructures by tuning the fabricating z-layer distance (Δd) during the two-photon layer-by-layer polymerization process, since Δd is inversely proportional to the cross-linking density.²¹ Both the BSA microstructures, fabricated using z-layer distance (Δd) of 0.1 μm and 0.4 μm , undergo swelling (Figure 3.1) when the surrounding pH is changed from 5 to 11 due to the presence of BSA protein. With an isoelectric point between pH 4.7 - 4.9, BSA is significantly more negatively charged at pH 11 than at pH 5.²⁵ 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.²⁶

pH 5	pH 11	Swelling Ratio ($A_{\text{pH } 11}/A_{\text{pH } 5}$)
(A) $\Delta d = 0.1 \mu\text{m}$		1.1 ± 0.0
(B) $\Delta d = 0.4 \mu\text{m}$		1.5 ± 0.0

Figure 3.1. Optical microscopic images illustrating the area swelling ratio of square BSA microstructures fabricated using a z-layer distance (Δd) of (A) 0.1 μm and (B) 0.4 μm , respectively. Area swelling ratio is calculated by dividing area at pH 11 ($A_{\text{pH } 11}$) with the area at pH 5 ($A_{\text{pH } 5}$).

As expected, BSA segments with high cross-linking density ($\Delta d = 0.1 \mu\text{m}$) demonstrate negligible swelling with a swelling ratio of (1.1 ± 0.0 ; Figure 3.1A). On the other hand, BSA segments with lower cross-linking density ($\Delta d = 0.4 \mu\text{m}$) has a swelling ratio of (1.5 ± 0.0 ; Figure 3.1B), which is $\sim 38 \%$ higher than that at $\Delta d = 0.1 \mu\text{m}$. Hence, it is evident that the precise modulation of cross-linking density of a BSA matrix is able to create distinct swelling properties, which forms the basis of our latter design on dynamic geometrical shape-shifting BSA microstructures. 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. Hereafter,

BSA segments fabricated at $\Delta d = 0.1 \mu\text{m}$ and $0.4 \mu\text{m}$ are termed as “skeletal frames” and “responsive BSA matrix”, respectively.

Circle-to-Polygon Shape-shifting Microstructures. For circle-to-polygon shape-shifting of BSA microstructures, the key criterion is the transformation of arc-to-corner/edges, in order to generate the well-defined corners and edges that make up a polygon from the initial arcs of a circle (Figure 3.2). Our approach lies at the induction of directed anisotropic swelling of responsive segmented BSA microstructures. We subsequently evaluate the arc-to-corner/edge transformation, and also the effectiveness of geometrical circle-to-polygon shape-shifting of our BSA microstructure using three critical parameters, namely (1) area of occupancy, (2) protrusion index and (3) edge fitting.

Firstly, area of occupancy (Figure 3.2A) denotes the extent to which the swelled microstructure conforms to the ideal intended shape, which directly indicates the effectiveness of geometrical shape-shifting. A_{ideal} is defined as the unoccupied area when a hypothetical isotropically-swelled microstructure overlaps with the ideal transformed polygon; while $A_{\text{structure}}$ is the experimentally obtained area within A_{ideal} that is occupied by BSA microstructure upon swelling. Area of occupancy is calculated as a percentage by which the protrusions generated by experimentally shape-shifted microstructures ($A_{\text{structure}}$) fill the area that is unoccupied by the isotropically-swelled microstructure (A_{ideal}) (Figure 3.2A), which can be illustrated as:

$$\text{Area of occupancy} = A_{\text{structure}}/A_{\text{ideal}} \times 100 \%$$

The larger the area of occupancy, the better a microstructure conforms its ideal shape and has more well-defined geometrical transition. For a successful shape-shifting, area of occupancy must be $> 50 \%$.

The second parameter we consider is termed the protrusion index (Figure 3.2B), which determines if a protrusion is classified as an arc or a corner. The protrusion index can be calculated as:

$$\text{Protrusion index} = r/R_c \times 100 \%,$$

where “ r ” is the radii of curvature of generated protrusions in experimentally transformed BSA microstructures and “ R_c ” is a reference radius of curvature of an isotropically swelled circular microstructure. A smaller “ r ” denotes a sharper protrusion, and vice-versa. A protrusion is defined as

a “corner” when the protrusion index ≤ 50 %. That is, a protrusion approximates to a corner when it has a radius of curvature that is at least 50 % smaller than that of an isotropically-swelled ideal circular microstructure (Figure 3.2B(3), (4)). On the other hand, protrusions with protrusion index > 50 % are classified as “arcs” (Figure 3.2B(2)).

Figure 3.2. (A) Schematics illustrating the area of occupancy of BSA microstructures (outline in blue) in an ideal shape (outline in black) when it undergoes anisotropic swelling to cover (1) 0 %, (2) 25 %, (3) 50 %, and (4) 75 % of the initial empty area, for circle-to-polygon shape-shifting, respectively. 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}). (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 3.6E; $R_c = 12.2 \mu\text{m}$; $\Delta d = 0.4 \mu\text{m}$; swelling ratio ~ 1.5).

(C) Schematics illustrating the contours of BSA microstructures with edge fitting of (1) 25 %, (2) 50 %, and (3) 75 %, to a hypothetical reference line (purple line). Edge fitting (edge fitting = $L_{\text{experimental}} / L_{\text{ideal}} \times 100 \%$) is determined by comparing the length of experimental and ideal edges.

The third parameter that we consider is edge fitting, which evaluates whether a microstructure's side contour resemble a straight line (edge) or an arc (Figure 3.2C). We define edge fitting as follows:

$$\text{Edge fitting} = L_{\text{experimental}} / L_{\text{ideal}} \times 100 \%$$

Where L_{ideal} denotes an ideal edge line, which is the longest possible straight line that can be fitted along the BSA microstructures, and $L_{\text{experimental}}$ denote regions of the microstructures that are in contact with the ideal edge line upon swelling. Microstructures with edge fitting $< 50 \%$ possess more rounded contours and has less contact with the ideal straight line (Figure 3.2C(1)). When edge fitting $> 50 \%$, microstructures possess flatter contours which are in larger contact with the ideal straight line (Figure 3.2C(2), (3)). This indicates that the higher the extent of edge fitting, the greater the microstructure contour approximates to a straight line. Hence, we define the “side” of a transformed polygon as an edge only if edge fitting $> 50 \%$.

Overall, in a successful circle-to-polygon shape-shifting event, we should observe (1) high conformity to the ideal polygon shapes with area of occupancy $> 50 \%$, as well as distinct arc-to-corner/edge transformation such that (2) corners have a protrusion index $\leq 50 \%$, and (3) edge fitting $> 50 \%$.

Using circle-to-triangle shape-shifting as an illustration, we first embed three equally spaced triangular skeletal frames into a responsive BSA circle (Figure 3.3A, B). When immersed in a pH 5 solution, the as-fabricated BSA microstructure remains circular due to negligible water absorption at its isoelectric point.²⁷ As the solution is changed from pH 5 to pH 11, the circular BSA microstructure becomes negatively-charged and interacts strongly with water via ion-dipole interactions. Consequently, the highly responsive BSA matrix undergo anisotropic swelling directed by the skeletal frames to effect rapid circle-to-triangle shape-shifting within 2 seconds via arc-to-corner/edge

transformations, as evident from the fulfillment of the three criteria where area of occupancy and edge fitting are both above 50 %, while protrusion index is <50 % (Figure 3.3F – H). In comparison, a plain circular BSA microstructure with no embedded skeletal frame simply undergoes ~ 1.5-fold isotropic swelling at pH 11 without exhibiting shape-shifting behavior (Figure 3.3E). We attribute such anisotropic swelling to the high mechanical constraints imposed by the embedded skeletal frames. Hence, regions of responsive BSA matrix attached to or near the skeletal frame experience a greater resistance to swell (lower swelling ratio) as imposed by the stationary skeletal frame (Figure 3.3A). On the other hand, regions of the same BSA segments further away from skeletal frames are less constrained and exhibit greater swelling.²³ The presence of such a swelling gradient in the responsive BSA matrix sandwiched between two adjacent skeletal frames generates a new parabolic swelling profile. Such circle-to-triangle transformation also exhibits high reversibility on decreasing pH 11 to initial pH 5 (Figure 3.4).

Figure 3.3. (A) Schematic illustrating the arc-to-corner conversions of responsive BSA matrices upon anisotropic swelling at pH 11. Circle-to-triangle shape-shifting of BSA microstructures embedded with three equally distributed (B) triangle-, (C) stick- and (D) block-shaped skeletal frames. (E) Control circular 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. Scale bar, 10 μm . Experimental comparison and schematics of (F) area of occupancy, (G) protrusion index and (H) edge fitting between shape-shifted BSA microstructures embedded with triangle-, stick- or block-shaped skeletal frames. A well-defined circle-to-polygon shape-shifting event occurs only when the transformed microstructure has area of occupancy $> 50\%$, protrusion index $\leq 50\%$ and edge fitting $> 50\%$.

In addition, the shape of skeletal frames has a direct impact on arc-to-corner/edge transformation and hence the precision of shape-shifting from circle-to-polygon. We demonstrate that triangular skeletal frame is superior in generating well-defined corners/edges when compared to skeletal frames of other shapes, such as sticks and blocks (Figures 3.3C, D). This is possibly due to sharper parabolic swelling profiles generated by triangular skeletal frames (Figure 3.3B(3)), which creates more oblique corners (protrusion index <50 %) than other skeletal frames of different shapes.

Different skeletal frame shapes		Area of occupancy, $A_{\text{structure}}/A_{\text{ideal}} (\%)$	Protrusion index, $r/R_c (\%)$	Edge fitting, $L_{\text{experimental}}/L_{\text{ideal}} (\%)$
<p>pH 5 pH 11</p> <p>(A) Triangular</p>		59 ± 2	50 ± 1	87 ± 4
<p>(B) Stick-shaped rectangular</p>		40 ± 2	84 ± 4	70 ± 8
<p>(C) Block-shaped rectangular</p> <p>—10 μm</p>		40 ± 2	69 ± 3	86 ± 6

Figure 3.4. Evaluation of area of occupancy, protrusion index and edge fitting of shape-shifted BSA microstructures embedded with three equally distributed (A) triangle-, (B) stick- and (C) block-shaped skeletal frames. The measurements highlighted in red denote the parameters that do not fulfill the requirement of circle-to-triangle shape-shifting.

As demonstrated in Figures 3c and 3d, BSA microstructures embedded with stick- and block-shaped skeletal frames possess broader and blunt parabolic swelling profiles (Figures 3.3C(3), D(3)) due to greater separation between adjacent skeletal frames, which prevents the formation of distinct edges and corners. A systematic investigation into the effect of skeletal frame's shapes (triangle, stick and block) on circle-to-polygon transformations (Figure 3.4) demonstrate that BSA microstructures

embedded with stick-shaped skeletal frames possess an area of occupancy, protrusion index, and edge fitting of $(40 \pm 1) \%$, $(84 \pm 4) \%$, and $(70 \pm 8) \%$ (Figure 3.4B), respectively. For block-shaped skeletal frames, the parameters are quantified accordingly at $(40 \pm 1) \%$, $(69 \pm 3) \%$, and $(86 \pm 6) \%$ (Figure 3.4C). Hence, the swelling behavior of microstructures embedded with both stick-shaped and block-shaped skeletal frames did not meet all the pre-defined criteria (Figure 3.2), and therefore, cannot be considered as successful and well-defined circle-to-triangle transformations. This highlights the superiority of triangle-shaped skeletal frames over skeletal frames of other shapes to effect well-defined circle-to-triangle shape-shifting. Hereafter, we only employ triangular skeletal frame for the fabrication of circle-to-polygon BSA microstructures due to its ability to create well-defined geometrical arc-to-corner/edge transformation.

Generation of a Library of Geometrical Shape-shifting Microstructures. Based on our design strategy above, we fabricate a library of circle-to-polygon shape-shifting BSA microstructures. We embed different numbers of triangular skeletal frames within the responsive BSA matrix to effect the corresponding number of arc-to-corner transformations; four, five and six equally spaced triangular skeletal frames are embedded in circular BSA microstructures (Figures 3.3C – E) to create square, pentagon and hexagon from circle at pH 11, respectively.

Using the concept of area of occupancy ($A_{\text{structure}}/A_{\text{ideal}} \times 100 \%$), our circle-to-polygon BSA microstructures are subsequently evaluated to determine the effectiveness of their geometrical shape-shifting. We quantify the experimental area of occupancies at 59 %, 71 %, 74 % and 73 % for the shape-shifting of initial circular BSA microstructure to triangle, square, pentagon and hexagon (Figure 3.5), respectively. Notably, all the circle-to-polygon shape-shifting BSA microstructures exhibit an area of occupancy of $> 50 \%$, which clearly indicates that the transformed microstructures approximate closely to their respective ideal shapes as a result of well-defined circle-to-polygon transitions.

Figure 3.5. Schematic representations (top) and optical microscopic images (bottom) of dynamic geometrical shape-shifting of circular BSA microstructures (A – D) at pH 5 to (E) triangle, (F) square, (G) pentagon, and (H) hexagon at pH 11. Ideal shapes of polygons (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.

The radius of curvature (RoC), and therefore the protrusion index, of all the shape-shifted BSA microstructures are quantified to distinguish between arc and corner for both circle-to-polygon transformations. This is one of the determining factors for achieving well-defined geometrical shape-shifting. We observe that the protrusion index for the shape-shifted triangle, square, pentagon and hexagon are 50 %, 45 %, 37 % and 33 %, respectively (Figure 3.6). The general protrusion index of $\leq 50 \%$ therefore indicate that protrusions generated on swelling at pH 11 can be identified as corners, clearly exemplifying the arc-to-corner transition which is crucial for circle-to-polygon transformation.

Figure 3.6. Schematic representations (top) and optical microscopic images (bottom) of dynamic geometrical shape-shifting of circular BSA microstructures (A – D) at pH 5 to (F) triangle, (G) square, (H) pentagon, and (I) hexagon at pH 11. Ideal circles (cyan) are fitted into generated protrusions of respective polygons at pH 11 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 $12.2 \mu\text{m}$. (K, L) Schematic illustrations of the anisotropic swelling for circle-to-triangle and circle-to-hexagon shape-shifting, respectively.

Furthermore, the newly-generated corners generally become sharper as the number of skeletal frames embedded increases from three (triangle; protrusion index $\sim 50\%$) to six (hexagon; protrusion index $\sim 33\%$). For the circle-to-triangle transformation, the relatively higher protrusion index can be attributed to the larger separation distance of adjacent skeletal frames. Regions of responsive BSA matrix attached to or near the skeletal frame experience a greater resistance to swell (lower swelling ratio) as imposed by the stationary skeletal frame (Figure 3.6K). On the other hand, regions of the same BSA segments further away from skeletal frames are less constrained and exhibit greater swelling ability. Consequently, the larger separation of adjacent skeletal frames in a circle-to-triangle shape-shifting microstructure creates broad parabolic swelling profiles which result in the formation of truncated corners with protrusion index $\sim 50\%$. In contrary, the close proximity of adjacent skeletal frames, such as in circle-to-hexagon BSA microstructures, leads to a sharper swelling profile which generate well-defined corners with a smaller protrusion index of $\sim 33\%$ (Figure 3.6L). The importance of skeletal frame in directing anisotropic swelling for geometrical shape-shifting is again emphasized when a control circular microstructure (Figure 3.6J), in the absence of skeletal frame, undergoes an isotropic swelling without geometrical shape change.

Figure 3.7. Schematic representations (top) and optical microscopic images (bottom) of dynamic geometrical shape-shifting of circular BSA microstructures (A – D) at pH 5 to (E) triangle, (F) square, (G) pentagon, and (H) hexagon at pH 11. Shapes of respective ideal polygons are outlined with blue dotted lines. Tangent lines on each side of transformed polygons (side length, $L_{experimental}$) are outlined in orange and ideal edge lengths (L_{ideal}) are in black. Edge fitting ($L_{experimental}/L_{ideal} \times 100\%$) is determined by comparing the length of experimental and ideal edges.

We also measure the edge fitting of the shape-transformed microstructures. Based on our experimental results (Figure 3.7), all the sides of our transformed polygons, such as triangle, square, pentagon and hexagon, are considered as edges due to their high conformity to a straight line (as edge fitting $> 50\%$), which is the basis for the definition of an edge.

Rules-of-Thumb for Geometrical Shape-shifting. Collectively, we propose two general rules-of-thumb for the fabrication and programming of pre-defined circle-to-polygon and shape-shifting into BSA microstructures. Firstly, arc-to-corner/edge conversion is crucial in circle-to-polygon shape-shifting. This is achieved by embedding equally spaced triangle-shaped skeletal frames to direct the formation of sharp corners in circular microstructures. Secondly, the number of embedded

skeletal frames determines the number of corners/edges formed from regions of responsive BSA matrices.

Figure 3.8. (A) Schematic depicting the circle-to-polygon shape-shifting of BSA microstructure on stimulation at pH 11. Optical microscopic images of dynamic geometrical shape-shifting of circular BSA microstructures to (B) triangle, (C) square, (D) pentagon, and (E) hexagon. (1, 2) Optical microscopic images of as-fabricated BSA microstructure at pH 5, and their transformed shape at pH 11, respectively. Circle-to-polygon shape-shifting efficacy expressed in terms of (F) area of occupancy, (G) protrusion index and (H) edge fitting.

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. We are able to precisely generate three, four, five and six corners as a result of the anisotropic swelling of responsive BSA matrix sandwiched between skeletal frames (Figure 3.8). We also observe that the

shape-shifted triangle, square, pentagon and hexagon fulfill all the three criteria set for well-defined circle-to-polygon shape-shifting with area of occupancy $> 50\%$, protrusion index $\leq 50\%$ and edge fitting $> 50\%$ (Figure 3.8F – H). Hence, it is clear that specific well-defined circle-to-polygon transformations can be programmed through defining the number of skeletal frames embedded in the BSA microstructures.

Furthermore, these precisely engineered BSA shape-shifting microstructures are the first demonstration of geometrical shape-shifting, which is unique to conventional shape-changing 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. The rapid, dynamic, well-defined and on-demand geometrical shape-shifting microstructures are essential for the future construction and study of reconfigurable “smart” materials.

3.3 CONCLUSIONS

We have demonstrated the fabrication of a large library of well-defined circle-to-polygon and polygon-to-circle shape-shifting bovine serum albumin (BSA) microstructures by adopting two general rules-of-thumb: (1) arc-to-corner conversions that are essential for circle-to-polygon transformation is achieved by employing triangular skeletal frames and (2) the number of corners created is controlled by the quantity of skeletal frames embedded. We define a successful shape-shifting event when the area of occupancy and edge fitting $> 50\%$ and the protrusion index $\leq 50\%$. By following these rules, we are able to achieve the successful shape-shifting of circles to triangles, squares, pentagons and hexagons when pH is tuned from pH 5 to pH 11. We can potentially apply these shape-shifting microstructures to construct the first shape-shifting 2D array which can be used to 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) by tuning the physical properties of the array.

3.4 MATERIALS AND METHODS

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. Ultrapure water (> 18.0 M Ω .cm) was obtained from a Sartorius arium® 611 UV ultrapure water system.

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.

Preparation of different pH solutions. pH solutions were prepared in volumes of 10 mL using 1 M hydrochloric acid and 1 M sodium hydroxide. Solution pH of 5 was prepared from serial dilution of 1 M hydrochloric acid and ultrapure water whereas solution pH of 11 was prepared by serial dilution from 1 M sodium hydroxide and ultrapure water.

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 Computer-aided Design (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 shape-shifting microstructures were fabricated with dimensions of $20.0\ \mu\text{m} \times 20.0\ \mu\text{m} \times 5.0\ \mu\text{m}$, $l \times w \times h$.

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.

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 $\times$ 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.

Data Analysis. Measurements of swelling ratio and pH-induced changes in area of as-fabricated BSA microstructures were conducted using ImageJ.

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4 Direct Metal Writing and Precise Positioning of Gold Nanoparticles within Microfluidic Channels for SERS Sensing of Gaseous Analytes**

ABSTRACT We demonstrate a one-step precision polyol photoreduction of Au³⁺ into monodisperse Au nanoparticles (AuNPs) forming densely packed, well-defined patterns for microfluidic surface-enhanced Raman scattering (SERS) detection of gaseous analytes. We achieve this using two-photon lithography from Au precursor comprising poly(vinylpyrrolidone) (PVP) and ethylene glycol (EG), where EG promotes higher reduction rate of Au³⁺ via polyol reduction. Hence, clusters of monodisperse AuNPs are generated along raster scanning of the laser, forming high particle density, well-defined structures. By varying PVP concentration, we tune AuNP size from 27.3 – 65.0 nm and density from $1.7 \times 10^2 - 9.7 \times 10^2$ particles/ μm^2 , corresponding to surface roughness of 12.9 – 67.0 nm, which is important for surface-based applications such as SERS. We find the microstructures exhibit SERS enhancement factor $> 10^5$, and demonstrate remote writing of well-defined Au microstructures within a microfluidic channel for SERS detection of gaseous molecules. We showcase in-situ SERS monitoring of gaseous 4-MBT and real-time detection of multiple small gaseous species with no specific affinity to Au. This one-step, laser-induced fabrication of AuNP microstructures ignites a plethora of possibilities to position desired patterns directly onto or remotely within most surfaces for future creation of multi-functional lab-on-a-chip devices.

4.1 INTRODUCTION

Direct metal writing using two-photon lithography is a laser-induced photoreduction process that leads to simultaneous nucleation of metal atoms into metallic nanostructures of precise patterns at ambient conditions. Consequently, conductive metallic nanostructures can be selectively imprinted onto designated areas easily. This is superior to conventional photolithography where fabrication is logistically challenging requiring clean room facilities and expensive photomask,¹⁻³ Laser direct metal writing is commonly achieved by mixing a metallic precursor with a polymer matrix or organic monomer, where the metallic precursor is photoreduced together with the polymer to form a metal-polymer composite.⁴⁻⁵ The polymer functions as a physical support to stabilize written nanostructures, allowing flexible and continuous metallic lines⁶ or disconnected nanoparticle (NP) layers⁷ to be fabricated as split-ring resonators, 3D structures,⁸⁻⁹ patterned surface-enhanced Raman scattering (SERS) substrates,¹⁰ and electrical micropatterns.¹¹ However, the presence of an insulating polymer results in decreased metal loading capacity (< 50 % wt)¹² and lowered conductivity,^{4, 8} reducing the efficiency of fabricated nanostructures in both nanoelectronics and photonics.

To resolve this issue, metallic nanostructures without a need for polymeric matrix have also been fabricated using direct metal writing recently.¹³ This is achieved by adding a surfactant into the metal precursor solution,¹⁴ to stabilize and facilitate nucleation of reduced metal atoms to form metal NPs. A photoinitiator may also be added to further promote NP synthesis and as a result, individual or interconnected metallic nanostructures can be generated.¹⁵ Unlike the polymer in the metal-polymer composite, the surfactant and photoinitiator on the NPs can be easily removed, making this technique promising for the fabrication of catalytic microreactors¹⁶ and designed SERS substrates for sensing and monitoring of reaction processes.¹⁷⁻¹⁸ However, many of these structures exhibit either sparsely distributed NPs¹⁹⁻²¹ (from $9.0 \times 10^1 - 3.6 \times 10^2$ particles/ μm^2)²² as the surfactant does not contribute to the photoreduction process, or densely packed but polydisperse NPs.¹²⁻¹³ While the effect of the photoinitiator has been demonstrated to create densely packed NPs, these NPs are generally inhomogeneous and random¹⁵ owing to the uncontrolled radical photoreduction. Size control over fabricated structures to maximize active surface area is especially important in catalysts to achieve

higher catalytic efficiency, as well as in SERS applications where plasmonic properties of the structures can be tuned to achieve higher sensitivity of detection²³⁻²⁵ and pave the way for the future creation of 3D photonic nanocrystals and optical antennas.²⁶⁻²⁷

Here, we demonstrate bottom up, in-situ two-photon polyol photoreduction of Au³⁺ into well-defined AuNP microstructures comprising densely packed, stable AuNPs via raster scanning of the laser, for SERS detection of gaseous analytes within a microfluidic channel. Our strategy aims to increase AuNP formation by addition of a viscous reducing agent, ethylene glycol (EG) which functions as polyol reducing agent²⁸ to achieve higher reduction rate of Au³⁺. The high viscosity of EG reduces diffusion rate of Au atoms²⁹⁻³⁰ and subsequently direct their nucleation into homogeneous, monodisperse AuNPs. This enables fabrication of precise AuNP microstructures comprising densely packed, quasi-spherical AuNPs with > 90 % increase in particle density compared to reported works,²² from $(1.7 \times 10^2 \pm 4)$ particles/ μm^2 to $(9.7 \times 10^2 \pm 65)$ particles/ μm^2 , By varying PVP concentration in the precursor solution, we are able to tune sizes of individual AuNPs from (27.3 ± 4.1) nm to (65.0 ± 15.9) nm and consequently, surface roughness of the microstructure from (12.9 ± 2.9) to (67.0 ± 16.6) nm. Owing to the unique direct metal writing technique to form AuNP microstructures, we are also able to perform remote metal writing. We demonstrate that as-designed AuNP microstructures can be fabricated *within* a flat microfluidic channel. The structures are stable and exhibit strong SERS performance where enhancement factor of the best performing platform is $> 10^5$, and can function as “on-off” indicators to monitor and detect diffusing gaseous analytes within 2 minutes. We further showcase the capability of the microstructures as a recyclable SERS gas detection channel to detect sequentially gaseous ethanol and acetone. Such precise positioning of as-designed AuNP microstructures ignites a plethora of possibilities, to position desired patterns directly onto or remotely within any transparent surface for the fabrication of next-generation multi-functional lab-on-a-chip devices.

4.2 RESULTS AND DISCUSSION

Direct Metal Writing and Characterization of AuNP Microstructures. We use two-photon lithography to achieve the in-situ, nanoscale polyol photoreduction of Au^{3+} into monodisperse, quasi-spherical Au nanoparticles (AuNP), where densely packed and well-defined AuNP microstructures are precisely formed via the raster scanning of the laser. To begin, we deposit Au precursor solution containing gold(III) chloride (HAuCl_4), poly(vinylpyrrolidone) (PVP, 10 % v/v), and ethylene glycol (EG) on a clean glass substrate functionalized with (3-aminopropyl)triethoxysilane (APTES). Our direct metal writing system is equipped with a femtosecond two-photon laser centered at 780 nm wavelength, which is raster-scanned at a speed of 1 $\mu\text{m/s}$ along a predefined x - y plane to reduce Au^{3+} to form Au microstructures to form the word “METAL”, demonstrating the versatility of our lithography technique. We subsequently wash the as-written substrate with water, 2-propanol and 0.5 M potassium iodide (KI) solution to remove excess reactants and PVP.³¹

The dark field and scanning electron microscopy (SEM) images show the written structures to be well-defined, with the structures exhibiting a yellow scattering color under the optical microscope (Figure 4.1A, B). The linewidth of written structures is measured at $(2.1 \pm 0.4) \mu\text{m}$ (Figure 4.1C). Upon closer inspection, each individual letter is revealed to comprise a close-packed cluster of NPs (Figure 4.1D), with average diameter of $(42.9 \pm 6.7) \text{ nm}$, and particle density of $(6.0 \times 10^2 \pm 1.0 \times 10^2) \text{ particles}/\mu\text{m}^2$ (Figure 4.1E), giving the microstructures an overall “rough” appearance.

Figure 4.1. Fabrication and characterization of Au structures. (A) Dark field image and (B) SEM images of direct laser written Au patterns. (C) A magnified SEM image demonstrates that the written structures are predominantly comprised of nanoparticles, (D, E) with average size distribution of the nanoparticles being $(42.9 \pm 6.7) \text{ nm}$.

We highlight that the amino-terminated glass surface is important to enhance the stability of as-fabricated microstructures to the glass substrate via a weak covalent bond between the amino group and gold.³²⁻³⁴ In contrast, structures written on non-functionalized glass substrates are usually poorly retained on the substrate surface (Figure 4.2).

Figure 4.2. Written Au microstructures on a non-functionalized glass substrate as observed (A) through the built-in camera on the Nanoscribe Photonic Professional just after fabrication and (B) through the dark field microscope after removing excess precursor solution, where most of the structures are observed to have fallen off the substrate.

The energy dispersive X-ray (EDX) analysis confirms that the written structures are primarily composed of Au, with the background comprising mainly Si and O which is typical of a glass substrate. The elemental map of the SEM image demonstrates that most of the Au present in the sample is concentrated on the fabricated microstructure (shown in light blue) whereas Si is mainly observed in the background (shown in purple) (Figure 4.3).

Figure 4.3. EDX spectrum of fabricated Au microstructures.

We perform X-ray photoelectron spectroscopy (XPS) analysis to further verify the surface composition of the clustered NP lines by identifying the elements present and their respective oxidation states. Due to the large scanning area (1 cm^2) and high density of AuNPs required to perform XPS (and later X-ray diffraction, XRD), we simulate the photoreduction reaction using a solar simulator³⁵ to generate AuNPs in bulk for the analyses (Figure 4.4, 4.5A).

Figure 4.4. SEM images of (A) AuNP generated via two-photon lithography and (B) AuNP generated via UV photoreduction process. Both samples contain 10 % PVP. The size difference in the nanoparticles between the two conditions is ~ 30 nm.

The 300 W Xenon lamp may appear to operate at a higher power output than the two-photon laser at 6 mW, however, it is noteworthy that the “laser power” of a two-photon laser refers to the average power of the pulsed laser, averaged over every pulse width and pulse-to-pulse duration, which is indicated by the repetition rate of the laser.³⁶ In our system, we use a laser with pulse width of 150 fs and repetition rate of 80 MHz, corresponding to a pulse-to-pulse duration of 12.5 ns. Hence, the actual power per pulse, termed the “peak power” of the two-photon laser operating at 780 nm wavelength, with 150 fs pulse width and at a repetition rate of 80 MHz is in fact 500 W, which is similar in power to the 300 W Xenon lamp (Please refer to Appendix). Hence, the 300 W Xenon lamp solar simulator is an adequate representative of the two-photon laser in generating AuNP in the bulk phase.

SEM images demonstrate that Au precursor can be similarly photoreduced under UV-light to form Au nanoparticles. These particles appear largely similar to those formed using two-photon lithography, however the average particle length and distribution of the UV-light photoreduced

nanoparticles are broader than those generated via two-photon lithography, ranging in size from 20 nm to 190 nm, and an average size of 73 nm. The difference in AuNP sizes could be a result of the difference in light intensity. Although the two-photon laser generates less photons per second (2.35×10^{16} photons) as compared to the Xenon lamp (7.73×10^{18} photons – 1.62×10^{19} photons from the 300 W Xenon lamp operating within a wavelength range between 200 – 420 nm (UV region)), the two-photon laser is tightly focused and hence confers significantly higher light intensity (2.55×10^{15} W/m² per pulse, and 3.06×10^{10} W/m² per second) than the diffuse light from the Xenon lamp (1.52×10^5 W/m²), resulting in higher and faster photoreduction rate of Au³⁺ (please refer to the appendix for detailed calculations). Hence, AuNPs fabricated with two-photon laser are smaller, and fabrication rate is observed to be instantaneous, whereas AuNPs fabricated with the Xenon lamp solar simulator are larger, and the fabrication process is slower, requiring ~ 15 minutes for the formation of AuNP (where precursor solution is observed to change colors from yellow to colorless, and then to purple).³⁷⁻

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The high-resolution Au 4f XPS spectrum of the AuNPs reveals only two sharp peaks at 87.0 and 83.3 eV, where the full-width half-maximum (FWHM) of each of these peaks is approximately 1.0. The difference between the 2 peaks is 3.7 eV, which is characteristic of the Au⁰ 4f doublet spaced at 3.65 eV (Figure 4.5B), indicating that the fabricated AuNPs composes of Au⁰. Furthermore, the XPS reveals the absence of N on the structures, and the presence of I,³⁹⁻⁴⁰ indicating that most of the surface N-containing PVP is successfully removed with KI (Figure 4.5C, D). This is a key advantage of surfactant-based Au microstructure fabrication over polymer matrix-based Au microstructure fabrication, in that while surfactants can be easily removed from the Au structures, the polymer matrix on the other hand, cannot be easily removed without compromising structure integrity. The ability to clean the surface of possibly interfering species is especially important in SERS applications to prevent cross-talking between analyte surface contaminants.

Figure 4.5. XPS spectra of UV photoreduced Au structure. Structures are washed with ethanol and potassium iodide after fabrication. (A) Low magnification SEM image showing uniform distribution of UV generated AuNP and the corresponding full XPS spectrum of the particles. (B) The presence of gold is observed, but the (C) absence of nitrogen indicates that most of the PVP has been successfully removed from the surface of the AuNP. (D) the element Iodine is observed as a result of the washing procedure.

The crystallinity of the fabricated AuNPs is also determined using (XRD). We observe five sharp diffraction peaks at 38.2° , 44.4° , 64.6° , 77.5° and 81.7° in the 2θ range of $20-85^\circ$ (Figure 4.6) in the XRD spectrum of the AuNPs. They are indexed to the (111), (200), (220), (311) and (222) reflections of fcc phase of metallic gold (JCPDS, card No. 04-0784), attesting the high crystallinity of the AuNPs.

Figure 4.6. XRD spectra of the nanoparticles demonstrate that the structures are made of crystalline Au^0 FCC lattice.

Our direct metal writing method using EG and PVP is highly robust in achieving quasi-spherical, crystalline AuNPs that can be precisely directed into well-defined and densely packed microstructures, which has not been demonstrated previously. The fabrication of highly packed AuNP microstructures arises from a laser-induced polyol reduction of Au^{3+} to form Au^0 . At the laser focal plane, Au^{3+} ions are excited^{38, 41-43} and subsequently reduced by EG in a polyol reduction process to form Au^0 (Figure 4.7). The presence of EG as a reducing agent promotes a higher reduction rate of Au^{3+} as compared to when no reducing agent is added. Furthermore, the high viscosity of EG serves to reduce the diffusion rate of Au atoms, leading to slower growth rates^{29, 44} and hence the formation of monodisperse, mainly quasi-spherical AuNPs.⁴⁵ On the other hand, PVP molecules function as a capping agent by competing with Au^0 atoms for the surface of nucleated AuNPs through charge-transfer type interactions⁴⁶ between Au and the carbonyl group in PVP,⁴⁷⁻⁴⁸ to prevent further metallic growth and promote the formation of AuNPs. The steric hindrance imposed by the bulky PVP molecule on PVP-capped AuNPs further prevents aggregation, allowing dense clusters of stable, monodispersed AuNPs to form within written microstructures.

Figure 4.7. The mechanism of direct gold writing in the presence of ethylene glycol and PVP via two-photon lithography to form Au nanoparticles which can be precisely positioned to form desired micropatterns

Effect of PVP on Au Structures. We systematically tune the size of AuNPs by varying the PVP concentration in the precursor solutions. Keeping the concentrations of Au(III) salt and EG constant, we prepare Au precursor solutions with increasing PVP concentrations of 0 %, 5 %, 10 %, 20 %, and 30 % v/v (termed P0, P05, P10, P20, and P30, respectively) and fabricate horizontal lines using the precursor solutions of varied PVP concentrations (Figure 4.8A). SEM images demonstrate that NP size decreases when PVP concentration increases from 0 % to 30 %. The average NP sizes are measured at (65.0 ± 15.9) nm, (52.3 ± 22.1) nm, (43.0 ± 6.7) nm, (37.4 ± 5.4) nm, and (27.3 ± 4.1) nm for P0, P05, P10, P20 and P30, respectively (Figure 4.8B – F).

Figure 4.8. (A) SEM image of written Au microstructures. (i) SEM images and (ii) nanoparticle size distribution of written structures when different amounts of PVP (B) 0 %, (C) 5 %, (D) 10 %, (E) 20 %, and (F) 30 % are added. Nanoparticle size is observed to decrease with increasing PVP concentration.

Beyond a PVP concentration of 30 %, the precursor solution is saturated and difficult to dissolve. The poor uniformity of the precursor solution results in the formation of NPs of

inhomogeneous sizes (Figure 4.9), hence, no further increase in PVP concentration to tune nanoparticle size is attempted.

Figure 4.9. The above shows the precursor solution of 40 % PVP concentration (P40). (A) The precursor solution turns cloudy during preparation, possibly from oversaturation of PVP, (B) and needs a longer time (~ 1 hour) to dissolve completely. However, during the writing process, the P40 precursor solution also turns cloudy again. This might be due to PVP precipitating out of the saturated precursor solution during the writing process upon laser agitation as a result of oversaturation, leading to both lowered net PVP concentration and poor uniformity of the precursor solution, and hence the formation of (C) (i) larger nanoparticles as seen from SEM images of P40 microstructures. (C) (ii) The average nanoparticle size is measured to be (47.0 ± 15.9) nm, similar to P10 microstructures.

The particle density also increases with increasing PVP concentration, from $(1.7 \times 10^2 \pm 4)$ particles/ μm^2 , to $(6.0 \times 10^2 \pm 1.0 \times 10^2)$ particles/ μm^2 , $(8.0 \times 10^2 \pm 40)$ particles/ μm^2 , $(8.9 \times 10^2 \pm 1.1 \times 10^2)$ particles/ μm^2 , and $(9.7 \times 10^2 \pm 65)$ particles/ μm^2 for P0, P05, P10, P20 and P30, respectively (Figure 4.10A – B). The particle density of our AuNP microstructures is higher than previously reported results of $9.0 \times 10^1 - 3.6 \times 10^2$ particles/ μm^2 , which we attribute to the presence of EG in our precursor solution that promotes higher reduction rate to Au^0 . In addition, the root-mean-squared surface roughness of the microstructures also increases with increasing PVP concentration, with P0 possessing the “smoothest” surface at (12.5 ± 2.9) nm, followed by P05, P10, P20 and P30

with decreasing surface roughness of (45.2 ± 3.6) nm, (47.9 ± 7.8) nm, (58.0 ± 10.3) nm, and (67.1 ± 16.6) nm, respectively (Figure 4.10C).

Figure 4.10. (A) Size distribution of nanoparticles, (B) particle density and (C) RMS roughness of fabricated AuNP microstructures with respect to different amount of PVP.

We note that while P05 - P30 microstructures mainly compose of nanoparticles, P0 microstructures are predominantly composed of fused, flat “smooth” Au structure with only the edges composing of sparse AuNPs (Figure 4.11A). As a result, P0 microstructures are wider ($6.0 \pm 1.3 \mu\text{m}$) and shorter (83.6 ± 37.9) nm as compared to P05 - P30 AuNP microstructures, which possess an average width of $\sim 2.0 \mu\text{m}$ and average height of ~ 286 nm (Figure 4.11B).

Figure 4.11. (A) SEM image of written Au structure when no PVP (P0) is added into the precursor solution. The written Au structure appears to possess two kinds of morphology: sparse nanoparticles and fused flat Au surface. Inset: the green region indicates the sparse Au nanoparticles that form along the edges of the written structure, whereas the orange region shows the fused flat Au surface that dominates most of the written structure. (B) Average height and (C) average width of written microstructures. Average height was measured using AFM and average width was measured using ImageJ, from SEM images of written microstructures of P40 microstructures.

The fused, “smooth” Au surface is a consequence of the absence of PVP in P0, which results in the aggregation and growth of photoreduced Au atoms with no size control to form large areas of fused, bulk Au structures instead of NP clusters like in P05 - P30.⁴⁹⁻⁵⁰ Such “smooth” surface is undesirable especially for SERS applications, where surface roughness is an important prerequisite for electromagnetic field enhancement.⁵¹⁻⁵² These results highlight the importance of PVP in controlling the overall morphology of Au microstructures, by preventing particle agglomeration and promoting the formation of AuNPs within written lines.⁵³⁻⁵⁴ The increase in NP density and surface roughness with increasing PVP concentration can be attributed to the higher capping efficiency of the

fabricated AuNPs, leading to the formation of smaller AuNPs,⁵⁵ hence enabling larger numbers of AuNP clusters to be generated within the same laser volume resulting in higher surface roughness.

Optical Properties of Au Structures. Dark field images of the respective structures demonstrate that under all conditions, written lines are well-defined (Figure 4.12A – E). We observe that P05 - P30 microstructures appear yellow under the dark field but P0 microstructures appear orange. The extinction spectrum of the P10 microstructures exhibit a peak at 570 nm which corresponds to the localized surface plasmon resonance (LSPR) of AuNPs,⁵⁶ indicating that the structures are plasmonically active within the visible-light range of 400 - 700 nm. On the other hand, the extinction spectrum of P0 microstructures also exhibits a weak peak at 570 nm, however, the spectrum is broadened which is likely due to the fused Au structure, and are hence only weakly plasmonically active (Figure 4.12F).

Figure 4.12. (A - E) Dark field images of P0 - P30 microstructures. While P05 - P30 microstructures appears yellow under the dark field microscope, P0 appears more orange colored, indicating a red-shift in the LSPR of P0 from P03 - P30 structures. (F) Extinction spectra of P10 Au microstructures compared to P0 Au microstructures. The broadening of the spectrum in P0 is due to the fused microstructure.

As AuNP clustering in the microstructures potentially leads to increased number of plasmonic hot-spots for SERS enhancement, we demonstrate that SERS properties of the AuNP microstructures

can be tuned by modulating the sizes of individual AuNPs. We begin by fabricating square and circle structures using P0 - P30 Au precursor solutions. The square and circle structures represent lines and arcs, respectively, which is the basis of all 2D designs. The fabricated ring and square microstructures are well-defined and uniformly written, where the diameter of each ring is 30 μm while the squares are written with lengths of 25 μm (Figure 4.14A(i), (ii)). We functionalize the microstructures with 4-methylbenzenethiol (4-MBT) via ligand exchange with PVP, as molecular probe for the SERS measurements. Raman spectra of the written microstructures show typical fingerprint of 4-MBT with clear identification of characteristic peaks at 1079 cm^{-1} and 1563 cm^{-1} . We attribute the peak at 1079 cm^{-1} to a combination of the ring-deformation, C-H in-plane bending, and thiol stretching modes respectively,⁵⁷ while the peak at 1563 cm^{-1} is attributed to the phenyl stretching mode (Figure 4.13, Table 1).⁵⁸

Figure 4.13. SERS spectra of (A) as-fabricated Au microstructures with PVP present and (B) Au microstructures after ligand exchange with 4-MBT. Two characteristic peaks at 1079 cm^{-1} and 1563 cm^{-1} are observed.

Table 2. Raman peak assignments of 4-methylbenzenethiol⁵⁸⁻⁶⁰

on AuNP lines (cm ⁻¹)	Band Assignment
1079	C-S stretching
1079, 1177	phenyl ring breathing
1177	C-H in-plane bending
1563, 1595	phenyl in-plane bending

The Raman image lights up homogeneously on the ring and square structures when 1079 cm⁻¹ is selected, clearly indicating largely consistent SERS intensities across fabricated microstructures (Figure 4.14B). A cross-sectional SERS profile plot of the P10 square microstructure further demonstrates that the most intense SERS signals are only recorded from the microstructures at (500 ± 128) counts/s, whereas in areas away from the microstructures, SERS intensities average around (6.4 ± 2.6) counts/s (Figure 4.14C). The average SERS intensity at 1079cm⁻¹ increases from P0 to P30, where P0 exhibits both the lowest and inhomogeneous SERS intensity of (62 ± 75) counts due to the presence of “smooth” fused areas and sparse AuNPs (Figure 4.15), followed by P05 ((492 ± 258) counts), P10 ((1084 ± 465) counts), P20 ((1150 ± 537) counts), and finally P30, which exhibits the highest SERS intensity at (1453 ± 446) counts (Figure 4.14D). The enhancement factor (EF) of P0 to P30 substrates based on the peak at 1079 cm⁻¹ are calculated at ~5.6 × 10⁴, ~9.5 × 10⁴, ~2.1 × 10⁵, 2.2 × 10⁵, and 2.8 × 10⁵ for fabricated microstructures P05, P10, P20, and P30, respectively (Figure 4.14E), comparable to reported literature values of 10⁴ - 10⁵ for similar AuNP sizes using wet chemistry methods.⁶¹⁻⁶³

Figure 4.14. SERS performance of written Au structures P0 - P30. (A) (i - ii) SEM images and (B) the corresponding Raman image showing an example of the P10 Au microstructure written for SERS detection (C) Cross-sectional SERS intensity profile along the red dotted line in the SEM image (inset). (D) The SERS intensity and (E) corresponding SERS enhancement factor of Au structures P0 - P30, which increases with decreasing nanoparticle size, increasing particle density as well as increasing surface roughness.

Figure 4.15. (A) SEM image of written Au structure when no PVP (P0) is added into the precursor solution. The blue region indicates the sparse Au nanoparticles that form along the edges of the written structure, whereas the red region shows the fused flat Au surface that dominates most of the written structure. (B) Raman image and corresponding SERS spectra of the written structure shows strong signals (120 ± 65) counts only at the edge of the structure, in the sparse nanoparticles region, whereas the flat fused region of the structure shows low SERS intensity of (4.7 ± 3.3) counts.

Generally, the SERS EF increases with PVP concentration. This is due to a greater extent of AuNP clustering as observed from the increasing particle densities and RMS roughness measurements when PVP concentration increases from P0 to P30 (Figure 4.17), indicating that we are able to achieve tunable SERS performance using our fabrication technique. This result highlights the stellar performance of our fabricated clustered AuNPs as potentially high performing SERS substrates. The tunable, strong SERS enhancement, coupled with the ability to precisely position the AuNPs on specific areas of the substrate puts this technique at a strong advantage for the future creation of tailored SERS platforms.

Figure 4.16. SERS EF generally increases with PVP concentration, which appears to be in conjunction with both (A) increasing particle density as well as the (B) increasing RMS roughness of the respective microstructures from P0 to P30.

Patterned Microfluidic Channel for In-Situ SERS Detection of Flowing Analytes. The ability to perform one-step, in-situ polyol photoreduction of Au^{3+} into densely packed, well-defined AuNP microstructures ignites a plethora of possibilities, to position desired patterns directly onto or remotely within any transparent surface. We demonstrate the versatility of our fabrication technique via the remote patterning of pre-designed AuNP microstructures *within* a microfluidic channel, to create a SERS detector for gaseous analyte molecules. While gases tend to diffuse and expand indefinitely in air, causing them to become diluted and hence difficult to detect using substrate-based platforms in air, a microfluidic SERS channel is able to confine gaseous molecules within the channel, bringing the molecules close to the SERS platform to be easily detected. Using this channel, we demonstrate that we are able to detect in real-time, the molecular fingerprints of small gaseous molecules both with, and without specific affinity to Au.

To begin, we fabricate a series of P10 Au microstructures within a flat, APTES-functionalized microfluidic channel ($l \times w \times h = 5.0 \text{ cm} \times 0.6 \text{ cm} \times 0.1 \text{ mm}$) (Figure 4.17). Although P30 microstructures exhibit the highest SERS EF, bubbles generated during lithography are observed to linger for longer periods, possibly due to a higher solution viscosity at high PVP concentration. Hence,

we select P10 to allow for easier dissipation of generated bubbles within the microfluidic channel with minimum compromise on SERS EF of the microstructures.

Figure 4.17. Writing AuNP microstructures within microfluidic channel for SERS sensing of gaseous analytes. (A) A camera image and (B) dark field image of the microfluidic channel written with Au metal structures; inset: a magnified optical image of the structures written within the tube.

We draw the precursor solution into the channel via capillary action and focus the laser on the inner wall to simultaneously reduce and drive the formation of AuNPs into pre-defined numbers within the microfluidic channel. The channel is then washed with KI to remove PVP to minimize interference of SERS signals between PVP and analyte (Figure 4.18). Such remote and precise formation of well-defined metallic Au structures within a microfluidic channel is otherwise not possible using pre-synthesized AuNPs in solution.

Figure 4.18. SERS spectra of (A) as-fabricated Au microstructures with PVP present and (B) Au microstructures after washing with 2-propanol, water and KI solution.

We first demonstrate the real-time, in-situ monitoring of gaseous flow using vapor 4-MBT as an analyte. We warm a solution of 0.5 M of 4-MBT in ethanol in a glass vial and begin the SERS measurement (Figure 4.19).

Figure 4.19. The set-up for the SERS detection of gaseous solvents; the desired solvent (ethanol or acetone) is placed within the glass bottle indicated by the red arrow, and is connected via a flexible teflon tubing to the structure-containing flat capillary tube on the microscope stage (indicated by blue arrow). To facilitate the diffusion process of the gaseous solvent molecules for detection, the solvent-containing glass vial is gently warmed using a Linkam™ stage at 50 °C for acetone and 70 °C for ethanol. The gaseous analytes diffusing through the microfluidic channel are then detected by SERS.

Within 2 minutes, we observe a faint outline of the microstructures in the Raman image, which becomes gradually more defined to reveal the written micro-pattern of “5 6 7 8 9”, indicating that 4-MBT molecules have diffused to the Au microstructure at 15 minutes (Figure 4.20A). A plot of SERS intensity against time demonstrates that SERS signal intensity of 4-MBT increases linearly with time (Figure 4.20B). At the 15 minute mark, the SERS signal intensity appears to be at a maximum, beyond which, we observe no change to the intensity (Figure 4.20C) or the Raman image, indicating that the microstructures are likely saturated with 4-MBT within 15 minutes.

Figure 4.20. The highly versatile lithography technique can be used to easily arrange AuNP structures into desired patterns. (A) In-situ monitoring of the diffusion of gaseous 4-MBT molecules through the microfluidic channel. Time-resolved Raman images demonstrate the gradual saturation of the SERS substrate with time to eventually reveal the design of the substrate. (B) SERS intensity is observed to increase with time and (C) A plot of SERS intensity over time demonstrates the increase in SERS intensity with plateau after 15 min.

To showcase the versatility of our microfluidic SERS channel as a universal sensor suitable for the detection of molecules with low Raman cross-sections,⁶⁴⁻⁶⁵ we further extend the SERS sensor to the detection of gaseous acetone followed by ethanol, both of which are common, small molecules that have no specific interactions with the AuNP microstructures. Acetone is heated for 15 minutes to allow the gaseous solvent to diffuse to the SERS microfluidic channel for detection first, followed by flushing with N₂ gas to remove residual solvent before carrying out the detection of gaseous ethanol.

The SERS spectrum of the written AuNP microstructures prior to gaseous acetone injection shows no peaks, demonstrating that the washing process to remove most of the PVP is successful. Subsequent SERS spectrum of gaseous acetone exhibits distinct Raman peaks at 1161 cm⁻¹ and 1530 cm⁻¹, corresponding to C-C-C bending, CH₃ wagging and scissoring modes of acetone, respectively (Table 2, Figure 4.21).

Table 2. Raman peak assignments of Acetone⁶⁶⁻⁶⁷

on AuNP lines (cm ⁻¹)	Band Assignment
1164	C-C-C bending CH ₃ rocking
1531	CH ₃ scissoring

Due to the difference in peak positions between the SERS and Raman spectra of acetone, we are unable to obtain accurate peak assignments based on literature alone, which mainly report liquid acetone Raman peak values. Hence, we performed Density Functional Theory (DFT) simulations of acetone on gold cluster to support our experimental acetone SERS spectra (Figure 4.21). The peak assignment was then obtained through the DFT simulations and supported by literature peak assignments.

Figure 4.21. Raman spectra showing (A) DFT-simulated gaseous acetone SERS spectra, experimental gaseous acetone SERS spectra, (C) experimental liquid acetone SERS spectra and (D) experimental liquid acetone Raman.

Raman measurement performed after flushing the channel with N₂ gas for 30 minutes exhibit no Raman peaks, demonstrating that most of the acetone has been successfully removed from the SERS platform. We repeat the detection process with ethanol and observe SERS peaks at 1278 cm⁻¹, 1338 cm⁻¹ and 1473 cm⁻¹, which correspond to the OH bending, CH₂ twisting, and CH₃ wagging modes of ethanol, respectively (Table 3).

Table 3. Raman peak assignments of Ethanol⁶⁸⁻⁶⁹

on AuNP lines (cm ⁻¹)	Band Assignment
1278	OH bending
1338	CH ₂ twisting
1473	CH ₃ wagging
1569	CH ₂ wagging

By switching between solvents, we demonstrate the versatility of a single microfluidic channel as such to potentially detect different types of small gaseous molecules that have no specific affinity to the AuNP microstructures (Figure 4.22).

Figure 4.22. (A) Schematic illustrating the SERS sensing of various gaseous analytes, including acetone and ethanol, passing through the microfluidic channel. (B) SERS spectra demonstrating the detection of gaseous acetone followed by flushing with nitrogen gas, and then the detection of ethanol using the microfluidic device.

The ability to remotely fabricate and precisely position AuNPs within a channel enables the creation of desired structures or specific words in an enclosed space, which is a main criterion to

perform gaseous detection, to create patterned SERS gas detectors. Besides the detection of 4-MBT and small gaseous molecules, the substrate can also potentially be functionalized with other compounds to impart molecular recognition ability to the substrate, allowing the patterns on these substrates to function as “on-off” indicators to identify the presence of gases through specific binding interactions, such as o-phenylenediamine for the detection of nitric oxide gas,⁷⁰ or APTES for the detection of airborne trinitrotoluene particulates.⁷¹

4.3 CONCLUSION

In this work, we have demonstrated the ability to simultaneously photoreduce Au³⁺ and drive the formation of AuNPs to create well-defined micro-patterns for SERS sensing, where the micro-patterns can be easily pre-programmed using a computer-aided design (CAD) software. By varying the PVP concentration in the Au precursor solution, we are able to tune the NP size, particle density, surface roughness and subsequently the SERS EF of the respective microstructures. We apply the fabrication technique to creating a patterned microfluidic channel for the detection and in-situ monitoring of gaseous analytes. The simple fabrication process coupled with the flexibility in design allows us to easily create a reusable, patterned SERS substrate for detection and real-time monitoring of analyte molecules and potentially reaction processes. Furthermore, as a one-step laser induced AuNP fabrication and structure formation route this technique also holds great promise for the creation of photothermal microreactor platforms, as well as split-ring resonators for transformation optics studies. The precision of the fabrication technique also allows for many applications to be easily integrated onto one single microchip to eventually create multi-functional lap-on-a-chip devices.

4.4 MATERIALS AND METHODS

Materials. Poly(vinyl pyrrolidone) (PVP, $M_w = 10\,000$ g/mol), ethylene glycol (EG, $\geq 99.8\%$), gold(III) chloride trihydrate (HAuCl_4 , $\geq 99.9\%$), 4-methylbenzenethiol (4-MBT, 98%) and (3-aminopropyl)triethoxysilane (APTES, $\geq 99.9\%$), were purchased from Sigma-Aldrich. Potassium

iodide and ethanol (ACS, ISO, Reag.) were purchased from EMSURE. Hydrochloric acid (HCl, 37 %) was purchased from VWR Chemicals. Acetone (AR grade) was purchased from Tedia Chemicals. 2-propanol (Certified ACS) was purchased from Fisher Scientific. All chemicals were used without further purification. Ultrapure water ($> 18.0 \text{ M}\Omega\cdot\text{cm}$) was obtained from a Sartorius arium611 UV ultrapure water system.

Preparation of Au precursor solutions. To a solution of 100 μL of EG and 20 μL of 600 mM HAuCl_4 , 0 μL , 10 μL , 20 μL , 40 μL , 60 μL and 80 μL of PVP solution (1 g in 50 mL H_2O) are added, and the mixtures are topped up with ultrapure water to 200 μL to make the precursor solutions of 0 %, 5 %, 10 %, 20 %, 30 % and 40 % (v/v) PVP respectively. The mixtures are then vortexed and sonicated at room temperature until they are fully dissolved to form a clear yellow solution. It is observed that at a PVP concentration of 40 %, the precursor solution turns cloudy, possibly from oversaturation of PVP, and needs a longer time (~ 1 hour) to dissolve completely. Post lithography, the as-written substrate is subsequently washed with water, 2-propanol and 0.5 M potassium iodide (KI) solution to remove excess reactants and PVP. The surface N-containing PVP is removed through ligand exchange with I^- ions, where I^- binds to Au through charge-transfer interactions.³⁹

We select PVP as a capping agent because it is cost effective, non-toxic and biodegradable,⁷² which is in alignment with the principles of green chemistry. In comparison, other polymeric capping agents such as polyethylene glycol (PEG) is expensive, non-biodegradable and a skin irritant,⁷³ and polyacrylic acid (PAA) classifies as toxic, corrosive and irritating to human health.⁷⁴ Polyvinyl alcohol (PVA) is another commonly used polymeric capping agent for two-photon direct metal writing. However, previous reports describing the two-photon fabrication of AuNPs using PVA demonstrate the formation of AuNPs that are generally polydisperse and irregularly shaped,²² instead of the quasi-spherical and monodisperse AuNPs that can be obtained with PVP as capping agent, hence we select PVP in our studies.

Functionalization of glass surfaces for lithography. Glass cover slips (Deckglasser cover glass, 22 mm $\times$ 22 mm), and flat microfluidic tubes ($l \times w \times h = 5.0 \text{ cm} \times 0.6 \text{ cm} \times 0.1 \text{ mm}$, VitroTubesTM, VitroCom) are functionalized for 5 minutes in 2 % v/v APTES in ethanol and then

rinsed with a 50 % water/ethanol mixture. The substrates are then blown dry with nitrogen gas and left to dry overnight in a 65 °C oven.

Lithography of Au microstructures. Lithography of the Au microstructures (P0 - P40) were carried out by the Nanoscribe® Photonic Professional. 100 µL of the respective Au precursor solutions (P0 - P40) are dropcasted on the functionalised glass substrates. The system is equipped with an erbium doped, femtosecond laser source, with a center wavelength of 780 nm, and pulse length ~ 150 fs. Structures for fabrication were designed using a Computer-aided Design (CAD) software, 3ds Max®. Writing conditions and sizes of the structures were programmed by the DeScribe software. All structures were fabricated with 6 mW laser power and scan speed of 1 µm/s. Objective lens used is a 100× oil immersion objective, N.A. 1.4.

During the lithography, small bubbles are observed as a result of absorption by fabricated Au nanoparticles. However, it is observed that the bubbles dissipate quickly upon generation, and hence generally do not compromise the stability and appearance of the written microstructures.

Fabrication of Au nanoparticles in bulk for XPS and XRD analyses. 100 µL of P10 Au precursor solution was dropcasted onto a glass substrate, and exposed to UV-light from a 300 W Xenon lamp Solar Simulator (Newport Corporation, Model: 69911, $\lambda = 200 - 420$ nm) for 15 minutes at ambient conditions. The solution is observed to decolorize and turn dark purple. The dark purple solution is washed to remove excess resist and redispersed in ethanol for characterization.

Functionalization of Au microstructures for SERS measurements. We perform a direct ligand exchange with 4-methylbenzenethiol (4-MBT). The structures are immersed in 15 mM of 4-MBT solution for 24 hours to ensure ample time for functionalization. We select 4-MBT as a Raman probe due to the strong coordination bond between the thiol group and metal, which favors the formation of a 4-MBT self-assembled monolayer (SAM) on the gold surface. This in turn allows for higher accuracy in assessing the number of surface molecules on the assembled Au nanoparticles that contribute to the SERS response.

Fabrication of Au microstructures inside capillary tube. The P10 Au precursor solution is drawn up the capillary tube via capillary action for lithography, and the excess is removed by draining

the liquid out and rinsing the tube with sufficient water and ethanol. Following which, we rinse the capillary tubes with 0.5 M KI and 1M HCl solution 3 times for 10 minutes each before immersing overnight in 0.5 M KI for ligand exchange with PVP on the surface of the written structures.

We select P10 as the precursor solution even though it does not exhibit the strongest SERS performance because we observe that bubbles generated during lithography will linger for longer periods of time in solutions with higher PVP concentration, which is possibly due to a higher solution viscosity. Hence, in order to allow for easier dissipation of generated bubbles in the capillary tube but at the same time maximise the SERS capability of the microstructures, we select P10 as the precursor solution.

Microfluidic flow cell setup. 15 mL of acetone or ethanol, or 0.5 M 4-MBT in ethanol is placed in a 20 mL glass vial and the vial is sealed with parafilm and connected through a flexible teflon tubing to the flat capillary tube (microfluidic channel). The vial is placed on a heat plate and heated gently to facilitate the evaporation and diffusion of the respective solvents into the SERS microfluidic channel. We begin the experiment with the detection of gaseous acetone. The acetone is gently warmed at 50 °C for 15 minutes before performing the SERS measurement. After which, the microfluidic device is flushed with N₂ gas flowing at 50 sccm for 30 minutes to remove acetone from the substrate. The SERS detection of ethanol is carried out in a similar fashion by heating the ethanol at 70 °C for 15 minutes before taking the SERS measurement. In performing the in-situ monitoring of 4-MBT, the 4-MBT/ethanol mixture is heated at 60 °C. SERS measurements are taken every 2 minutes until no further change to SERS signal intensity or Raman image is observed.

SERS measurements of fabricated structures. Raman x-y imaging was performed by the Ramantouch microspectrometer (Nanophoton Inc, Osaka, Japan) using a laser wavelength of 785 nm and excitation power of 0.39 mW. The laser was focused onto the structures using a Nikon LU Plan objective (50×, N.A. 0.55). For each line, the samples were measured with an exposure time of 1 s.

Density Functional Theory (DFT) simulations. The calculation of acetone Raman peaks on Au cluster (10 atom pyramid) was carried out using the B3LYP (default) exchange-correlation

functional, as implemented in the Gaussian 09 computational chemistry package, for which the LANL2DZ basis set was employed.

Materials characterization. Scanning electron microscopy (SEM) was carried out with a JEOL-JSM-7600F microscope, and dark field spectroscopy was carried out with an Olympus BX51BD microscope. The extinction spectra were obtained using a UV-Vis spectrometer, Cary 60 UV-Vis from Agilent Technologies. Energy dispersive X-ray (EDX) analysis was performed with AZtecEnergy Oxford Instruments. Au microstructure width, AuNP density and AuNP sizes were measured using ImageJ. For AuNP size, the histograms were plotted by measuring 100 AuNPs. X-ray photoelectron spectroscopy (XPS) spectra were measured using a Phoibos 100 spectrometer with a monochromatic Mg X-ray radiation source. All XPS spectra were fitted using XPS Peak 4.1 (freeware accessible at <http://www.phy.cuhk.edu.hk/~surface>). Atomic force microscopy (AFM) RMS roughness and structure height measurements were carried out using the Bruker Dimension ICON with NanoScope V controller from Bruker. A $1.0\ \mu\text{m} \times 1.0\ \mu\text{m}$ window was used for each RMS roughness measurement. Reported roughness and height values were averaged over at least 10 measurements. Tapping mode (non-contact mode) image was acquired using silicon probes (Tap300Al-G with 30-nm aluminium reflex coating) from BudgetSensor. Data analysis was carried out using WSxM Scanning Probe Microscopy Software, a free program from Nanotec Electrónica S.L36.

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4.7 APPENDIX

Calculations to compare two-photon laser with the solar simulator:

Two-photon pulsed laser:

$$\text{Repetition rate} = 80 \text{ MHz} = 80\,000\,000 \text{ Hz} = 80\,000\,000 \text{ s}^{-1} = [1/(80\,000\,000)] \text{ s} = 12.5 \times 10^{-8} \text{ s}$$

$$\text{Peak laser power} = (\text{average laser power}) / (\text{repetition rate} \times \text{pulse width})$$

$$\begin{aligned}\text{Peak laser power} &= (0.00600 \text{ W}) / [(80\,000\,000 \text{ s}^{-1}) \times (150 \times 10^{-15} \text{ s})] \\ &= 500 \text{ W}\end{aligned}$$

Number of photons generated

$$\text{Energy of 1 photon operating at } 780 \text{ nm} = h\nu$$

$$= (6.63 \times 10^{-34} \text{ J}\cdot\text{s}) \times [(3.00 \times 10^8 \text{ m/s}) / (780 \times 10^{-9} \text{ m})]$$

$$= 2.55 \times 10^{-19} \text{ J}$$

$$\text{Average energy per second} = \text{average laser power} \times \text{time}$$

$$= 0.00600 \text{ W} \times 1 \text{ s} = 0.00600 \text{ J}$$

$$\text{Number of photons generated per second} = 0.00600 \text{ J} / 2.55 \times 10^{-19} \text{ J}$$

$$= 2.35 \times 10^{16} \text{ photons}$$

Intensity

$$\text{Intensity} = \text{peak laser power} / \text{area}$$

$$\text{Intensity} = 500 \text{ W} / [\pi \times (250 \times 10^{-9})^2] = 2.55 \times 10^{15} \text{ W/m}^2 \text{ (per pulse)}$$

$$\text{Average intensity} = 0.00600 \text{ W} / [\pi \times (250 \times 10^{-9})^2] = 3.06 \times 10^{10} \text{ W/m}^2 \text{ (per second)}$$

300 W Xenon solar simulator (wavelength range between 200 – 420 nm):

Number of photons generated

$$\text{Energy per second} = \text{power} \times \text{time}$$

$$= 300 \text{ W} \times 1 \text{ s} = 300 \text{ J}$$

$$\text{Energy of 1 photon operating at } 200 \text{ nm} = h\nu$$

$$= (6.63 \times 10^{-34} \text{ J}\cdot\text{s}) \times [(3.00 \times 10^8 \text{ m/s}) / (200 \times 10^{-9} \text{ m})]$$

$$= 9.95 \times 10^{-19} \text{ J}$$

$$\begin{aligned}\text{Number of photons generated per second} &= 300 \text{ J} / 9.95 \times 10^{-19} \text{ J} \\ &= 3.02 \times 10^{20} \text{ photons}\end{aligned}$$

Because the resist measures only 0.8 cm in diameter, whereas the opening of the Xenon lamp measures 5 cm in diameter, the resist only receives a small fraction of UV light.

$$\begin{aligned}\text{Ratio of resist area to total area} &= [\pi \times (0.4 \times 10^{-2})^2] / [\pi \times (2.5 \times 10^{-2})^2] \\ &= 0.026\end{aligned}$$

$$\begin{aligned}\text{Therefore, number of photons received by resist in 1 second} &= 3.02 \times 10^{20} \text{ photons} \times 0.026 \\ &= 7.7 \times 10^{18} \text{ photons}\end{aligned}$$

$$\begin{aligned}\text{Energy of 1 photon operating at 420 nm} &= h\nu \\ &= (6.63 \times 10^{-34} \text{ J}\cdot\text{s}) \times [(3.00 \times 10^8 \text{ m/s}) / (420 \times 10^{-9} \text{ m})] \\ &= 4.74 \times 10^{-19} \text{ J}\end{aligned}$$

$$\begin{aligned}\text{Number of photons generated per second} &= 300 \text{ J} / 4.74 \times 10^{-19} \text{ J} \\ &= 6.33 \times 10^{20} \text{ photons}\end{aligned}$$

$$\begin{aligned}\text{Therefore, number of photons received by resist in 1 second} &= 6.33 \times 10^{20} \text{ photons} \times 0.026 \\ &= 1.6 \times 10^{19} \text{ photons}\end{aligned}$$

Intensity

Intensity = total power / area

$$\text{Intensity} = 300 \text{ W} / [\pi \times (2.5 \times 10^{-2})^2] = 1.52 \times 10^5 \text{ W/m}^2$$

Calculation of enhancement factor (EF)

$$EF = \frac{I_{SERS}}{I_{NORMAL}} \times \frac{N_{NORMAL}}{N_{SERS}}$$

where N_{SERS} and N_{Normal} denote the Raman intensity and number of molecules probed in the bulk phase analyte sample respectively and I_{SERS} and I_{Normal} refers to the SERS intensity and number of molecules probed on the SERS substrate. We chose the peak at 1079 cm^{-1} to calculate the EF.

Figure 4.23. Normal Raman spectrum of 1 M 4-MBT in ethanol. The highlighted peak at 1079 cm^{-1} is used for calculation of enhancement factor

$$I_{\text{normal}} = 1673 \text{ counts}/1800 \text{ s}$$

$$= 0.9294 \text{ cps (based on 1 M 4-MBT solution, 50x objective lens, line scan, 1800 s/line)}$$

N_{normal} = number of molecules within the measured laser spot

$$= \text{Volume of solution} \times \text{concentration of molecules} \times \text{Avogadro's number}$$

$$\text{Volume of solution} = \pi \times x \times y \times z \quad (x = 1.25/2 = 0.625 \text{ } \mu\text{m}, y = 1.67/2 = 0.835 \text{ } \mu\text{m}, z = 6.77 \text{ } \mu\text{m})$$

$$= \pi \times 625 \text{ nm} \times 835 \text{ nm} \times 67700 \text{ nm}$$

$$= 1.11 \times 10^{11} \text{ nm}^3$$

$$N_{\text{normal}} = 1.11 \times 10^{11} \text{ nm}^3 (=1.11 \times 10^{-16} \text{ m}^3) \times 1 \text{ M} (=1000 \text{ mol/m}^3) \times 6.022 \times 10^{23} = 6.68 \times 10^{10} \text{ molecules}$$

N_{SERS} = number of molecules on the surface of the nanoparticles within the measured laser spot

$$N_{\text{SERS}} = \text{surface area of single nanoparticle} \div \text{surface area of 4-MBT}$$

$$\text{Surface area of 4-MBT} = 2.30 \times 2.30 \text{ \AA}^2 / \text{molecule} = 0.0529 \text{ nm}^2 / \text{molecule}$$

For 0 % PVP

Particles area:

$$I_{\text{SERS}} = 120$$

$$\begin{aligned} \text{Surface area of 1 nanoparticle (assume sphere)} &= 4\pi r^2 \\ &= 4 \times \pi \times ((65.9)/2)^2 \\ &= 1.36 \times 10^4 \text{ nm}^2 \end{aligned}$$

$$\begin{aligned} \text{Number of molecules on 1 nanoparticle} &= 1.36 \times 10^4 \text{ nm}^2 \div 0.0529 \text{ nm}^2 / \text{molecule} \\ &= 2.58 \times 10^5 \text{ molecule/nanoparticle} \end{aligned}$$

Packing density of the nanoparticles = 68.9 % packed with nanoparticles. Assuming this holds true for all area with particles, the number of nanoparticles exposed to the laser is:

Number of nanoparticles exposed to laser $\div$ spot area:

$$\begin{aligned} &= (\pi \times x \times y \times 0.689) / \pi r^2 \\ &= (\pi \times 625 \text{ nm} \times 835 \text{ nm} \times 0.689) / \pi \times ((65.9)/2)^2 \\ &= 331 \text{ nanoparticles} \end{aligned}$$

Number of molecules in laser volume (on 331 nanoparticles)

$$\begin{aligned} &= 331 \text{ nanoparticles} \times 2.58 \times 10^5 \text{ molecule/nanoparticle} \\ &= 8.55 \times 10^7 \text{ molecules} \end{aligned}$$

$$\text{EF} = [120/0.9294] \times [6.68 \times 10^{10} / 8.55 \times 10^7] = 1.01 \times 10^5$$

Flat area:

$$I_{\text{SERS}} = 4.7$$

The number of 4-MBT molecules exposed to the laser is:

$$\begin{aligned} &= (\pi \times 625 \text{ nm} \times 835 \text{ nm}) \div 0.0529 \text{ nm}^2 \\ &= 1.64 \times 10^6 \div 0.0529 \text{ nm}^2 \\ &= 3.10 \times 10^7 \end{aligned}$$

$$\underline{\text{EF} = [4.7/0.9294] \times [6.68 \times 10^{10} / 3.10 \times 10^7] = 1.1 \times 10^4}$$

$$\text{Average EF} = (1.01 \times 10^5 + 1.1 \times 10^4) / 2 = 5.6 \times 10^4$$

For 5 % PVP

$$I_{\text{SERS}} = 492$$

$$\begin{aligned} \text{Surface area of 1 nanoparticle (assume sphere)} &= 4\pi r^2 \\ &= 4 \times \pi \times ((52.3)/2)^2 \\ &= 8.59 \times 10^3 \text{ nm}^2 \end{aligned}$$

$$\begin{aligned} \text{Number of molecules on 1 nanoparticle} &= 8.59 \times 10^3 \text{ nm}^2 \div 0.0529 \text{ nm}^2 / \text{molecule} \\ &= 1.62 \times 10^5 \text{ molecule/nanoparticle} \end{aligned}$$

$$\begin{aligned} \text{Laser spot area} &= \pi \times x \times y \\ &= \pi \times 625 \text{ nm} \times 835 \text{ nm} \\ &= 1.64 \times 10^6 \text{ nm}^2 \end{aligned}$$

Due to the close-packed nature of the fabricated microstructures, laser penetration depth ~ 3 layers thick,⁶⁴ hence number of nanoparticles in laser spot area

$$= 3 (1.64 \times 10^6 / \pi r^2)$$

$$= 3 (1.64 \times 10^6 / [\pi \times ((52.3)/2)^2])$$

$$= 3 \times 763$$

$$= 2.29 \times 10^3 \text{ nanoparticles}$$

Number of molecules in laser volume (on 2.29×10^3 nanoparticles)

$$= 2.29 \times 10^3 \text{ nanoparticles} \times 1.62 \times 10^5 \text{ molecule/nanoparticle}$$

$$= 3.71 \times 10^8 \text{ molecules}$$

$$\mathbf{EF} = [492/0.9294] \times [6.68 \times 10^{10}/3.71 \times 10^8] = 9.53 \times 10^4$$

For 10 % PVP

$$I_{\text{SERS}} = 1084$$

Surface area of 1 nanoparticle (assume sphere) = $4\pi r^2$

$$= 4 \times \pi \times ((42.9)/2)^2$$

$$= 5.78 \times 10^3 \text{ nm}^2$$

Number of molecules on 1 nanoparticle = $5.78 \times 10^3 \text{ nm}^2 \div 0.0529 \text{ nm}^2/\text{molecule}$

$$= 1.09 \times 10^5 \text{ molecule/nanoparticle}$$

Laser spot area = $\pi \times x \times y$

$$= \pi \times 625 \text{ nm} \times 835 \text{ nm}$$

$$= 1.64 \times 10^6 \text{ nm}^2$$

Due to the close-packed nature of the fabricated microstructures, laser penetration depth ~ 3 layers thick,⁸ hence number of nanoparticles in laser spot area

$$= 3 (1.64 \times 10^6 / \pi r^2)$$

$$= 3 (1.64 \times 10^6 / [\pi \times ((42.9)/2)^2])$$

$$= 3 \times 1134$$

$$= 3.40 \times 10^3 \text{ nanoparticles}$$

Number of molecules in laser volume (on 3.40×10^3 nanoparticles)

$$= 3.40 \times 10^3 \text{ nanoparticles} \times 1.09 \times 10^5 \text{ molecule/nanoparticle}$$

$$= 3.71 \times 10^8 \text{ molecules}$$

$$\text{EF} = [1084/0.9294] \times [6.68 \times 10^{10}/3.71 \times 10^8] = 2.10 \times 10^5$$

For 20 % PVP

$$I_{\text{SERS}} = 1150$$

Surface area of 1 nanoparticle (assume sphere) = $4\pi r^2$

$$= 4 \times \pi \times ((37.4)/2)^2$$

$$= 4.39 \times 10^3 \text{ nm}^2$$

Number of molecules on 1 nanoparticle = $4.39 \times 10^3 \text{ nm}^2 \div 0.0529 \text{ nm}^2/\text{molecule}$

$$= 8.31 \times 10^4 \text{ molecule/nanoparticle}$$

Laser spot area = $\pi \times x \times y$

$$= \pi \times 625 \text{ nm} \times 835 \text{ nm}$$

$$= 1.64 \times 10^6 \text{ nm}^2$$

Due to the close-packed nature of the fabricated microstructures, laser penetration depth ~ 3 layers thick,⁸ hence number of nanoparticles in laser spot area

$$= 3 (1.64 \times 10^6 / \pi r^2)$$

$$= 3 (1.64 \times 10^6 / [\pi \times ((37.4)/2)^2])$$

$$= 3 \times 1492$$

$$= 4.48 \times 10^3 \text{ nanoparticles}$$

Number of molecules in laser volume (on 4.48×10^3 nanoparticles)

$$= 4.48 \times 10^3 \text{ nanoparticles} \times 8.31 \times 10^4 \text{ molecule/nanoparticle}$$

$$= 3.72 \times 10^8 \text{ molecules}$$

$$\mathbf{EF = [1150/0.929] \times [6.68 \times 10^{10}/3.72 \times 10^8] = 2.22 \times 10^5}$$

For 30 % PVP

$$I_{\text{SERS}} = 1453$$

Surface area of 1 nanoparticle (assume sphere) = $4\pi r^2$

$$= 4 \times \pi \times ((27.3)/2)^2$$

$$= 2.34 \times 10^3 \text{ nm}^2$$

Number of molecules on 1 nanoparticle = $2.34 \times 10^3 \text{ nm}^2 \div 0.0529 \text{ nm}^2/\text{molecule}$

$$= 4.42 \times 10^4 \text{ molecule/nanoparticle}$$

Laser spot area = $\pi \times x \times y$

$$= \pi \times 625 \text{ nm} \times 835 \text{ nm}$$

$$= 1.64 \times 10^6 \text{ nm}^2$$

Due to the close-packed nature of the fabricated microstructures, laser penetration depth ~ 3 layers thick,⁸ hence number of nanoparticles in laser spot area

$$= 3 (1.64 \times 10^6 / \pi r^2)$$

$$= 3 (1.64 \times 10^6 / [\pi \times ((27.3)/2)^2])$$

$$= 3 \times 2802$$

$$= 8.41 \times 10^3 \text{ nanoparticles}$$

Number of molecules in laser volume (on 8.41×10^3 nanoparticles)

$$= 8.41 \times 10^3 \text{ nanoparticles} \times 4.42 \times 10^4 \text{ molecule/nanoparticle}$$

$$= 3.71 \times 10^8 \text{ molecules}$$

$$\mathbf{EF = [1453/0.929] \times [6.68 \times 10^{10}/3.71 \times 10^8] = 2.81 \times 10^5}$$

5 Generating an Ideal Metallic Resist for Fabricating a Large Array of 2D Gold Microstructures

ABSTRACT. We demonstrate in this chapter the fabrication of a stable aqueous Au precursor solution that will enable us to write well-defined metal structures with sub-micron thickness over long hours (~ 4 hours). Our strategy is to add surfactant poly(vinylpyrrolidone) (PVP) and dimethylsulfoxide (DMSO) into the Au precursor solution. We optimize the written structures by tuning the surfactant concentration, laser power and scanning speed. We find that when the laser power is 6 Mw, scanning speed 5 $\mu\text{m/s}$ and DMSO: PVP ratio of 1:2, structures are tightly-packed, well-defined with a width of $1.19 \pm 0.1 \mu\text{m}$ per line. To showcase the robustness of the resist, we fabricate a large array of concentric squares and rings to demonstrate the robustness of the resist and the reliability to fabricate structures consistently even after 4 hours of writing. Such robust fabrication method is promising for the fabrication of metamaterials such as split ring resonators, chiral polarizers and photothermal platforms. By varying the type and amounts of additives added into the precursor solution, the morphology of the resulting structure can be tuned to accommodate different applications, such as porous, rough surface for SERS platforms, or smooth, connected structures for microelectronic systems.

5.1 INTRODUCTION

In the laser direct writing of metallic structures, the precision and morphology of fabricated structures are largely dependent on the fabrication parameters and composition of the photoresist used.¹ For instance, fabrication at higher laser powers or slower scanning speeds generally result in more densely packed, well-defined structures due to the longer dwelling time of the laser.²⁻⁴ The composition of the photoresist can be varied by incorporating additives, for instance, the addition of a monomer or polymer into the metallic precursor solution enables metallic nanoparticles to be embedded within the polymer matrix to form a metal-polymer composite with a smooth surface.⁵⁻⁶ Conversely, addition of a surfactant enables the formation of nanoparticle clusters, giving the fabricated structure a rough appearance.⁷⁻⁸ In many cases, a photoinitiator is also added into the precursor solution to form porous, interconnected metallic nanostructures due to uncontrolled radical photoreduction.⁹⁻¹⁰ Hence by simply varying the type and amounts of additives added into the precursor solution, the morphology of the resulting structure can be tuned to accommodate different applications, such as porous, rough surface for SERS platforms,¹¹⁻¹² or smooth, connected structures for microelectronic systems.¹³⁻¹⁴

However, a complete systematic study into the effect of various parameters such as type and concentration of additives, laser power and scanning speed on the morphology and density of the written structure has not been performed. In order to fabricate consistently well-defined metallic structures, a comprehensive, quantitative protocol detailing optimized parameters for the fabrication process is essential. Furthermore, this protocol must also offer comprehensive solutions to tune the surface morphology of fabricated metallic structures. Another important factor is that the precursor solution must be robust to fabricate consistent microstructures even after long hours of lithography. A common problem among aqueous-based precursor solutions is the evaporation of water over time,¹⁵ which results in increasingly poor uniformity of precursor solution¹⁶ due to inhomogeneous solubility of contained solutes, limiting the size and number of structures that can be written from the same precursor solution. The need to change precursor solutions periodically to sustain long hours of

lithography is time consuming and technically inconvenient, hence there is a need to fabricate a robust resist that can undertake long hours of consistent writing.

Here, we demonstrate in this chapter the fabrication of a stable aqueous Au precursor solution that will enable us to write well-defined metal structures with sub-micron thickness over long hours (~ 4 hours). Our strategy is to add surfactant poly(vinylpyrrolidone) (PVP) and dimethylsulfoxide (DMSO) into the Au precursor solution. DMSO is hygroscopic which serves to form polar bonds with water molecules,¹⁷ preventing drying of the resist, and reduces Au crystallization rate, allowing large Au crystals to form. PVP aids in structure shaping and size control and functions as a capping agent to deter uncontrolled growth of Au.¹⁸⁻¹⁹ We optimize the written structures by tuning the surfactant concentration, laser power and scanning speed. We find that when the laser power is 6 mW, scanning speed 5 $\mu\text{m/s}$ and DMSO: PVP ratio of 1:2, structures are tightly-packed, well-defined with a width of $1.19 \pm 0.1 \mu\text{m}$ per line. To showcase the robustness of the resist, we fabricate a large array of concentric squares and rings to demonstrate the robustness of the resist and the reliability to fabricate structures consistently even after 4 hours of writing. Such robust fabrication method is promising for the fabrication of metamaterials such as split ring resonators, chiral polarizers and photothermal platforms.

5.2 RESULTS AND DISCUSSION

We demonstrate the ability to perform direct metal writing of Au structures via two-photon lithography. On a clean glass substrate functionalized with (3-aminopropyl)triethoxysilane (APTES), we deposit a Au precursor solution containing 60 mM of gold(III) chloride (HAuCl_4) in water and ethylene glycol (EG), in the ratio of 3:2 EG:water. At the focus plane of the laser, Au^{3+} gets excited and is subsequently reduced by ethylene glycol to form Au^0 .²⁰⁻²¹ The amino groups of APTES aid in anchoring the fabricated structures to the glass substrate via a weak covalent bond.²²

We begin by writing a simple line pattern. Dark field images (Figure 5.1A) demonstrate that the written structures possess a yellow scattering colour, and low magnification scanning electron microscopy (SEM) images (Figure 5.1B) reveal the structure to be generally well-written, with the inset showing a magnified image of the surface morphology of the lines. The lines appear to be ~ 2.5 μm wide, and surface of the lines appear to have an indistinct morphology. Energy dispersive X-ray (EDX) analysis determines that the written structures are primarily composed of Au, whereas the background comprises of mainly Si as expected of a glass substrate (Figure 5.1C).

Figure 5.1. (A) Dark field image and (B) corresponding SEM image of fabricated Au lines. Inset of (B) shows a magnified region of the written structure. (C) EDX spectrum of the written structures demonstrates that they are composed of mainly Au. This observation is supported by (D) XPS spectrum that demonstrates that the surface of the written lines are predominantly Au^0 and (E) XRD spectra that reveals the structures to be predominantly crystalline FCC lattice.

We perform X-ray photoelectron spectroscopy (XPS) analysis to further confirm the surface composition by determining the elements present as well as their oxidation states, and X-ray diffraction (XRD) analysis to determine the crystallinity of the fabricated Au structure. As a large scanning area (1 cm^2) is required to perform both XPS and XRD, we decide to simulate the photoreduction reaction using a solar simulator to generate Au particles in bulk for the analyses. We do this by exposing the Au precursor solution to UV-light from a 300 W Xenon lamp for 15 minutes before washing off the excess resist. SEM images demonstrate that Au precursor can be similarly photoreduced under UV-light, but taking on the form of particles instead of ordered structures.

The high-resolution XPS spectrum of the nanoparticles reveals only 2 sharp peaks at 86.9 and 83.2 eV, where the full-width half-maximum (FWHM) of each of these peaks is approximately 1.0. The difference between the 2 peaks is 3.7 eV, which is characteristic of the Au^0 4f doublet spaced at 3.65 eV (Figure 5.1D). This result suggests that the fabricated particles are largely composed of Au^0 , indicating that the photoreduction of Au^{3+} salt to form Au^0 structures is highly efficient. In the XRD spectrum, we observe five sharp diffraction peaks at 38.2° , 44.4° , 64.6° , 77.5° and 81.7° in the 2θ range of $20\text{--}85^\circ$ (Figure 5.1E), which are indexed to the (111), (200), (220), (311) and (222) reflections of fcc phase of metallic gold (JCPDS, card No. 04-0784) attesting the high crystallinity of the Au structures.

At the focus plane of the laser, Au^{3+} gets excited and is subsequently reduced by ethylene glycol to form Au^{2+} via a polyol reduction process. Au^{2+} then undergoes a fast disproportionation step to form Au^{3+} and Au^{1+} , followed by a slow disproportionation step from Au^{1+} to form Au^{2+} and Au^0 atoms. Finally, Au^0 atoms undergo nucleation followed by aggregation to form the observed Au microstructures.²³

Effect of additives on structure of Au lines. In general, the fabricated Au microstructure lines are $\sim 2\text{ }\mu\text{m}$ in width, and the surface of written lines appear to consist of indistinct nano-features (Figure 5.1B, 5.3A). In order to modulate the physical properties of fabricated structures, we introduce

additives into the Au precursor solution to tune the surface morphology and linewidth of written Au microstructures.

We select two common compounds, poly(vinylpyrrolidone) (PVP) and dimethylsulfoxide (DMSO) as additives, and create 3 different precursor solutions: PVP-only precursor solution, DMSO-only precursor solution and precursor solution with both PVP and DMSO, which we term PS-P, PS-D, and PS-PD respectively.

We begin by adding PVP (1 g in 50 mL water, MW = 10 000 g/mol) to the Au precursor. The morphology of the of the written structure fabricated from PS-P changes from indistinct nano-features to afford numerous aggregated, quasi-spherical nanoparticles (Figure 5.2A, 5.3B), with an average nanoparticle length of (59.9 ± 13.3) nm (Figure 5.2B). On the other hand, Au microstructures fabricated from 10 % DMSO precursor solution (PS-D) are observed to comprise of large crystallites fused together (Figure 5.3C).

Figure 5.2. The effect of PVP on the morphology of the written Au lines. (A) The morphology of written Au structures appears to compose of mainly nanoparticles when PVP is added to the Au precursor solution. (B) The size distribution of fabricated nanoparticles measured across 100 nanoparticles.

PVP is a bulky capping agent which can adsorb onto Au atoms via a charge-transfer bond between Au and the carbonyl group on PVP, to efficiently control the extent of growth and prevent aggregation of Au through the steric hindrance that PVP imposes.²⁴ As a result, numerous nanoparticles are generated instead of the fused Au structure with indistinct surface features that is

obtained when no additives are added. DMSO is a well-known cyroprotectant which also serves to increase viscosity of the precursor solution. Hence, reduced Au atoms experience decreased mobility, resulting in reduced nucleation and growth rates which enables the formation of large Au crystals.²⁵

When both PVP and DMSO are added in the ratio of 1:1, the cumulative effects of both additives are observed in structures fabricated from the PS-PD precursor solution; written lines appear to adopt both the characteristics of structures fabricated from PS-P and PS-D such that resulting Au structures consist of large, misshapen nanoparticles that are tightly packed along the written structure (Figure 5.3D).

Figure 5.3. The effect of additives (PVP and DMSO) on the morphology of the written Au lines. (A) The morphology of written Au structures appears indistinct in the absence of additives. However, structures fabricated from (B) PS-P appear to compose of mainly nanoparticles. On the other hand, structures fabricated from (C) PS-D contain large crystallites. Mixing both (D) DMSO and PVP in a PS-PD precursor solution results in a close-packed structure of moderate sized crystallites.

In comparison with PS-D, lines written with PS-PD Au precursor are also thinner and more consistent in width at $(1.17 \pm 0.1) \mu\text{m}$ (Figure 5.4A) as compared to $(2.7 \pm 0.6) \mu\text{m}$ for structures written from PS-D (Figure 5.4B).

Figure 5.4. Comparing the width of structures written with (A) PS-PD Au precursor solution and (B) PS-D Au precursor solution. (A)(i) SEM image of structures written with PS-PD Au precursor solution and (A)(ii) corresponding measured widths across 50 points and SEM image of (B)(i) PS-D Au precursor solution and (B)(ii) corresponding measured widths across 50 points.

The mechanism behind the fabrication of Au microstructures with large, densely packed nanoparticles and a consistent, narrower linewidth from PS-PD precursor solution can be understood by considering that PVP functions as a capping agent²⁶ and that DMSO increases solution viscosity.²⁷ Upon laser irradiation, Au^{3+} is reduced to Au^0 atoms via a polyol photoreduction process in the presence of EG. The nucleation of the atoms and subsequent growth of the microstructure is retarded by DMSO, resulting in the formation of large, densely packed nanoparticles.²⁸ PVP adsorbs onto the growing Au nanoparticles and microstructure to prevent further growth, creating densely packed Au microstructures with narrow and consistent line width.

We also observe that as compared to PS-P, or precursor solutions containing no additives, which turn slightly cloudy after ~ 1.5 hours of lithography, the PS-PD precursor solution is more stable and remains a clear solution even after 4 hours of lithography. This could be due to the presence of

hygroscopic DMSO, which binds water molecules via dipole-dipole interactions which effectively reduces water evaporation rate.²⁹ The stability of the precursor solution and ability to form consistent structures are important factors to formulate a universal recipe for metallic photoresists. Hence from herein, we use PS-PD Au precursor for the fabrication of Au microstructures.

Optimization of DMSO to PVP ratio. We vary the DMSO to PVP concentration in the PS-PD precursor solution to compare precision of lithography and the robustness of the precursor solution in achieving continuous written lines and well-defined structures. We design a simple concentric square ($l = 25 \mu m$) and circle pattern ($d = 30 \mu m$) and fabricate 10 sets of the pattern from PS-PD Au precursor solutions containing DMSO: PVP ratio of 2:1, 1.5:1, 1:1, 1:2 and 1:3. We observe that when DMSO concentration is greater than PVP concentration, at the ratio of DMSO: PVP = 2:1 and 1.5:1 (Figure 5.5A, B), fabricated structures appear to exhibit poor adhesion to the substrate surface, as indicated by the dark field images of the written structures (Figure 5.5A(i), B(i)). SEM images of the structures adhered to the substrate surface demonstrate that the written concentric circle and square structures are generally incomplete (Figure 5.5A(ii, iii), B(ii, iii)) and the written lines appear to have low Au particle density (Figure 5.5A(iv), B(iv)). With increasing PVP concentration, both the adhesion of the structures to substrate (Figure 5.5C(i) - E(i)) as well as the density of written lines appear to increase (Figure 5.5C(ii, iii, iv) - E(ii, iii, iv)).

Figure 5.5. (i - iv) Dark field images, corresponding SEM images of written structures and high magnification of SEM images demonstrating structure density, when the ratio of DMSO: PVP are (A) 2:1, (B) 1.5:1, (C) 1:1, (D) 1:2 and (E) 1:3. We observe that structures written with Au precursor containing DMSO:PVP ratio of 1:2 appear to be the most well defined, with the highest structure density.

The poor adhesion observed between fabricated Au microstructures and the APTES functionalized substrate at high relative concentrations of DMSO (DMSO:PVP = 2:1, 1.5:1) is likely due to the formation of hydrogen bonds between DMSO and APTES, hence less APTES is available to bind to the Au microstructures to anchor them to the substrate.³⁰ Furthermore, DMSO is able to form molecular complexes with low-coordinated active Au adatoms generated during the

photoreduction of Au^{3+} , effectively stabilizing these adatoms and hence effectively slowing down the nucleation and crystallization rate of Au.³¹⁻³² At DMSO:PVP = 1:3, we observe the precursor solution to turn cloudy within 30 minutes of lithography. Fabricated structures appear densely-packed, well-adhered to the substrate but poorly defined, with excessive Au growth along written lines resulting in inconsistent widths and Au particulates deposited across the substrate (Figure 5.5E). This could be because higher PVP concentration reduces the solubility of Au salt in the precursor solution, resulting in Au salt precipitating easily out of the solution to form a cloudy precursor solution. This in turn leads to the deposition of arbitrary Au particulates around the substrate and the fabrication of structures with poor precision. We also observe that at the DMSO:PVP ratios of both 1:1 and 1:2, individual structures are generally well-written, complete and well-adhered on the substrate surface where written individual lines are dense and close-packed with Au particles (Figure 5.5C, D). However, the resist of 1:1 DMSO:PVP is less robust than the resist of 1:2 DMSO:PVP in producing consistently well-defined structures, as 5 out of the 20 written structures are partially incomplete (Figure 5.5C). Hence from here on, we use PS-PD Au precursor with ratio of 1:2 DMSO:PVP (Figure 5.5D).

Optimization of laser power and scanning speed. Next, we vary both the scanning speed and laser power during fabrication to maximize both particle density and consistency of writing to obtain complete, well-defined structures. Using the same pattern of concentric circles and squares, we begin by fixing the scanning speed at 5 $\mu\text{m/s}$ and gradually increasing the laser power from 4.4 to 5.2, 6.0, 6.8, and 7.6 mW (Figure 5.6A).

We observe from both dark field and SEM images that as laser power increases gradually from 4.4 to 7.6 mW, the completeness of the structures generally increases (Figure 5.6A, B). However, at laser powers above 6.0 mW, structures appear to be less defined, with arbitrary “string-like” structures appearing between written lines (Figure 5.6A(iv) - (v)). High-magnification SEM images further demonstrate that as laser power increases, written lines appear more dense and compact (Figure 6B), but above laser power of 6.0 mW the particle density of written lines remain similar (Figure 5.6B(iv) - (v)).

As laser power increases, more Au^{3+} ions can be effectively photo-reduced to form Au^0 within the same duration of laser exposure. Hence, both the compactness of written lines and structure completeness generally increases with increasing laser power. However, at high laser powers beyond 6.0 mW, the fast photo-reduction and nucleation of Au decreases the precision of the lithography process and leads to the formation of undesirable structural by-products. This problem can potentially be rectified by increasing the scanning speed to reduce the dwell time of the laser at a single spot. However, high laser powers could result in the burning and hence destruction of written structures.³³ Hence, to prevent burning or the growth of undesirable structures, but at the same time maximize the writing efficiency of the structures, we select the laser power of 6.0 mW.

Figure 5.6. (A) Dark field images of written structures with increasing laser power from 4.4 to 7.6 mW, with corresponding (B) SEM images depicting the appearance and density of written structures. (C) Dark field images of written structures with increasing scan speeds from 1 to 9 $\mu\text{m/s}$, similarly with corresponding (D) SEM images depicting the appearance and density of written structures. We observe that structures written with 6 mW laser power at scan speed of 5 $\mu\text{m/s}$ appears to be more well-defined with high density of written lines.

We then vary the scanning speed during writing to further optimize the lithography process. Here, we fix the laser power at 6.0 mW and increase the scanning speed from 1.0, 3.0, 5.0, 7.0 to 9.0 $\mu\text{m/s}$. From both the dark field and SEM images, we observe that written structures are generally complete from as scanning speed increases from 1.0 to 5.0 $\mu\text{m/s}$. However, beyond 5.0 $\mu\text{m/s}$, the structures appear to be less complete with increasing scanning speed to 9.0 $\mu\text{m/s}$ (Figure 5.6C, D). At slow scanning speeds of $< 5.0 \mu\text{m/s}$, the longer dwell time of the laser at any single spot results in an increase in amount of Au^{3+} ions that are effectively photo-reduced to form Au^0 .³⁴ Hence, structures at scanning speeds of 1.0 and 3.0 $\mu\text{m/s}$ have wider written lines and higher Au density within each line (Figure 5.6D(i) - (ii)). However, the longer laser dwell time at slower scanning speeds also could result in burning of the structures. Large bubbles usually form within the precursor solution when burning occurs, which could remove parts of the written structure from the substrate. This result of this is observed in both the dark field and SEM images where some parts of the structures are lifted from the substrate (Figure 5.6C, D(i) - (ii)).

At fast scanning speeds $> 5.0 \mu\text{m/s}$, the reverse occurs. The laser dwell time at a single spot is now shorter, and hence the amount of Au^{3+} ions that are effectively photo-reduced to form Au^0 is also reduced. As a result, with increasing scanning speeds of 7.0 and 9.0 $\mu\text{m/s}$, both the completeness of the written structures and the compactness of individual lines are observed to decrease (Figure 5.6D(iv) - (v)). At 5.0 $\mu\text{m/s}$, the structure appears to be both complete and individual lines are compact, hence we select the writing parameters of 6.0 mW and 5.0 $\mu\text{m/s}$ to fabricate Au structures.

Fabrication of a large array of Au microstructures. We showcase the high robustness of the optimized Au precursor solution and precision of fabrication using the optimized parameters by writing 30 sets of concentric circle and square patterns over 3.5 hours. Dark field images demonstrate that even after nearly 4 hours of lithography, written structures are still well-defined, well-adhered on the substrate surface and most of them complete (Figure 5.7A).

Figure 5.7. (A) Dark field images showcasing the structures written over a period of 3.5 hours (B) magnified dark field images of the individual concentric square and circle, (D, E) SEM images and (F) AFM 3D image demonstrating the consistently well-defined lithography and high structure density across various structures.

High magnification dark field images further demonstrate the precision of the writing process where each line and the gaps between each line is consistent in width of $\sim 1 \mu\text{m}$ (Figure 5.7B, C). Magnified SEM and AFM images demonstrate both right angles in square structures and arcs in circles to be written with high precision, and individual written lines to be compact with high Au particle density with line widths of $1.19 \pm 0.1 \mu\text{m}$ (Figure 5.7D - F)

5.3 CONCLUSIONS

In conclusion, we have created a robust Au precursor solution containing DMSO:PVP in the ratio of 1:2, and optimized the writing parameters to fabricate highly precise, well-defined Au microstructures even after long hours of lithography (~ 4 hours). The formation of the precursor solution is simple and excess precursor post-lithography can be easily removed through washing with IPA and water. The optimized laser power for writing is 6.0 mW, at scanning speed of $5.0 \mu\text{m/s}$. Under this condition, the structures are well-defined and individual lines are compact with Au particles. The ability to write such large arrays of well-defined Au microstructures can potentially be applied to the fabrication of microelectronics, metamaterials and patterned SERS substrates.

5.4 MATERIALS AND METHODS

Materials. Poly(vinyl pyrrolidone) (PVP, $M_w=10\ 000$ g/mol), ethylene glycol ($\geq 99.8\%$), gold(III) chloride trihydrate ($\geq 99.9\%$), dimethylsulfoxide (DMSO) (99.9 %, ACS Reag.) and (3-aminopropyl)triethoxysilane ($\geq 99.9\%$), were purchased from Sigma-Aldrich; isopropyl alcohol (HPLC grade) was purchased from J.T. Baker; and ethanol (ACS, ISO, Reag.) was purchased from EMSURE. All chemicals were used without further purification. Ultrapure water ($> 18.0\ M\Omega\ cm$) was obtained from a Sartorius arium611 UV ultrapure water system.

Preparation of Au precursor solutions. To a solution of 100 μL of ethylene glycol and 10 μL of 600 mM HAuCl_4 , DMSO and PVP solution (1 g in 50 mL H_2O) are added in specified ratios, and the mixtures are topped up with ultrapure water to 200 μL to make the precursor solutions of DMSO:PVP = 2:1, 1.5:1, 1:1, 1:2, 1:3 respectively, where 1 unit = 5 μL . The mixtures are then vortexed and sonicated for a sufficient amount of time at room temperature until they are fully dissolved to form a clear yellow solution.

Functionalization of glass surfaces for lithography. Glass cover slips are functionalized for 5 minutes in 2 % APTES in ethanol and then rinsed with a 50 % water/ethanol mixture. The substrates are then blown dry with nitrogen gas and left to dry overnight in a 65 °C oven.

Lithography of Au microstructures. Lithography of the Au microstructures were carried out by the Nanoscribe® Photonic Professional. 100 μL of the respective Au precursor solutions are dropcasted on the functionalised glass substrates. The system is equipped with an erbium doped, femto second laser source, with a center wavelength of 780 nm, and pulse length of 100 – 200 fs. Structures for fabrication were designed using a Computer-aided Design (CAD) software, 3ds Max®. Writing conditions of the structures were programmed by the DeScribe software. All structures in Figures 5.1, 5.2, 5.3 were written with 4 mW laser power and scan speed of 0.5 $\mu\text{m/s}$, whereas the structures in Figure 5.4, 5.5 were written with 4mW laser power and scan speed of 3 $\mu\text{m/s}$, Figure 5.6 was written

with varied laser power and scanning speed as specified, and structures in Figure 5.7 were written with 6 mW laser power and scan speed of 5 $\mu\text{m/s}$

Materials characterization. Scanning Electron Microscopy (SEM) was performed with a JEOL-JSM-7600F microscope, and dark field spectroscopy was performed with an Olympus BX51BD microscope. Energy Dispersive X-ray (EDX) analysis was performed with AZtecEnergy Oxford Instruments. Nanoparticle sizes were measured using ImageJ. The histograms were plotted by measuring 100 nanoparticles. Linewidth measurements (40 data points) were also carried out using ImageJ. X-ray Photoelectron Spectroscopy (XPS) spectra were measured using a Phoibos 100 spectrometer with a monochromatic Mg X-ray radiation source. All XPS spectra were fitted using XPS Peak 4.1 (freeware accessible at <http://www.phy.cuhk.edu.hk/~surface>). Atomic Force Microscopy (AFM) RMS roughness measurements were carried out using the Bruker Dimension ICON with NanoScope V controller from Bruker. Tapping mode (non- contact mode) image was acquired using silicon probes (Tap300Al-G with 30 nm Aluminium reflex coating) from BudgetSensor. Data analysis was carried out using WSxM Scanning Probe Microscopy Software, a free program from Nanotec Electrónica S.L36.

5.5 REFERENCES

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6 Summary and Outlook

6.1 SUMMARY

Two-photon lithography is an attractive and promising technique to fabricate high resolution 2D and 3D, micro- to milli-meter sized structures with sub-100 nm resolution, well beyond the diffraction limit of light. This technique is highly versatile and can be extended to the fabrication of microstructures of various composition such as polymers, metals and composites.

In this thesis, we focused on 2 key areas: two-photon polymerization and two-photon photoreduction. In two-photon polymerization, we introduced shape-shifting as a key feature of stimuli-responsive, smart polymers. Currently, most shape-shifting structures are fabricated via photolithography and range from millimeter to centimeter sizes. These large sizes could be challenging in applications such as plasmonic studies, lab-on-a-chip microdevices and intravenous drug delivery.

Via two-photon lithography, we fabricated in chapter 2, sub-50 μm sized, shape-shifting 3D microstructures with quantitative, directional bending. Our technique for achieving controlled directional responsiveness is to incorporate a stiff, highly cross-linked reinforcement layer by modulating the cross-linking density of Bovine Serum Albumin hydrogel microstructures spatially at nanometer resolution. We calculated the Young's moduli of fabricated microstructures from the atomic force microscopy measurements and determined them to be inversely proportional to the increase in laser writing distance at the z-axis. Hence, by tuning the z-layer distance at the nanoscale, the cross-linking density, and subsequently the swelling extent of the microstructures can be precisely controlled. Using this concept, we fabricated bi-layer microstructures, where one layer possesses a high cross-linking density and hence swells to a smaller extent, and the other layer possesses a low cross-linking density and hence swells to a larger extent. Upon water uptake, the bi-layer structure bends towards the highly cross-linked layer. We apply this design to the fabrication of shape-shifting

microstructures with stimulus-inducible chirality, as well as a free-standing 3D micro-trap that opens and closes in response to pH change.

Another challenge usually faced in shape-shifting structures is that such structures are usually fabricated as single macrostructures with directional shape change, such as bending or twisting, which limit their versatility. Hence, in chapter 3, we demonstrate the circle-to-polygon, geometrical shape-shifting of BSA microstructures fabricated via two-photon lithography. We used a strategy similar to chapter 2, where rigid “skeletal frames” with high cross-linking density are incorporated within low cross-linked responsive BSA matrices with nanometer-scale precision to achieve the directed, pH-responsive anisotropic swelling of the microstructure. By controlling the orientation, shape and number of the incorporated skeletal frames, we demonstrate well-defined shape-shifting from circles to various shapes such as triangles, squares, pentagons and hexagons with precise arc-to-corner transformations.

In chapter 4, we highlight that while two-photon photoreduction of metals is usually carried out within a polymer to form metal-polymer composites, the presence of the polymer matrix could interfere with analyte SERS signals resulting in inaccuracy of detection, or exhibit unremarkable conductivities as an electrical platform. Conversely, surfactant-based fabrication of metallic structures allows for greater flexibility of application as the surfactant can be easily removed or replaced with other ligands post-fabrication. However, although direct metal writing has been explored extensively for the past decade, most fabricated structures comprise sparse, or dense and irregularly shaped nanoparticles. Hence, we demonstrate the direct metal writing of Au^{3+} into monodisperse Au nanoparticles (AuNPs) forming densely packed, well-defined AuNP microstructures for microfluidic surface-enhanced Raman scattering (SERS) detection of gaseous analytes. We achieve this using two-photon lithography from Au precursor comprising poly(vinylpyrrolidone) (PVP) and ethylene glycol (EG), where EG promotes higher reduction rate of Au^{3+} via polyol reduction. By varying PVP concentration, we demonstrate that the physical properties of the AuNP microstructure can be tuned, such as AuNP size from 27.3 – 65.0 nm and density from $1.7 \times 10^2 - 9.7 \times 10^2$ particles/ μm^2 , corresponding to surface roughness of 12.9 – 90.5 nm, which is important for surface-based

applications such as SERS. We find that consequently, the SERS enhancement factor can also be tuned from 10^4 to $> 10^5$. By fabricating AuNP microstructures within a microfluidic channel, we demonstrate the remote in-situ SERS monitoring of gaseous 4-MBT and real-time detection of multiple small gaseous species with no specific affinity to Au.

In chapter 5, we recognize that the precision and morphology of two-photon fabricated metallic structures are largely dependent on the fabrication parameters and composition of the photoresist used, however, there is a lack of investigation into the how the type and combination of additives, as well as laser power, scanning speed and surfactant concentration affect surface morphology of the resulting structure. Hence, we formulate a universal metallic precursor recipe for the fabrication of dense, well-defined metallic microstructures consistently over > 4 hours of lithography. We also recognise that additives such as PVP and DMSO can tune the surface morphology of Au microstructures from aggregated AuNP to large fused Au chunks, and demonstrate that a PVP:DMSO ratio of 2: 1 produces dense, well-defined microstructures. By varying the laser power from 4.4 to 7.6 mW and scanning speed from 1.0 to 5.0 $\mu\text{m/s}$, the fabrication of Au microstructures can be optimized at 6.0 mW and 5.0 $\mu\text{m/s}$ to create highly precise, well-defined Au microstructures even after long hours of lithography (~ 4 hours). The ability to write such large arrays of well-defined Au microstructures can potentially be applied to the fabrication of microelectronics and metamaterials.

6.2 OUTLOOK

Two-photon lithography has proven to be a highly versatile and efficient technique for the precise fabrication of 2D and 3D microstructures. The high intensity laser enables the fabrication of microstructures from various materials, such as polymers, composites and even metals. We have demonstrated the ability to create pH-responsive, shape-shifting BSA microstructures by simply modulating the cross-linking density within the structure, and have also demonstrated the formation of densely packed, well-defined Au microstructures of tunable surface morphology, physical and optical properties. Hence, a promising advancement in this field will be to combine both two-photon polymerization and two-photon photoreduction into a single structure – not by forming a metal-

polymer composite, but by fabricating metallic microstructures over polymeric shape-shifting microstructures to form stimuli-responsive SERS platforms.

By incorporating Au microstructures on the tips of the BSA micro-trap pillars in chapter 2, a pH-responsive SERS platform can be generated, where “closing” of the trap at high pH produces a SERS response which is otherwise not observed at low pH when the trap is “open”. Alternatively, Au microstructures can be incorporated onto the edges of the geometrical shape-shifting microstructures as seen in chapter 3. By positioning individual microstructures close to one another, yet not in contact, a SERS response will be generated only upon swelling and shape-shifting of the microstructures at high pH.

A real life application of this dynamic SERS sensing platform would be environmental pH regulation. Maintaining the pH of the surrounding environment is crucial in many biochemical reactions, especially enzymatic reactions¹⁻² as most enzymatic reactions proceed most efficiently at an optimum pH value. Since the pH-dependent reaction kinetics of most enzymes follow a Gaussian “bell-shaped” curve,³ the effectual pH range of enzymatic reactions is usually narrow, beyond which, the enzyme experiences pH-denaturation effects and will not be able to catalyse reactions further.⁴ Hence, maintaining the pH of surrounding environment in enzymatic reactions is highly crucial to ensure that reactions proceed at an adequate pace. By positioning the pH-responsive SERS platform within a microfluidic channel as demonstrated in chapter 4, a calibration curve detailing the changes in SERS intensity against pH can be plotted, enabling pH changes in flowing analytes through the microfluidic channel, or to reactions happening within the channel to be easily monitored based on the SERS intensity read-out. The versatile nature of two-photon lithography also allows fabrication to be extended to other stimuli-responsive materials,

Finally, we will like to highlight possible future directions in the fabrication of metallic microstructures, by either extending the resist formula in chapter 5 to fabricating other metallic microstructures such as silver, copper and platinum, or creating bimetallic structures by incorporating two different metallic salts within the same precursor solution, or by fabricating each metallic microstructure separately. Such microstructures composed of two metals can potentially demonstrate

synergistic effects which may offer new properties,⁵⁻⁷ to further cement the possibility of creating multi-functional lab-on-a-chip platforms. The incorporation of two different metallic salts within the same precursor solution is especially exciting as new alloys can be potentially created, which may exhibit new physical, chemical and optical properties that are yet to be discovered.

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7 List of Publications

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