2011

Top-down Particle Fabraction by Layer-by-Layer Assembly and Microcontact Printing

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TOP-DOWN PARTICLE FABRACTION BY LAYER-BY-LAYER ASSEMBLY AND MICROCONTACT PRINTING

By

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A thesis submitted to the Department of Chemical and Biomedical Engineering in partial fulfillment of the requirements for the degree of Master of Science

Degree Awarded:
Summer Semester, 2011
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ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Jingjiao Guan, for his patience, support, encouragement and guidance in this project. I am deeply grateful to Dr. Guan for leading me into scientific world, for spending his valuable time to guide my research and writing.

I would like to thank Dr. Teng Ma and Dr. Anant Paravastu for spending their valuable time to be my committee members. I would also like to express my gratitude to Dr. Ma’s guidance in writing, Dr. Paravastu’s help in the group study and all the professors who taught me here. I must say that during my study with these professors, I really learned a lot, not only in scientific research, but also in my life philosophy.

I am grateful to Liuqi Yu and Kansheng Chen in Dr. Peng Xiong’s group in Department of Physics for their help in photolithography. Their experience greatly saved my time and energy in exploring the experimental details of photolithography.

I am deeply grateful to my family, for their support during my study. I really appreciate the patience and love they gave to me since I came to this world.

I dedicate to this work to my parents.
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Microparticles are widely used for biomedical applications. Top-down techniques are able to generate particles with non-spherical shapes, precisely defined geometries, and complex structures and compositions. These properties allow well-defined interactions between microparticles and biomolecules, cells, and tissues, and are therefore desirable for drug delivery and biomedical imaging applications. However, biomedical application of microparticles is currently limited by lack of robust, versatile and inexpensive production methods. We intend to solve this problem by developing a novel top-down approach for fabricating microparticles.

In this approach, a thin film composed of multiple layers of polyelectrolytes is deposited on a stamp carrying micrometer-sized topological surface structures via layer-by-layer assembly. The multilayer film is then partially transferred onto a substrate by microContact Printing to form isolated pieces of films. When the substrate is coated with a water soluble sacrificial film, dissolving the film with water releases the films as free microparticles. We have used this method to produce microparticles with various shapes, sizes, numbers of layers, and compositions. We have also found that particles consisting of only a few layers (≤ 10 layer) fold significantly in water, but the folding can be inhibited either by increasing the number of layers or by covalently crosslinking the component materials. Meanwhile, spin-assisted layer-by-layer assembly has been utilized for the first time to fabricate multilayer particles in this work. In addition, we have developed a series of methods for producing microparticles with complex structures. Moreover, we have developed a method capable of producing a particle array that consisted of multiple types of particles arranged in a well-defined pattern. Taken together, this approach is simple, inexpensive, and highly versatile. It thus possesses great potential to be useful for biomedical applications especially drug delivery and biomedical imaging.
CHAPTER 1  BACKGROUND AND INTRODUCTION

1.1 Introduction

Microparticles are particulate structures ranging from 1 to 1000 µm in size. They are widely used and studied for various biomedical applications. Many of the targeted applications require microparticles that contain multiple component materials organized in well-defined and complex structures in order to be both functional and biocompatible. Production of such microparticles poses a great challenge to existing manufacturing technologies. This work aims to address this challenge by developing a novel particle fabrication approach with a unique set of advantages, in particular, for drug delivery, biomedical imaging applications. This chapter provides a background and introduction to the research. It starts with a review of key particle characteristics with respect to the abovementioned applications. An overview of some mainstream and emerging methods for particle fabrication is followed. Finally, two specific techniques, based on which our new approach is developed, are introduced.

1.2 Key characteristics of microparticles for drug delivery and biomedical imaging

Performance of a microparticle system for any biomedical applications is largely determined by interactions between the microparticles and biological entities including biomolecules, cells and tissues. The interactions are mainly dictated by several characteristics of the microparticles, notably including size, shape, surface properties, and component materials, which are briefly reviewed below.

1.2.1 Size

Size has been found to significantly influence cellular uptake, degradation, and in vivo destination of microparticles (1-6). For example, particles intended for blood circulation for an extended period of time must be smaller than the width of capillaries, which are approximately 10 µm in diameter (7). Nevertheless, particles with diameters of 1-5 µm are mainly trapped in the liver though blood circulation (8). In the pulmonary system, particles smaller than 3 µm are
exhaled and those with a larger size usually accumulate in the upper airways (8). In terms of cellular uptake, particles with a size larger than 500 nm are phagocytosed by macrophages and those with a smaller diameter can be endocytosed by phagocytic or non-phagocytic cells (6, 9). Hydrolysis is a major mechanism for \textit{in vivo} degradation of biodegradable particles (10). Particle size affects degradation by influencing penetration of water and clearance rate of degradation products, which can catalyze degradation reaction (1-2).

\subsection*{1.2.2 Shape}

Microparticles produced by conventional methods are mostly spherical in shape. While this shape is desirable for inhibiting agglomeration due to the minimized surface-to-volume ratio, non-spherical shapes can offer unique advantages by allowing manipulation of cell/tissue-particle interactions and drug release. Shape significantly influences ability of particles to bind to cells/tissues and cellular uptake. For example, a plate-shaped microparticle can provide a larger binding area than a microsphere with the same volume, resulting in a higher binding force between the particles and tissues without sacrificing drug-loading capacity of the particles (11). Moreover, it was reported that local particle shape, especially the initial contact point between a particle and a macrophage determined whether the macrophage would initiate phagocytosis or simply spread on the particles (12). Transport of microparticles in the blood vessels, gastrointestinal tract, and airways can also be affected by their shape. For example, Netti and colleagues have demonstrated by theoretical modeling that particle shape could significantly influence the way in which particles interact with tumor capillaries (13). Also, considering that internalized particles can be actively transported along microtubules and actin filaments as organelles (14), it is possible that particle shape can affect intracellular trafficking. With respect to drug release, it has been demonstrated that non-spherical particles could affect drug release profiles (15).

\subsection*{1.2.3 Surface properties}

Surface properties of microparticles are critical to their applications for drug delivery and biomedical imaging because interactions between particles and cells/tissues typically start at the surface of the particles. For drug delivery, one of the most widely used strategies to control the surface properties of particles is to coat the particles with a hydrophilic polymer brush in order to reduce immunological recognition (16). Polyethylene glycol (PEG) is the most widely used
among the polymers. For example, intravenously administered liposomes grafted with PEG has been demonstrated to have an increased circulating half life (17). Moreover, di-block copolymers of polylactide-co-glycolide (PLGA) and PEG have been synthesized to form particles with a hydrophilic PEG surface and a hydrophobic PLGA core (18-20). For in vivo drug delivery to solid tumors, PEG-coated nanoparticles are found to preferentially accumulate in the tumor vasculature due to the enhanced permeability and retention effect (21-22). In addition to PEG coating, surface properties such as surface charge and hydrophobicity influence in vivo distribution of particles and their uptake by macrophages (23). For example, nanoparticles with a positive surface charge preferentially accumulated in tumors and retained for a longer time than negatively charged particles (24). Also, DeSimone and colleagues demonstrated that cylindrical particles with a positive surface charge exhibited an enhanced rate of endocytosis compared to negatively charged particles of the same size and shape (25). In addition, surface grafting of certain molecules allows enhanced interactions between the particles and specific types of cells or tissues. A number of such molecules including antibodies, carbohydrates, peptides and other small molecules have been grafted to particles to target a variety of tissues including the brain, liver, intestinal M cells, and tumors (26-27).

1.2.4 Component materials

Microparticles for drug delivery and biomedical imaging are typically composed of structural materials and therapeutic and/or imaging agents. High degree of biocompatibility is typically required for the structural materials of a particle formulation intended to be administrated into human body. Food and Drug Administration (FDA)-approved polymers such as PLGA are therefore widely used to prepare microparticles for drug delivery (28). It is also desirable for the structural materials to be functional in various ways. For example, Bae et al. developed pH-sensitive polymers, e.g., poly (L-histidine)-PEG and poly (L-lactide)/PEG-polysulfonamide (PLLA/PEG-PSD) (29-30). Particles made of these polymers allowed pH-dependent release of carried drug. In many cases, physiochemical properties of the therapeutic/imaging materials determine the types of the structural materials to be used. For example, DNA is a primary therapeutic agent in a particle formulation for gene delivery. Due to the anionic nature of DNA, positively charged molecules such as chitosan (31) and PEI (32) are typically used to condense DNA into particles. Adoption of structural materials is also affected by a particular particle
fabrication approach. For example, photolithography is originally developed based on the use of silicon-based materials. As a result, silicon-based microparticles have been fabricated by this method for drug delivery even though silicon is unsuitable for systemic administration due to its non-biodegradability \(^\text{(33)}\). In another example, use of ultraviolet (UV) light to solidify light-sensitive structural materials raised the risk of damaging therapeutic molecules in the particles \(^\text{(34)}\). One of the most significant challenges for developing next-generation microparticles for biomedical applications is to precisely control quantities and locations of component materials within a particle. Such type of particles promises to achieve highly repeatable and controllable drug delivery and medical imaging. A particle fabrication technique with this capability does not exist currently.

1.3 Methods for fabrication of particles

The methods for particle production can generally be divided into two groups based on how particles are formed from the starting materials, as shown in Figure 1.1. One is bottom-up approach, characterized by assembling smaller parts such as molecules into larger entities in a macroscopic environment. The other, known as top-down approach, is featured by offering a spatial control of the formation of particles at a microscopic scale.

1.3.1 Bottom-up methods

Most of the conventional particle fabrication methods adopt the bottom-up approach. Examples include suspension emulsion \(^\text{(35)}\), spray drying \(^\text{(36)}\) and phase separation \(^\text{(37)}\). This group of methods has generated a wide range of micro/nanoparticles including capsules \(^\text{(38)}\), vesicles \(^\text{(39)}\), and liposomes \(^\text{(40)}\) for biomedical applications. While this approach is capable of producing particles of various component materials, with diverse structures, in large quantities and at relatively low costs, it is generally incapable of generating particles with highly uniform sizes, well-defined non-spherical shapes, and composed of certain biomedical materials. Moreover, this approach typically does not allow precise and versatile control of quantities and locations of component materials within each particle.
1.3.2 Top-down methods

Various top-down methods have been developed to make particles. The methods can be grouped according to key techniques involved as briefly reviewed below.

1.3.2. a Particle fabrication by photolithography

Photolithography relies on illuminating a light-sensitive film with UV light through a photomask to create typically micrometer-sized two-dimensional (2-D) features (41-42). The pattern of the photomask defines the geometries and 2-D shapes of the particles. In principle, particles in any 2-D shapes can be produced by this approach as exemplified by the particles in the shapes of 26 letters of the Latin alphabet (43). This approach has been used to fabricate particles using various materials including silicon (44), polymethyl methacrylate (PMMA) (45) and hydrogels (46). In addition, particles composed of two layers of photoresist with different shapes were prepared via two rounds of photolithography (43). Compared to other top-down methods for particle fabrication, perhaps the most notable drawback of this approach is its requirement for cleanroom-based facilities, which are expensive and not accessible to many researchers.
Moreover, this approach commonly suffers from exposure of delicate biomolecules to ionizing UV radiation.

1.3.2. b  Particle fabrication by molding and printing

A mold or stamp with topological surface structures can be used to produce microparticles via a molding or printing process. This group of methods is represented by nanoimprint lithography and soft lithography.

**Nanoimprint lithography.** Nanoimprint lithography typically uses a mold with nanometer-sized surface features (47). Notably, DeSimone et al. developed an approach, known as “PRINT” (Particle Replication in Non-wetting Templates) (48-50), to produce micro/nano particles. In this method, a liquid precursor containing functional cargo materials was molded against a mold on a substrate and then solidified. The mold was then removed, leaving the particles on the substrate. Particles produced by were featured by a tightly controlled size (from 20 nm to > 20 μm), various shapes (spheres, cylinders, discs, toroids, etc), structural materials (hydrogel materials, biodegradable polypeptides, titanium, barium titanate, and tin oxide), broad range of cargo materials (therapeutics, proteins, oligonucleotides, siRNA, and imaging contrast agents), controllable elastic modulus (stiff or deformable), and versatile surface chemistries (antibodies, PEG and metal chelators). In a similar method, Roy et al. used synthetic and biological macromers (peptides) as structural materials to produce nanoparticles carrying antibodies and nucleic acid (51). Although nanoimprint lithography is capable of generating particles with well controlled shapes, sizes and compositions, it suffered from the use of harsh conditions such as exposure of UV light or elevated temperature for solidification of the structural materials. Moreover, the particles produced by this approach were limited to a monolithic structure, in which the cargo agents were simply dispersed in the structural materials. In addition, the loading capacity of the particles was limited by the need for a significant portion of structural materials for particle formation. Similar to nanoimprint lithography, a hard photoresist mold was used to produce hydrogel microparticles (46).

**Soft Lithography.** Soft lithography consists of a group of techniques that use a soft stamp with topological surface features for micro/nanofabrication (52). A typical technique is microContact Printing (μCP) as shown in Figure 1.2. It relies on coating a stamp with a thin layer of material known as ink and transferring the material on the protruding areas of the stamp to a substrate by
conformal contact (52). This method has been used to pattern a wide variety of materials, including proteins (53-56), DNA (57-59), colloidal crystals (60), thermoplastics (61) and polyelectrolytes (62), polymeric nanocrystals (63-65), metal and semiconductor nanoparticles (66-69) and dendrimers (70-73). µCP is characterized by mild operating conditions, ease of use, and high reproducibility. It is used as cornerstone component in this work for particle fabrication by defining the lateral size and shape of microparticles.

Guan and coworkers used µCP and other soft lithographic techniques to fabricate polymeric microparticles (61, 74-75). In their methods, isolated polymeric structures of different 2-D shapes were first generated on micropillars or in microwells of a poly(dimethyl siloxane) (PDMS) stamp. The polymeric structures were then printed onto a substrate covered by a water-soluble sacrificial layer. Free particles were obtained by adding water to dissolve the sacrificial layer. In addition, reservoir-containing, capsule-like, and self-folding particles of biodegradable and biocompatible materials such as PLGA and chitosan were fabricated by the methods. These particles hold potential for various advanced drug delivery applications such as enhanced tissue binding and unidirectional drug release (74). However, the methods have only been used to produce particles from thermoplastics, thermosets and a chemically cross-linked polyelectrolyte,
but not from water-soluble polymers such as DNA. For many microparticle formulations for drug delivery and biomedical imaging, water-soluble materials such as polyelectrolytes are necessary to construct the microparticles.

1.3.1. c Particle fabrication by layer-by-layer assembly

LbL assembly is a technique characterized by sequential deposition of different materials on a substrate to form a multilayered structure as shown in Figure 1.3 [76-78]. Polyelectrolytes constitute the first group of materials used in LbL assembly and remain the most widely used. Polyelectrolytes can be ionized at certain pH, thus allowing strong electrostatic attraction between oppositely charged molecules [79]. While this interaction is mainly responsible for assembly of polyelectrolyte multilayer, secondary interactions especially hydrogen bonding can also play a critical role [80]. Layer formation between polyelectrolytes with opposite charges is repeatable until a desired layer number is achieved. The thickness of each single polyelectrolyte layer is typically 0.1-0.7 nm, depending on the properties of the polyelectrolytes, ionic strength [81] and pH [79] of the solution. Since this technique typically requires mild operating conditions, LbL assembly has been widely used to incorporate biological materials such as proteins [82-85], enzymes [86-88], DNA [89-91], cell membrane and viruses [92-93] into multilayers for biomedical applications. As a result, LbL assembly is employed in this thesis as a key component method for particle fabrication. Rubner et al. produced microparticles based on photolithography and layer-by-layer assembly [94-97]. In their approach, polyelectrolyte multilayer was deposited on a substrate at an array of isolated areas defined by a patterned photoresist film. However, release of the particles required a pH-sensitive interaction or a scraping method which limits types of materials that can be used and poses the particles to potential damage by the mechanical force, respectively.

1.3.1. d Microfluidic synthesis and lithography

Microfluidic allows continuous production of particles in a micrometer-sized environment. Current microfluidic methods for particle production can be categorized into two groups: “oil-in-water emulsion method” and “one phase fabrication method”, depending on the number of liquid phases involved. In the oil-in-water emulsion method, more than two streams of liquid precursors in different phases were brought together at a T-junction [98] or a flow-focusing nozzle [99-101]. Particles were formed as a result of physical or chemical interactions among the
different precursors (102). The flow-focusing (FF) method has been used to generate monodispersed solid and gel particles in both spherical and non-spherical shapes (103). The one-phase approach was developed by Doyle and colleagues (104). It integrated microscope projection lithography and microfluidic. Particles were formed by crosslinking liquid resin in a microchannel with UV light passing through a photomask. Recently, Doyle group fabricated 3-D particles by introducing a 3-way valve in a multileveled microfluidic device (105). In general, the microfluidic approach offers spatial control of precursors at the micrometer scale in a continuous fashion. Formation of the particles from the precursors is based on either self assembly or lithography. Particles with a range of sizes, shapes, structures, and component materials have been produced using this approach. However, production of particles with well defined non-spherical shapes requires in situ solidification typically induced by UV illumination, which is harmful to biological molecules such as DNA and proteins (34). Moreover, this approach has not been demonstrated with the capability for generating polyelectrolyte particles with well defined non-spherical shapes and structures.

Figure 1.3: Schematic presentation of layer-by-layer assembly.

1.3.1. e  Particle reshaping by stretching (106)
In this approach, spherical thermoplastic particles were embedded in a polyvinyl alcohol (PVA) film. The film was heated or exposed to a solvent of the particle materials to soften the particles. The film was then stretched mechanically, resulting in simultaneous deformation of the embedded particles. The final shapes of the particles were determined by the properties of the film and particles such as particle viscosity, film thickness, particle-film wetting property and other parameters including method of liquefaction and aspect ratio of stretching the film prior to particle liquefaction. By carefully controlling these variables, polystyrene and PLGA particles with over 20 distinct shapes have been prepared. These particles of different shapes were later used to demonstrate a critical role of particle shape in affecting phagocytosis (12).

1.4 Techniques used for developing a new particle fabrication approach

This thesis work seeks to invent a new approach to fabricating microparticles with a unique set of abilities to control particle size, shape, structure and composition for drug delivery and biomedical imaging applications. The new approach will be established on an integration of µCP and LbL assembly. µCP and LbL assembly have been combined for micropatterning by Hammond and coworkers (107). However, microparticles have not been produced using this technique. Our approach utilizes an existing particle-releasing method in addition to µCP and LbL assembly. This integration, as we will demonstrate in this thesis, offers significant synergistic benefits for particle fabrication.
CHAPTER 2  FABRICATION OF STAMPS

2.1 Introduction

µCP requires use of a stamp bearing micrometer-sized surface features. PDMS is the most widely used stamp material. PDMS stamp with specific topographical features is usually generated by a lithographic method such as photolithography and e-beam lithography. In this chapter, photolithography was employed to fabricate PDMS stamps.

2.2 Fabrication process and results

The fabrication process includes two steps: 1) production of a master by photolithography and 2) fabrication of a PDMS stamp as shown in Figure 2.1. A 4-inch chrome/quartz photomask was first designed using Microsoft PowerPoint and produced by Photosciences Inc. (Torrance, CA). To produce the master, a silicon wafer was sonicated in acetone, methanol and isopropanol, each for 5 min. The wafer was then baked at 96 °C for 15 min to remove the moisture. It was followed by depositing a thin film of photoresist (AZ5206e, Hoechst Celanses Corporation) on the wafer by spin coating (5500 rpm, 30 sec). The photoresist film on the wafer was then baked at 96 °C for 30 min. The wafer and photomask were mounted in a mask aligner (Carl Suss MJB3, PENN). Certain areas of the photoresist film were exposed to UV light passing through the photomask. The AZ5206e photoresist had a positive tone, meaning the photoresist exposed to UV became dissolvable in its developer solution. The photoresist film at the exposed area was then removed by washing with developer (AZ351 and water at a volume ratio of 1:5) for 20 sec, finally creating a master bearing photoresist features.

To produce a PDMS stamp using the master, PDMS liquid precursor was prepared by mixing Dow Corning Sylgard 184 prepolymer and curing agent at a 10:1 weight ratio. The mixture was degassed in a vacuum desiccator. It was then poured onto the master. Curing of the liquid precursor was accelerated at an elevated temperature of 37 °C for 24 hr. A photograph of a master covered by PDMS is shown in Figure 2.2A. The solidified PDMS was then peeled off from the master as a stamp. Figure 2.2B shows a PDMS stamp bearing reversed “FSU” features.
Figure 2.1: Schematic representation of the process for preparing a PDMS stamp.
Figure 2.2: (A) Photograph of PDMS casted on a master. (B) Optical micrograph of reversed “FSU” pattern of a PDMS stamp.
CHAPTER 3  FABRICATION OF MICROPARTICLES OF A SIMPLE STRUCTURE

3.1 Introduction

This chapter describes fabrication of multilayer microparticles with a simple multilayer structure using the new approach. It starts with introducing basic concept of the technique and is followed by a summary of materials used in this study, a detailed description of a typical fabrication process and its results. It also includes studies on the effect of layer number and chemical crosslinking on structural stability of the particles.

3.2 Basic concept

Figure 3.1 shows the conceptual processes for preparing multilayer particles using two distinct types of PDMS stamps. One carries an array of isolated protruding surface features called micropillars (Figure 3.1A). The other carries isolated recessed surface features called microwells, which are surrounded by ridges (Figure 3.1B). For either stamp type, a stamp is first coated with a thin layer of Poly(allylamine hydrochloride) (PAH) due to hydrophobic interaction between PAH and PDMS (107). It is important to set the pH of the PAH solution around 10. At this pH, the majority of the amine groups in PAH are unprotonated. As a result, there is a strong hydrophobic interaction between PAH and PDMS (107), leading to the adherence of a thin layer of PAH on the PDMS stamp. The positively charged amine groups in the PAH allow subsequent LbL assembly by electrostatic attraction to form a multilayer film on the stamp as demonstrated by Hammond et al (107). For a micropillar stamp, the multilayer film on the micropillars is transferred on a PVA-coated glass slide by µCP as demonstrated by Guan et al (61). Since PVA is soluble in water, the individually isolated pieces of multilayer films on PVA can be released from the substrate by water as free microparticles. For a microwell stamp, the PAH monolayer on ridge area of the stamp is removed first by µCP. The isolated pieces of multilayer film are then formed in the microwells by LbL assembly. The film in the microwells can be printed out onto a PVA-coated substrate and released in water by pressing and deforming the soft PDMS stamp.
(61). Both processes allow fabrication of microparticles that strictly replicate the surface features of a PDMS stamp. Moreover, LbL assembly permits precise and quantitative control of the materials to be incorporated in the particles simply by controlling the number of layers. In addition, the particles can be released in a mild condition by using a PVA sacrificial layer.

Figure 3.1: Schematic representation of preparing multilayer microparticles by integrating layer-by-layer assembly and microContact Printing using (A) a micropillar stamp and (B) a microwell stamp.
3.3 Materials

A prominent advantage of LbL assembly is its applicability to a wide variety of materials such as polyelectrolytes (108), metal nanoparticles (109-110), magnetic nanoparticles (111), quantum dots (112) and proteins (113) assembled by various types of interactions such as electrostatic attraction (114), covalent bond (115), and hydrogen bond (116). Polyelectrolytes constitute the most widely used class of materials for LbL assembly. Moreover, many of the polyelectrolytes have unique properties for biomedical applications. It was therefore used in this study to validate the concept of this new technique. The polyelectrolytes used in this study are listed in Table 3.1. Molecular structures, charges and relevance to biomedical applications are also included in the table.

To better visualize the multilayer microparticles, PAH was labeled with rhodamine-B-Isothiocyanate (RITC) (Sigma) and fluorescein isothiocyanate (FITC) (VWR). PAH-RITC/PAH-FITC was produced by mixing RITC/FITC and PAH molecules at a molar ratio of 1:1000 in Na₂CO₃/NaHCO₃ buffer for 3 hr (0.5% PAH, wt/wt, buffer pH = 9.1). Labeled PAH was purified by dialysis (Molecular weight cut-off: 20,000 Dalton, Thermo Scientific) against deionized (DI) water for 24 hr to remove unreacted RITC or FITC molecules (118).

3.4 Fabrication procedures

Two different procedures were used to deposit polyelectrolytes on a stamp in this study. One relied on soaking the stamp in a polyelectrolyte solution, which is commonly used for LbL assembly. The other method was based on spin coating, which was developed by Cho et al (119). Both micropillar and microwell stamps were used in the soaking method. One of the stamps that were used contained micropillars of 7 µm in diameter, 8.2 µm in center-to-center distance arranged in a square lattice. The stamp was soaked into PAH solution (0.5% in water, wt/wt, pH = 10) for 15 min, and washed in DI water for 1 min. It was then soaked in PSS, PDAC, PSS, PAH-FITC and PSS solutions (0.5% in water, wt/wt, 0.15 M NaCl in DI water, and no pH adjustment) alternatively, each for 15 min. After the deposition of each layer, the stamp was washed in DI water for 1 min. As a result, a 6-layer film with a structure of PAH/PSS/PDAC/PSS/PAH-FITC/PSS was formed on the stamp surface. Meanwhile, a sacrificial layer for particle release was prepared by spin-coating a 3% wt/wt PVA solution (Molecular weight: 31,000, 97% hydrolyzation, Sigma) on a glass slide at 3,000 rpm for 30 sec.
As a result, the glass slide was covered with a thin film of PVA. The multilayer film on the stamp was exposed to water vapor generated by a 65 °C water bath for 5 sec right before being brought into contact with the PVA-coated slide. A slight pressure was applied on the stamp manually to ensure a complete conformal contact between the stamp surface and the PVA film. After being contact for 45 sec, the stamp was peeled off from the slide. The particles were released either by exposing slide to water vapor or by adding water directly on the slide.

Spin coating is a common used method for preparing a thin film on a flat substrate. It allows easy control of film thickness by adjusting spin rate, properties of the liquid precursor, and/or solute concentration. Spin coating has been combined with LbL assembly to prepare multilayer films in a process known as spin-assisted LbL assembly (119). It permits a faster deposition rate than the conventional soaking method. However, it has not been applied to produce microparticles. Therefore, we applied this method to the production of microparticles for the first time.

Our spin-assisted method for particle production is shown in Figure 3.2A. A 1 × 1 cm² PDMS stamp was first coated with a layer of PAH using the soaking method. 1 mL polyanion such as PSS was dropped on the stamp mounted on an SCS G3P spin coater (Specialty Coating System, Indianapolis, IN) and spun at 1,500 rpm for 90 sec. 3 mL DI water was then added onto the stamp and spun at 1500 rpm for 90 sec to wash away unbounded polyelectrolyte. Repeating these two steps with polyelectrolytes of opposite charges led to formation of a multilayer film on the stamp. The same printing method as used in the soaking method was employed to transfer the pieces of films from the pillars to a PVA-coated slide.

This spin-assisted LbL assembly method can substantially reduce time needed for depositing each layer than the conventional soaking method. In a soaking method, it usually takes approximately 15 min for the polyelectrolyte molecules to bind to the substrate (119). However, in the spin-assisted LbL assembly, concentration of the polyelectrolyte is increased rapidly on the substrate surface due to fast evaporation of water (120). The increased concentration accelerates the rate for polyelectrolyte molecules to bind to the substrate. As a result, deposition of each layer requires much shorter time than the conventional soaking method.
Table 3.1: Polyelectrolytes used for preparing multilayer particles.

<table>
<thead>
<tr>
<th>Name of materials</th>
<th>Structure</th>
<th>Note</th>
</tr>
</thead>
</table>
| Poly(allylamine hydrochloride) | \[
\text{NH}_2 \]
\[
\text{HCl} \]
| Polycation. Used as the first layer in this technique. Labeled with RITC and FITC for fluorescence visualization. |
| Poly(sodium 4-styrene sulfonate) | \[
\text{O}^\text{3-} \text{Na}^+ \]
\[
\text{O=S=O} \]
| Polyanion. |
| Poly(diallyldimethyl ammonium chloride) | \[
\text{Cl}^- \]
\[
\text{H}_3\text{C} \]
\[
\text{CH}_3 \]
| Polycation. |
| Polyethyleneimine (branched) | | Polycation. Used as a structural and functional material in gene delivery (32) |
| Poly(acrylic acid) | | Polyanion |
| Deoxyribonucleic acid | Helix double strand | Polyanion. Used as a therapeutic, structural and functional material in gene delivery. Can be stained by YOYO-1 fluorescence dye. |
| Poly-L-Lysine | | Polycation. Used as a structural material in gene delivery (117). |
Figure 3.2: Schematic representation of process for the fabrication of multilayer microparticles by combining spin-assisted LbL assembly and microContact Printing.
3.5 Results

Figure 3.3 shows the micropillar PDMS stamp, the 6-layer microparticles on a PVA-coated slide and particles released by water vapor, and the stamp after printing. The high similarity between the stamp (Figure 3.3A) and the microparticles on PVA (Figure 3.3B and C) reflect the capability of this method for producing microparticles with the same geometries as the surface features of the stamp. The gray-scale brightness and fluorescence intensity within each microparticles were also uniform, indicating the thickness of the microparticles were largely homogenous. Transfer of the multilayer film from the stamp onto the PVA-coated slide was confirmed by the dark pillars after printing in contrast to the bright area around the pillars as shown in Figure 3.3D. The microparticles were released by water vapor as shown in Figure 3.3E and F. Clearly the particle retained their size and shape.

3.6 Effect of layer number on structural stability of microparticles

It is well known that thickness of a multilayer prepared by LbL assembly is proportional to the number of layers (121). Number of layers of a multilayer particle is therefore an important parameter that affects thickness and presumably mechanical properties of the particle. We fabricated microparticles composed of various numbers of layers and found that layer number played an important role in dictating structural stability of the particles. Figure 3.4A shows 7 µm-wide single-layer PAH-RITC particles on PVA. Once being exposed to water vapor, the particles disintegrated as shown in Figure 3.4B presumably because of lack of force to hold the polymer chains together. The particles disappeared after adding water likely because the polyelectrolyte molecules were completely dissolved. Figure 3.4C shows 2-layer PAH-RITC/PSS particles in water. The particles did not dissociate but folded significantly, reflecting an improved structural stability compared to the single-layer particles. Figure 3.4D shows 6-layer PAH/PSS/PAH-RITC/PSS/PDAC/PSS particles on PVA and in water. Most of the particles folded into a triangular shape with larger lateral sizes than the folded 2-layer particles. Figures 3.4E and F show particles with 12 and 30 layers (PAH/PSS/PDAC/PSS/PAH-RITC/(PSS/PDAC)_6/PSS and PAH/PSS/PDAC/PSS/PAH-RITC/(PSS/PDAC)_12/PSS, respectively) in water. Both types of particles did not fold but curved with a decreasing level of curvature as layer number increases. These results indicate that thicker particles are more structurally stable in water than thinner ones. Moreover, the results imply that particle curvature can be controlled by varying the number of layers.
Figure 3.3: Phase-contrast micrograph of (A) the PDMS stamp. Phase-contrast (B) and fluorescence micrograph (C) of 6-layer PAH/PSS/PDAC/PSS/PAH-FITC/PSS microparticles on PVA. (D) Fluorescence image of PDMS surface after printing. Phase-contrast (E) and fluorescence micrograph (F) of the microparticles released by water vapor.
Figure 3.4: Fluorescence images of 7 µm-wide particles with different layers. (A) single-layer PAH-RITC particles on PVA, (B) disintegrated single-layer PAH-RITC particles released by water vapor, (C) collapsed 2-layer PAH-RITC/PSS particles in water, (D) folded 6-layer (PAH/PSS/PAH-RITC/PSS/PDAC/PSS/PDAC) particles released in water, (E) curved 12-layer (PAH/PSS/PDAC/PSS/PAH-RITC/(PSS/PDAC)$_6$/PSS) particles released in water, and (F) slightly curved 30-layer (PAH/PSS/PDAC/PSS/PAH-RITC/(PSS/PDAC)$_{12}$/PSS) particles released in water.

3.7 Enhancing structural stability of microparticles by chemical crosslinking
Although microparticles with a small number of layers have been revealed with poor structural stability in water, such type of particles may be desirable for certain envisioned biomedical applications. For example, particles with fewer layers are presumably softer than the particles with more layers. The soft particles would allow conformal adhesion to highly curved surface of cells or other biosurfaces such as internal wall of capillaries. Also, such particles may allow higher degree of mass transport across the particles than thicker ones. This feature may be useful for preparing bioadhesive drug-delivery devices. We therefore seek to retain the structural stability of the particles with a small number of layers using crosslinking approach. Glutaraldehyde (GA) is a commonly used chemical crosslinking agent (122). A GA molecule contains two aldehyde groups that can form covalent bonds with amino groups. It was used in this study to crosslink PAH in microparticles composed of single layer, 3 layers, and 6 layers, respectively.

Single-layer particles were prepared with PAH-RITC. After being coated with PAH-RITC, the stamp was soaked in GA solution (5% in DI water, wt/wt) for 1 hr. The stamp was then rinsed with water for 30 sec, dried by nitrogen stream, and printed onto a PVA-coated glass slide. The particles were released first by water vapor with fully retained shape as shown in Figure 3.5A, indicating that the structural stability of the particles was enhanced by the crosslinking reaction. Water was later added to completely release the particles, which shrunk significantly as shown in Figure 3.5B. However, individual particles were clearly resolvable as in contrast to the disintegrated single-layer particles without being crosslinked in Figure 3.4B. The enhancing effect of GA in stabilizing the particles was further proved using 3-layer (PAH/PSS/PAH-RITC) and 6-layer ((PAH/PSS)2/PAH-RITC/PSS) particles as shown in Figures 3.5C-F. Both types of particles became less folded or curved as a result of GA crosslinking. In particular, the crosslinked 6-layer microparticles resembled 30-layer microparticles in water.
Figure 3.5: Fluorescence images of microparticles. Single-layer GA-crosslinked PAH-RITC microparticles released (A) by water vapor and (B) in water. Uncrosslinked (C) and GA-crosslinked (D) 3-layer (PAH/PSS/PAH-RITC) microparticles in water. Uncrosslinked (E) and GA-crosslinked (F) 6-layer ((PAH/PSS) \textsubscript{2}/PAH-RITC/PSS) microparticles in water.

3.8 Microparticles of different sizes and shapes
The approach developed in this thesis is able to produce multilayer microparticles with various sizes and shapes determined by the surface features of the PDMS stamps. Figure 3.6A shows 1 \( \mu \text{m} \) -wide 5-layer (PAH/PSS/PAH-RITC/PSS/PDAC) circular particles released by water vapor. In principle, nanometer-sized multilayer particles can be produced with this method by using a stamp with smaller surface features. Figure 3.6B and C show 5-layer (PAH/PSS/PAH-RITC/PSS/PDAC), \( 2 \times 5 \ \mu \text{m}^2 \) rectangular particles with rounded corners and 3 \( \mu \text{m} \) -wide square particles released by water vapor. Figure 3.6D shows released GA-crosslinked 5-layer (PAH/PSS/PAH/PSS/PAH) “FSU” particles produced using a microwell stamp, demonstrating that particles with arbitrary 2-D shapes can be produced using this method. These particles can be used to investigate the role of particle shape in particle-cell integrations.

Figure 3.6: Fluorescence micrographs of 5-layer PAH/PSS/PAH-RITC/PSS/PDAC (A) 1\( \mu \text{m} \) -wide circular particles, (B) \( 2 \times 5 \ \mu \text{m}^2 \) rectangular particles, (C) 3 \( \mu \text{m} \) –wide square particles, and (D) 5-layer composition “FSU” particles.
CHAPTER 4  FABRICATION OF MICROPARTICLES WITH COMPLEX STRUCTURES AND A PATTERNED MICROPARTICLE ARRAY

4.1 Introduction

In recent years, microparticles with complex structures have attracted more and more attentions for biomedical applications. For example, Desai’s group fabricated microparticles with an asymmetric, reservoir-containing structure by photolithography (123). Such microparticles promised to allow more efficient delivery of drug than the conventional microspheres. Similarly, Guan et al. fabricated drug-delivery microparticles with various structures by soft lithography (74). Moreover, Doyle produced microparticles for multiplexed detection using the microfluidic method (124). However, these methods are not suitable to produce microparticles using polyelectrolytes with complex structures. In addition, conventional lithographic fabrication methods are only used to produce identical particles in a single batch. We believe an ability to create distinct populations of particles in a well defined pattern can offer new application opportunities for the microparticles. In this chapter, we introduce fabrication of four types of microparticles with complex structures and a patterned microparticle array by extending the technique developed in the last chapter.

4.2 Fabrication of butterfly-like particles

µCP allows transfer of particles onto a PVA film more than one time. A double printing of two different types of particles on PVA with partial overlaps would produce particles resembling a butterfly with two wings. Figure 4.1 shows the process for the fabrication of such microparticles composed of two overlapped circular particles. It is essential to ensure that the top layer of the first printed particles is positively charged and the bottom layer of the secondly printed particles is negatively charged. As a result, electrostatic attraction between these two layers can hold the two multilayer particles together. The 1 µm-wide pillar stamp was used to fabricate the butterfly-like particles. The first printed particles consisted of 3 layers (PAH/PSS/PAH-FITC) with anionic PAH-FITC as the top layer on PVA. The secondly printed particles consisted of 4 layers (PAH/PSS/PAH-RITC/PSS) with the cationic PSS as the bottom layer. The two groups of particles were labeled with different fluorescence dyes. Figure 4.2A shows butterfly–like
microparticles on PVA, showing the two groups of particles overlapped partially. Figure 4.2B shows the microparticles released by water vapor. Obviously the two groups of particles were bounded together. In principle, this method allows multiple printings of component particles with different compositions and structures in a modular fashion. A wide range of end-product microparticles can thus be produced with integrated multiple functionalities through a reproducible process.

Figure 4.1: Schematic representation of process for the fabrication of butterfly-like microparticles by double printing.
Figure 4.2: Fluorescence images of (A) butterfly-like microparticles on PVA and (B) released particles by water vapor.
4.3 Fabrication of dotted microparticles

µCP is typically performed by printing a stamp on a flat substrate such as the PVA-coated glass slide used in this study. However, µCP can also be conducted by printing a stamp on another stamp as shown in Figure 4.3. As a result, a multilayer film on one stamp (stamp A) formed by LbL assembly can be transferred to another stamp (stamp B) that is covered by a multilayer film. If stamp A carries surface features that are smaller than those on stamp B, a dotted pattern can be generated on stamp B. Transfer of the dotted film on stamp B onto PVA and subsequent release will produce dotted microparticles.

The 1 µm micropillar stamp was used as stamp A and the 7 µm micropillar stamp as stamp B in this study. A 3-layer (PAH/PSS/PAH-RITC) film was formed on stamp A and a 6-layer (PAH/PSS/PDAC/DNA-YOYO-1/PDAC/PSS) film on stamp B. Then stamp A was brought into contact with stamp B for 90 sec. Stamp B was placed on a PVA-coated slide for 45 sec. Figures 4.4A and B show dotted microparticles on PVA and released by water vapor.

Cationic PAH-RITC and anionic PSS were the last layers on stamp A and B respectively. Therefore, the electrostatic attraction was presumably responsible for holding the dots on the pads. It is also important to ensure that there were fewer polyelectrolyte layers on stamp A than on stamp B. As a result, we believe the multilayer film on stamp A (film A) would be weaker than that on stamp B (film B), allowing breakage of only film A at the edge of the micropillars rather than film B. Otherwise, film B would have been transferred onto stamp A.

This method allows incorporating functional materials into a particulate carrier in a designed pattern. Such type of particles has been produced by Doyle and coworkers and used for multiplex bioanalysis (124). This method is applicable to a variety of functional materials such as fluorescent molecules, quantum dots, magnetic nanoparticles, and cell/tissue-targeting ligands. Moreover, multiple printings can be performed to construct microparticles containing more components in a more complex structure. It is important to note that the abovementioned double-printing method performed on a PVA-coated slide also allows fabrication of dotted microparticles by printing 7 µm and 1 µm particles on PVA sequentially. However, two populations of particles including both dotted 7 µm particles and 1 µm particles would be produced simultaneously. For certain envisioned applications, such a mixture of particles is not desirable.
Figure 4.3: Schematic representation of process for the fabrication of dotted microparticles.
Figure 4.4: Fluorescence images of (A) dotted microparticles on PVA and (B) released particles by water vapor.
4.4 Fabrication of porous microparticles

Fabrication of the above dotted microparticles required transfer of materials from the 1 µm pillar stamp onto the 7 µm pillar stamp by µCP. The transfer can be reversed, i.e., from the 7 µm stamp to the 1 µm stamp as shown in Figure 4.5. It is equivalent to the removal of the multilayer film on the 7 µm pillars by the 1 µm pillar stamp at the contact areas. As a result, 7 µm-wide microparticles with 1 µm pores can be produced.

To ensure efficient transfer from multilayer film onto the 1 µm pillar stamp. The stamp was treated with an oxygen plasma (HARRICK PLASMA, 680 torr, 3 min, power level: high) before being brought into contact with 7 µm pillar stamp coated by 4-layer (PAH-RITC/PSS/PDAC/PSS/PDAC) film. Effect of the plasma treatment was confirmed by the change of contact angle of water on a flat PDMS from 109° to 20° under the same plasma condition. The two stamps were kept in contact for 90 sec. Regular µCP was then performed on a PVA-coated slide to obtain porous particles as shown in Figures 4.6A and B.

The porous microparticles hold potential to be useful for cell tracking and tissue engineering. In cell tracking, the particles can be grafted with cell-binding agents, and functional materials such as magnetic nanoparticles can be incorporated into the particles for MRI imaging. The particles can thus bind to cells and be tracked by MRI as proposed by Rubner et al (94). The pores in the particles can offer a unique advantage by allowing mass transfer between the tagged cell and environment. As a result, normal cellular activities are less impaired than using the particles with same sizes but without pores. For tissue engineering, one porous particle can, in principle, binds to two single cells with its two faces. Without the pores, the particle forms barrier between the two cells. Since intercellular communications via direct contact are critical to the health and function of cells, the pores may reduce this problem by allowing intimate interactions between the two cells.
Figure 4.5: Schematic representation of process for fabrication of the porous particles.

- 1 µm pillar PDMS
  Oxygen plasma treated

- Stamp with 4 layers of polyelectrolyte

- Stamp-on-stamp printing

- Peel off stamp

- Printing onto PVA film

- Hydrophilic surface
Figure 4.6: Fluorescence images of (A) porous microparticles on PVA and (B) released particles by water vapor.
4.5 Fabrication of a patterned microparticle array

We have extended the stamp-on-stamp printing method introduced in section 4.3 to the fabrication of an array of particles of multiple types organized in a well defined pattern as shown in Figure 4.7. Two types of stamps were used in this method. One was the 1 µm pillar stamp and the other contained an array of protruding stripes of 3 µm wide and 6 µm in edge-to-edge distance. A 6-layer (PAH/PSS/PDAC/PSS/PDAC/PSS) film was deposited on the 1 µm pillar stamp. Meanwhile, single-layer PAH-FITC and PAH-RITC was deposited on two 3 µm-stripe stamps respectively. The PAH-FITC on a stripe stamp was then transferred onto the film-coated 1 µm pillar stamp by µCP. As a result, some of the 1 µm pillars were coated with PAH-FITC. It was followed by a second printing using the PAH-RITC-coated stripe stamp. The second printing was oriented perpendicularly to the first one with respect to direction of the stripes. Consequently, some pillars were coated with PAH-RITC. Moreover, pillars at the intersections of two printings are coated by both PAH-FITC and PAH-RITC. The multilayer film on the stamp was then printed on a PVA-coated slide as microparticles. This array of microparticles consisted of four types of microparticles: the original 6-layer particles, particles containing the 6 layers plus PAH-FITC (green fluorescence), particles containing the 6 layers plus PAH-RITC (red fluorescence), and particles containing the 6 layers plus PAH-FITC and PAH-RITC (appear yellow by overlaying red and green fluorescence). The different particles are arranged in a highly ordered fashion determined by the surface features of the stamps and the orientations of printing. Figure 4.8A shows the array of particles on PVA with different colors. Released particles are shown in Figure 4.8B.

McShane and coworkers developed a method for patterning an array of multiplex, individually isolated multilayer thin films based on photolithography and LbL assembly (96). However, their method required cleanroom-based facilities and involved UV exposure. Moreover, they have not used the methods to make free microparticles. In contrast, our method is simple and inexpensive. Moreover, the stamp-on-stamp printing can be performed multiple times using stamps with different surface features to incorporate various types of functional materials into the microparticles. One envisioned application of this particle array is for multiplex labeling of therapeutic cells, which can be useful for quantifying in vivo fates of the cells in a cell therapy.
Figure 4.7: Schematic representation of process for fabrication of a highly ordered microparticle array consisting of four different types of particles.
Figure 4.8: Fluorescence images of (A) an array of differentially colored microparticles with a highly ordered pattern on PVA and (B) released microparticles by water vapor.

CHAPTER 5  CONCLUSIONS AND FUTURE WORKS

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5.1 Conclusions

We have developed a novel top-down approach to the fabrication of polyelectrolyte microparticles by integrating LbL assembly, µCP, and a particle release method. This approach allows production of microparticles with well defined sizes, shapes, and a multilayered structure. The structural stability of the microparticles increases with number of layers and can be enhanced by chemical crosslinking. Moreover, the new approach permits preparation of microparticles with a variety of complex structures and a patterned microparticle array. Taken together, the new approach is simple, inexpensive, and highly versatile. It thus holds great potential to be useful for various biomedical applications.

5.2 Future works

Among the potential biomedical applications of this new particle fabrication approach, we are particularly interested in using it to produce microparticles for tracking cells and studying cell-particle interactions.

5.2.1 Cell tracking

Microparticles carrying magnetically and optically active agents are useful for in vivo tracking of cells (125). Our new technique allows incorporation of multiple functional materials into single particles with precise control of the locations and quantities of each component. Figure 5.1A shows a design of a multilayer microparticle. The particle contains two layers of magnetic nanoparticles for MRI imaging and one layer of quantum dots for optical imaging. The magnetic nanoparticles and quantum dots will be positioned in the interior of the microparticles. One face of the microparticles will be made of a cell-binding layer, which can adhere to cell surface. The other face will be made of a cell-repelling layer, which does not adhere to cell surface. As a result, one particle can only bind to one cell as shown in Figure 5.1B.

5.2.2 Cell-particle interactions

Mitragotri et al. demonstrated that particle shape played an important role in determining cell-particle interactions (12). Rubner et al. showed multilayer thin-film particles that could bind to
cells (94-97). However, the roles of geometry and mechanical properties of the particles in the cell-particle interactions were not studied in details in these studies. Our new technique allows fabrication of ultrathin particles with precisely controlled geometries and presumably mechanical properties. We will fabricate particles with various sizes, shapes, layer numbers, and mechanical properties and study the interactions of the particles with different types of cells including macrophages and cancer cells. The information will be useful for designing drug delivery and cell tracking microparticles.

Figure 5.1: (A) Design of a multifunctional microparticle for cell tracking. (B) Schematic representation of adherence of a designed microparticle to a cell.
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Education

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- A novel Top-down Particle Fabrication for Biomedical Engineering, Poster in Department of Chemical and Biomedical Engineering, Florida State University. 2011.
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