Predefinable quantum-dot barcodes

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Predefinable Quantum-Dot Barcodes

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Abstract

Modern biological research requires cost-effective and high-throughput methods to interrogate plenty of biomolecules simultaneously with high efficiency. The suspension multiplexed assays of using barcoded microparticles have obvious advantages over microtiter plates and microarrays for high-throughput screening applications, including less consumption of biological reagents, optimized bioconjugation between the microparticles and biomolecules and higher throughput capability. As a next-generation platform to perform high-throughput screening, the barcoded microparticles have broad applications in drug discovery, gene profiling and diagnostic therapy. Particularly, the barcodes formed by quantum dots (Qdots) attract great attention due to their high coding capacity. However, in the current state of the art, all methods proposed fail to create stable, consistent and repeatable Qdot barcodes for practical usage, rendering these Qdot barcodes non-predefinable in their spectra.

This work focuses on the development of predefinable Qdot barcodes in microcapsules which are templated from microfluidic double emulsion droplets. A microfluidic device was developed to form the microcapsules with precise morphology controls, especially, ensuring the Qdot-loaded aqueous cores are fully enclosed in hard polymer shells without leakage. Two types of predefinable barcodes were developed. Firstly, colorimetric barcodes with pre-defined fluorescent profiles were formed by loading Qdots in the aqueous cores of microcapsules, which were stable to ambient fluids due to protection of the hard polymer shells. An image-based decoding method was demonstrated to identify the colorimetric barcodes, in which

graphical information of the Qdot-loaded aqueous cores was used to promote the coding capacity. Secondly, easily discernible fluorescent-pattern barcodes with a high coding capacity were formed without using photomasks by loading the quantum dots in different colors in the aqueous cores of multi-component microcapsules. Furthermore, mechanical stability of the microcapsules was examined for processing the assay samples with the barcodes. The methods to tailor the surfaces of barcoded microcapsules with functional groups were also presented. The predefinable Qdot barcodes formed in the microcapsules can be used as a general tool to conduct various screening applications.

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List of Abbreviations

Qdot	quantum dot
HTS	high-throughput screening
PS	polystyrene
PDMS	polydimethylsiloxane
FRET	fluorescence resonance energy transfer
PE	polyelectrolyte
TOPO	tri-octylphospine oxide
PAH	poly(allylamine hydrochloride)
PSS	poly(sodium 4-styrenesulfonate)
PU	polyurethane
AA	acrylic acid
PAA	poly(acrylic acid)
HDDA	1,6-hexanediol dimethacrylate
TPGDA	tripropylene glycol diacrylate
TMPTA	trimethylolpropane triacrylate
ETPTA	ethoxylated trimethylolpropane triacrylate
DRIE	deep reactive ion etching
IPA	isopropyl alcohol
PMMA	poly(methyl methacrylate)
Daracure 1173	2-hydroxy-2-methylpropiophenone

Span 80	sorbitan monooleate
PVA	poly(vinyl alcohol)
APTS	3-aminopropyltriethoxysilane
CES	carboxyethylsilanetriol disodium salt
XPS	x-ray photoelectron spectroscopy
FTIR	Fourier transform infrared spectroscopy
PBS	phosphate buffered saline
EDC	1-ethyl-3-(3-dimethylaminopropyl)
FAM	6-carboxyfluorescein
oligo	oligonucleotide

Chapter 1 Introduction

The investigation of interactions among different biomolecules and their combinations requires high-throughput methods to perform multiplexed assays. In the last three decades, the methods of using microtiter plates and microarrays have been developed, in which up to thousands of individual assays can be carried out simultaneously. Currently, barcoded microparticles attract a great attention for microparticle-based suspension assays which can be used to conduct the multiplexed assays with a higher throughput capability and lower costs.

1.1 High-throughput screening

Instead of traditional laboratory assays which are performed individually and manually, modern biological and pharmaceutical research requires high-throughput methods to screen a large number of compounds, inclusive of gene, protein and other biomolecules, with a high efficiency [1-6]. Usually, the high-throughput screening (HTS) refers to the testing of 10,000 to 100,000 compounds per day. The interactions of different compounds, roles of particular biological processes and mutations of genes can be investigated by HTS, which provides a preliminary understanding for further investigations. The methods to perform HTS not only extend the capability of research but also have highly commercial values in pharmaceutical industry. Therefore, HTS

attracts a great attention. Numerous efforts have been made to develop methods or platforms to perform HTS with higher throughput capabilities and lower costs.

A HTS method takes account of interdisciplinary factors on biology, chemistry, engineering and informatics to produce reliable, sensitive and cost-effective results [5]. To conduct the multiplexed immunoassays automatically, a HTS method should follow a simplified protocol involving steps as few as possible. The assay volumes are minimized to run HTS economically with optimizations of reagent concentrations to obtain sufficient sensitivities to target compounds, high reproducibility of results and accuracies of the positive and negative controls. The specificities of target compounds, such as the protein stability, needs to be considered to provide suitable reaction environments. The responses of the probe compounds to the target compounds should be clearly defined by detectable optical or electrical signals, which can be recorded in a short time to collect sufficient data to make a report with bioinformatic analysis. All above factors associate with each other and work as a whole system to perform HTS.

The design of a HTS method usually begins with developing new liquid-handling approaches and, especially, reactors to run the assays. The HTS requires loading reagents into corresponding reactors in precise volumes. The cross contaminations between different compounds should be avoid during reagent handling processes. The HTS reactors that carry out the assays with low reagent consumptions should be assembled in a compacted manner to handle the reagents easily and to collect the results quickly. Balances among the reaction conditions, such as temperature, reaction volume and concentrations of target molecules, are required, since not all of the assays with specific compounds run optimally in the same condition. Most of all, the HTS

assays in scale of thousands or even more should be registered to identify different compounds after being loaded in these reactors for data processing and analysis.

1.2 Trends in high-throughput screening

The increasing requirements of high-throughput capabilities for biological and diagnostic research stimulated the developments of the HTS methods to interrogate more and more compounds simultaneously. Microtiter plates and microarrays have been invented and developed as standard tools. Microparticle-based suspension assays have been proposed and have attracted a great attention as the next-generation platform for HTS [7-9].

Figure 1.1 Trends in HTS. (a) 96-well, 384-well and 1536-well microtiter plates [10]. (b) Gene microarray (Agilent Technologies Pet. Led.) [11]. (c) Microparticle-based suspension assays [9].

1.2.1 Microtiter plate

The most straightforward HTS method is to conduct the individual assays in a number of small wells in a rectangular array on a flat plate, which is called as microtiter plate. Usually, the microtiter plate is fabricated by injection moulding of polystyrene (PS) which has a low autofluorescence and a high transmission from UV (~300 nm) to infrared (~1600 nm) for optical detections of assay productions. Titanium dioxide is added in PS to make white microtiter plates for luminescence detection, and carbon is added in PS to make black microtiter plates for fluorescence detection. The surface of PS can be attached with proteins or cells passively after proper treatments with buffers or plasma discharge. As shown in Figure 1.1(a), the widely used 96-well plate is a typical microtiter plate. Some microtiter plates have 384 or even more wells in volume ranged from sub-microliter to milliliter. The different assays in these wells are registered by their own coordinates of locations on the microtiter plate. The wells of microtiter plate are separated from each other, the liquid-phase assays can be carried out individually without interference.

Mechanical systems or robotic arms are designed to handle the liquid reagents on the microtiter plate with a relatively high speed up to ten doses per second. Taking advantage of sophisticate automatic devices which can accurately control the volume of liquid, the air pressure to drive liquid or the time to open valve, the volumes of the liquid reagents loaded into the wells are highly repeatable. These mechanical systems are usually expensive with the usage of precisely mechanical moving parts and electronic controllers. Since the 96-well microtiter plate has been used as a standard tool for biological research and clinical diagnostics, plenty of commercial instruments are designed for the 96-well microtiter plate to measure the fluorophore-labeled assay

productions by non-imaging optical sensors, such as photo diode and photomultiplier tube. Fluorescence or luminescence emission intensities or spectra are recorded one by one from these wells on the microtiter plates.

1.2.2 Microarray

Large-scale genome projects are conducted all over the world including genome mapping and sequencing of various organisms and species. The most famous and successful one is the *Human Genome Project* completed in 2003 [12]. The microarray technology offered all of these projects an efficient bioinformatic tool for automatic and miniaturized biological detection. The determinations of complicated functions of proteins are also conducted with the microarrays with less time [13]. Generally, the microarrays are flat glass or silicon substrates, as shown in Figure 1.1(b), which are easily modified by silane compounds with functional groups, such as amine and carboxyl groups. Various probe biomolecules are anchored on a substrate in a spot array for solid-phase assays which are conducted simultaneously by immersing the microarray in a target sample. The types of these probe biomolecules are also registered by their coordinates of locations on the microarray as the microtiter plate.

The microarrays have different throughput capabilities according to their probe-spot densities. The most general approach to fabricate a microarray is to load the probe-molecule solutions as micro- or nano-droplets on a substrate in an array, as shown in Figure 1.2(a). The robotic arm with a fine needle array load the probe-molecule droplets on a substrate by contact printing [14, 15], which can make high-density probe spots as high as 1600 per square centimeter [13]. Another method of loading the probe-solution droplets on a substrate is to use a piezoelectric actuator

Figure 1.2 Microarray fabrication. (a) Contact printing by robotic arm [16]; (b) Piezoelectric actuator [17]; (c) Microarray made by photolithography synthesis [18]; (d) Bead-based microarray [19].

[17, 20-23], as shown in Figure 1.2(b), which is derived from the ink-jetting technology in printers [24]. The biological reagents are reserved in a nozzle array, and refilled in these nozzles through microchannels from corresponding reservoirs beside the nozzle array. The biological reagents are expelled as droplets in precise volumes down to picoliters by fine controlled displacements of a piezoelectric actuator which compresses the air or elastomer above the nozzle array. The array of probe-molecule spots loaded on a substrate is in the same dimension as the nozzle array.

Totally different from above two droplet-loading methods, a large number of different compounds on a glass or silicon substrate can be synthesized simultaneously by photolithography [18], as illustrated in Figure 1.2(c). With helps of solid-phase synthesis and photo-removable protecting group, different compounds made from several identical subunits grow on the substrate according to the masks used in very photolithography step. Take advantage of this photolithography patterning method, the spatial limitation between different compounds can be reduced down to micrometers in high-degree miniaturization.

Figure 1.2(d) shows a bead-based microarray manufactured by *Illumina Pet Ltd*. A fluorescent coding technique offers a solution to sort a large number of different DNA probes on the bead-based microarray by fluorescence color sequences but not their coordinates of locations. Firstly, microbeads labeled covalently with DNA probes are dispensed randomly on a microarray. Then these microbeads are sorted by a series of sequential hybridizations with their own fluorescence-labeled DNA targets. This method can produce the high-density microarrays due to the use of the small microbeads in diameter of 5 μm [25]. A tip of 500 μm -diameter optical imaging fiber acid-etched with 6,000 microwells in a controlled depth is used as the substrate to

support the microbeads. Suppose two colors are used to combine the fluorescence color sequences and the number of fluorescence color sequences (coding length) is 10, 2^{10} different DNA probes can be sorted. Gunderson *et al.* [19] reported the error checking codes to this method and provided a direct functional quality control of each microarray with median error rate less than 10^{-4} per bead.

All the above microarrays use fluorophores to determine the interactions between the probe molecules on the microarrays and the target molecules in the sample. The fluorescence signals from the microarrays are recorded by the CCD camera in fluorescent images. The data collection method is highly efficient for HTS.

1.2.3 Microparticle-based multiplexed suspension assay

Different from the microtiter plates and the microarrays which perform HTS assays on the two-dimensional (2D) well arrays or substrates with location-registered probe molecules, the microparticle-based suspension assay performs HTS by tracking the barcodes of the microparticles which carry different probe molecules in small volume samples [7-9], as illustrated in Figure 1.1(c). The microparticles with the barcodes are used as the solid supports for the probe molecules. Each type of probe molecules has its specific barcode for identification. These encoded microparticles with probe molecules are pooled together for multiplexed detection of the different target molecules. After hybridization, the probe molecules capture the target molecules specifically. The target molecules are labeled by fluorophore regardless of their types for target detection. Finally, the barcodes are identified with recording the target signals. Numbers of different analytes can be interrogated simultaneously. Generally, these encoded microparticles are made in small sizes of several micrometers. Their

surfaces can be tailored with different functional groups for optimal conjugation to various biomolecules, which has an advantage over the microarray functionalized under the same surface chemistry. In addition, the using of encoded microparticles for multiplexed assays allows flexible adding or reducing certain probe molecules with their corresponding barcodes to improve experiment designs. Furthermore, the binding rate between the probes on the microparticle and the targets in the sample can be accelerated by vortex mixing. To produce high-quality data, the probe molecules need to be design properly to improve the sensitivities and specificities of the multiplexed assays [26].

1.3 Motivation and Objectives

The microparticle-based multiplexed suspension assay has significant advantages over the microtiter plates and the microarrays to perform HTS. Particularly, the barcodes formed by Qdots are very promising due to their potentially high coding capacity. However, all the existing Qdot barcodes have their own inherent drawbacks, such as the complicated formation process and the low stability to ambient fluids. Moreover, they all suffer from a common problem that hinders their practical usages, that is the fluorescent profiles of the existing Qdot-barcoded microparticles are not the same as those of the Qdot mixtures before being incorporated into the microparticles to form the barcodes. In another word, the fluorescent profiles used to identify the barcodes cannot be pre-determined directly by those of the Qdot mixtures. As a result, the fluorescent profiles of those Qdot-incorporated microparticles require a comprehensive interrogation to check their specificities as unique barcodes, which is an unacceptable in cost and lack the feasibility for identifying numerous Qdot

mixtures individually. Furthermore, the fluorescent profiles of existing Qdot-incorporated microparticles are not stable, lacking necessary repeatability for the microparticles incorporated with an identical Qdot mixture as a barcodes. In the current state of the art, the Qdot barcode technology still remains at a proof-and-concept stage.

The main objective of this work is to develop pre-definable Qdot barcodes with high coding capacities. Polymer microcapsules templated from microfluidic double emulsion droplets are used as the barcode carriers. Taking advantage of microfluidic techniques, the morphology of microcapsules including their sizes, number of cores and even contents of cores can be controlled precisely. Stable and repeatable colorimetric barcodes are formed with pre-defined fluorescent profiles by loading the aqueous Qdot mixtures as the cores in the microcapsules. In addition, maskless fluorescent-pattern barcodes are formed in multi-component microcapsules which contain the aqueous cores with different Qdot contents. To bring the Qdot barcode technology from the proof-and-concept stage to practical applications, this work also investigates the mechanical stability of the barcoded microcapsules and the methods to functionalize the barcoded microcapsules for practical usages.

1.4 Scope of dissertation

Chapter 1 introduces the background, motivation and objective of this dissertation. The microtiter plates, microarrays and barcoded microparticles used to conduct the multiplexed assays are introduced with comparisons, which show the advantages and significances of the barcode technologies for HTS applications.

Chapter 2 is a literature review on the existing barcode technologies. The barcoding strategies using Qdots are discussed with details including barcode formations, performances, encoding algorithms and identification methods.

Chapter 3 is a methodological description for the strategies of using the microcapsules to form the colorimetric and fluorescent-pattern barcodes. A preliminary study on the microcapsule formation by the microfluidic double emulsion is presented.

Chapter 4 describes the formation of microcapsules, including the design and fabrication of the microfluidic device, the microfluidic operation and the formation of fully closed microcapsules.

Chapter 5 describes the formation of the colorimetric barcodes of using Qdots in single-component microcapsules and an image-based decoding method for express identification of the barcodes.

Chapter 6 describes the formation of the fluorescent-pattern barcodes in the multi-component microcapsules without using photomasks.

Chapter 7 investigates the suitability of the Qdot barcodes formed in the microcapsules for biological assays. The mechanical stability of the microcapsules and the method to functionalize the microcapsules are investigated.

Chapter 8 summarizes the work in this dissertation. The future work on the quality control of the Qdot barcodes, promoting the coding capability and using a flow cytometry for identifying the barcodes for HTS are discussed.

Chapter 2 Literature Review

2.1 Overview of barcoded microparticles

The barcodes can be formed using hollow [27, 28] or color [29] patterns, microstructures [30-33] and fluorescent emissions [34-36], as well as use of rare elements [37], magnetic elements [38], Raman spectra [39, 40], holograms [41, 42], DNA-based monomers [43] and optical crystals [44, 45]. These barcodes can be categorized into two main types: (1) graphical barcodes that are identified by deciphering the specific geometric patterns, and (2) optical barcodes that are identified by specific spectra or non-imaging optical signals.

2.1.1 Graphical barcodes

The graphical barcodes are mainly created by transferring the patterns from photomasks to the microplates by microfabrication techniques or photolithography. The method of using the microfabrication techniques creates the graphical barcodes by making microstructures on the microplates. Firstly, the photoresist patterns are made by lithography on a substrate. By depositing a layer of metal [28] or polysilicon oxide [33] on the photoresist patterns, the microplates with the patterns are formed above the patterned photoresist. Then, the microplates with the patterns are released from the substrate by dissolving the photoresist, as shown in Figure 2.1(a and b).

Figure 2.1 Graphical barcodes (a) Dot-pattern barcodes made on metal microplates [28]. (b) Structure-pattern barcodes made on silicon-oxide microplates [33]. (c) Dot-pattern barcodes made by flow-focusing photolithography [27]. (d) Color-pattern barcodes made by magnetic photonic crystals [29]. (e) Hologram-pattern barcodes [42]. (f) 1D-pattern barcodes made by alternant metal deposition [30]. (g) Fluorescent-pattern barcodes made by rare earth elements [37]. (h) Bar-pattern barcodes made by magnetic elements [38].

The method of photolithography in polydimethylsiloxane (PDMS) microchannels to form the pattern barcodes is developed from the fabrication of multifunctional non-spherical Janus microparticles in the PDMS microchannels by UV-induced polymerization of polymer precursors [46-49]. The polymer precursors are prepared by mixing monomers and photoinitiators. After filling the PDMS microchannel with the liquid-state polymer precursor, a region above the microchannel defined by a photomask is exposed to UV light to cure the polymer precursor to create the microplates with hollow patterns, as shown in Figure 2.1(c). Since oxygen molecules in the PDMS matrix inhibit the UV-induced polymerization [50], a thin layer of polymer precursor at the PDMS inner wall of the microchannel cannot be cured. The un-cured polymer precursor acts as lubricant to help the cured microplates being flashed downstream [51]. The hollow-dot patterns can be readout easily by a flow-focusing device [27, 47]. Besides of the area to carry the patterns for barcoding, the microplates should have extra areas to carry the probe molecules for bioassays. Similar to the hollow-pattern barcodes, color-pattern barcodes [29], shown in Figure 2.1(d), are also created by photolithography of the polymer precursor in the PDMS microchannel [29]. The polymer precursor is added with color-tunable magnetic crystals [52]. Before UV-induced polymerization, the colors of patterned areas are manipulated by the strengths of magnetic fields. After UV exposure, the color-tunable magnetic crystals are fixed in the cured polymer matrix, and the microplates are created with predefined color patterns for encoding.

Other graphical barcodes include the grating patterns on the microplates [42] (Figure 2.1(e)) or glass rods (*Illumina VeraCode Technology*) [53] to create holograms, one-dimensional (1D) patterns made by alternant metal depositions [30] (Figure 2.1(f)),

fluorescent patterns made by doping the rare earth elements in glass rods [37] (Figure 2.1(g)) and magnetic patterns [38] (Figure 2.1(h)).

These graphical barcodes are stable to ambient factors, such as temperature and assay buffers, in a long time. The coding capacity of 2D-pattern barcodes is quite high in scale of millions. Numerous graphical barcodes can be identified simultaneously in their images. However, the graphical barcodes have some drawbacks. The fabrication of these graphical barcodes by the microfabrication techniques requires complex procedures with low production rates. These graphical barcodes are hard to be made in small sizes down to several micrometers because of the usage of photomask to form the 2D patterns. Due to the minimum feature size of photomask (2~10 μm), the pattern-barcode microplates are usually made in size of 100~200 μm to carry the patterns for barcoding and offer sufficient binding areas for probe molecules. But the small size is a quite importance to conduct the multiplexed assay within small-volume samples, which should be at least two orders of magnitude larger than the encoded microparticles for 100-plex assay [54]. Furthermore, the expensive photomasks and precise alignment instruments set a huge economic hurdle to synthesize millions of these 2D-pattern barcodes.

2.1.2 Optical barcodes

The best developed and commercialized optical barcode is the fluorescent barcode formed by doping organic fluorescence dyes into polymer microparticles internally [34, 36, 55]. The polymer microparticles are swelled by an organic solvent with pores, through which the dye molecules penetrate into their outer surfaces. When these microparticles are re-dispersed in water, the pores are closed up to lock the dyes in

Figure 2.2 Optical barcodes (a) Multiplexed signals from organic fluorescent dyes in two colors (Luminex xMAP technology) [36]. (b) Qdot-encoded PS microparticles [35]. (c) PS microparticles made of different styrene monomers and their Raman spectra [56]. (d) Reflection spectra from colloidal microparticle made of nanoparticles in different sizes [45].

the microparticles. The microparticles doped with different mixtures of fluorescent dyes are identified according to their fluorescence profiles multiplexed by colors and intensities as shown in Figure 2.2(a). The coding capacity of this fluorescent barcode can be increased by using more colors and higher concentrations of dyes. However, the organic fluorescence dyes have broad emission bands about 100 nm. The multi-color emissions from the mixture of organic fluorescence dyes have significant overlaps, which limits the number of discernible barcodes. In addition, the organic fluorescence dyes of different colors have different absorption bands. Multiple narrow-

spectrum excitation sources are required for sequential exciting the fluorescent dyes in different colors on in a series at different wavelengths. In the current state of the art, a maximum of 500 barcodes using organic fluorescence dyes in three colors are created by *Luminex xMAP technology* [57]. The fluorescent barcodes in the small microparticles can be identified by a specially designed flow cytometer in high speed (2,000~40,000 per second) with simultaneous target detections.

Figure 2.3 (a) The emission spectra of organic fluorescence dyes. (b) The emission spectra of Qdots.[58]

The using of Qdots is promising to create millions of the fluorescent barcodes [35]. Typically, Qdots are semiconductor crystals made in size of 2~60 nm [58, 59]. Their electronic properties mainly depend upon their sizes and shapes. Since the smaller

crystals have larger band gaps between their exciting state and resting state, the smaller Qdots emit fluorescence of shorter wavelengths under the same excitation. The Qdot emission peaks are tunable by controlling their sizes. As indicated in Figure 2.3, the Qdots have narrow emission bands (~20 nm) and wide absorption bands from UV. The Qdots with different emission peaks can be excited by UV light simultaneously. Compared with organic fluorescence dyes, the Qdots have higher quantum efficiencies with a higher photostability, resulting higher emission intensities and less photobleaching. Therefore, multiplexing the emission peaks and the quantities of Qdots can create more discernible combinations for barcoding than the organic fluorescence dyes.

Other optical barcodes are created by Raman spectra of PS microparticles made of different styrene monomers [39, 56, 60] (Figure 2.2(c)), reflection spectra of colloidal microparticles made by nanoparticles in different sizes [45, 61, 62] (Figure 2.2(d)), and fluorescent nanostructures made of DNA-based monomers [43].

Compared with the graphical barcodes, the optical barcodes can be incorporated in the microparticles in quite small sizes. For example, the microparticles encoded by using organic fluorescent dyes are in a small size of 5.6 μm , which are compatible well with the flow cytometer. These microparticles with the optical barcodes can be made in a batch of millions or even more with low costs. However, the actual coding capacity of these optical barcodes is quite lower than the graphical barcodes. Although the using of Qdots is very promising to create millions of barcodes, the existing strategies to form the Qdot-encodes microparticles fail to achieve such high coding capacity with stable and repeatable fluorescent profiles, which is further described in Section 2.2.

2.2 A review of existing Qdot barcodes

A review on the existing strategies to form the Qdot-encoded microparticles is made on their fabrication methods, barcode formation algorithms and decoding methods, which provides a reference for designing new barcoding strategies to circumvent their problems of instability to ambient fluids, low repeatability and unpredictable changes of fluorescent profiles.

2.2.1 Qdot incorporation methods

Figure 2.4 Qdot incorporation methods. (a) Surface embedding. (b) Surface coating. (c) Matrix doping.

As illustrated in Figure 2.4, the existing strategies to form the Qdot barcodes incorporate the Qdots into the microparticles by three methods, including surface embedding, surface coating and matrix doping. The methods to form the Qdot-incorporated microparticles directly determine their optical properties to create the barcodes. The methods of surface embedding and coating can create Qdot barcodes by commercially available microparticles with a pre-definable manner. The method of matrix doping can form the Qdot-barcoded microparticles following a simple procedure with a high production rate. However, all these methods create the Qdot

barcodes with the drawback of unstable fluorescent profiles, which limits their practical applications for HTS.

a. Surface embedding

The concept to form the barcodes of using Qdots is firstly demonstrated by embedding the Qdots over the swelled PS microparticles with precisely controlled ratios [35]. By multiplexing fluorescent colors and intensity levels, ten intensity levels and six colors can create one million unique codes, theoretically. By dispersing the PS microparticles in a solvent mixture of chloroform and propanol, porous structures are made over the outer surfaces of PS microparticles. The Qdots dispersed in the solvent penetrate into the porous structures due to hydrophobic interaction. The porous structures separate Qdots spatially and avoid fluorescence resonance energy transfer (FRET) among the Qdots. Then, the porous structures are closed by filling polymerized monomers of 3-mercaptopropyl-trimetroxysilane to embed the Qdots at the top layer of these PS microparticles, as illustrated in Figure 2.4(a). The fluorescence intensity of the Qdot-embedded microparticle is linearly proportional to the amount of the Qdots on the microparticles. Mesoporous structures are made on the microparticles to provide more surface areas for Qdot immobilization [63]. To enhance the hydrophobic interaction between the Qdots and the polymer porous structures, the Qdots can be coated with a hydrophobic layer of tri-octylphosphine oxide (TOPO).

The method of surface embedding creates the encoded microparticles following a simple protocol. The microparticles are commercially available in small size of 5 μm as the organic-dye-encoded microparticles. However, the Qdots embedded at the outer surfaces of microparticles may be leaked to organic environments. The estimated

amount of Qdots on the microparticles may be not accurate. The Qdots are embedded over the microparticles in a low volume fraction, which limits the numbers of intensity levels. Furthermore, the fluorescent emissions of Qdot-embedded microparticles change in different bioassay buffers [64], which lack the necessary stability and repeatability as specific labelings for identifying the multiplexed immunoassays.

b. Surface coating

The surface coating method uses oppositely charged polyelectrolyte (PE) layers to sandwich the water-dispersed Qdots over the nanoparticles by layer-by-layer self-assembly [65-67]. The water-dispersed Qdots are treated with negatively charged carboxyl groups. Firstly, the nanoparticles are coated by poly(allylamine hydrochloride) (PAH) with positively charged amine groups. Secondly, the water-dispersed Qdots of a certain color are immobilized on the nanoparticles through electrostatic interaction. Thirdly, the extra positively charged amine groups sites are covered by the negatively charged poly(sodium 4-styrenesulfonate) (PSS). The above three steps form a layer of sandwiched Qdots. The nanoparticles are rinsed after every step to remove the extra PE molecules or Qdots. Several Qdot layers in different colors create a specific fluorescent profile as a barcode as illustrated in Figure 2.4(b). The PE layers in thickness of ~20 nm avoid the distant-dependent FRET between the Qdots, which may cause the unpredictable changes to the fluorescent profiles.

Besides PS and silica nanoparticles, magnetic nanoparticles are also coated with Qdots for barcoding. The encoded magnetic nanoparticles can be manipulated by magnet for selected separation of biomolecules [67]. The Qdot barcodes made by the method of surface coating can be defined clearly by the number of Qdot layers of each color. However, similar to the surface embedding method, the surface coating method

also suffers from the drawbacks, including the leakage of Qdots, the low-volume-fraction incorporation of Qdots and the changes of Qdot fluorescence emissions in different bioassay buffers. In addition, the fabrication procedure of layer-by-layer self assembly of PE-Qdot layers is complicated and time-consuming.

c. Matrix doping

The matrix doping method is to fix the Qdots internally in the polymer matrix of the microparticles as illustrated in Figure 2.4(c). The Qdot mixture for barcoding is firstly mixed within PS polymer precursor in liquid state. Then the PS polymer precursor with the Qdots is emulsified as microdroplets in an unsolvable media such as water bath. The polymer precursor droplets are solidified as microparticles by solvent evaporation [68] or thermal polymerization [69] to form the Qdot-doped microparticles.

The matrix doping method can make Qdot-doped microparticles in mass production up to 160,000 per second with a low cost. The Qdots are incorporated inside the polymer microparticles in relatively high volume fractions, which can create more intensity levels for barcoding. Moreover, the Qdots are locked inside the microparticles without any leakage. However, the long-time heating used to solidify the polymer precursor microdroplets decreases the quantum efficiencies of the Qdots [69], which makes the fluorescence spectra of Qdot-doped microparticles unpredictable. In addition, the shrinkage of the polymer microparticles caused by solvent evaporation induces FRET in their reduced volumes [68, 70]. Although, the Qdot-doped microparticles may still have specific spectra, a batch of them lacks the necessary repeatability to form an identical barcode. Because of the unpredictable

change caused by FRET and reduction of Qdot quantum efficiencies, their spectra need an interrogation to identify their specificities as unique barcodes.

2.2.2 Barcode formation methods

The Qdot barcodes formed by multiplexing colors and intensity levels is colorimetric barcodes which are identified by the fluorescent profiles of the encoded microparticles. Both of the fluorescent spectrum and the combination of fluorescent intensities at several wavelengths can be regarded as the fluorescent profiles. Two methods are designed to form the colorimetric barcodes in the microparticles. If the microparticles have a narrow size distribution, these monodispersed microparticles are encoded with external references which calibrate the intensity levels for decoding. If the microparticles have a broad size distribution, although the polydispersed microparticles are prepared in the same procedure, the big microparticles must carry more Qdots than the smaller ones with higher brightness. The polydispersed microparticles use internal references of their total emission intensities to define intensity levels individually.

a. Barcodes formation in monodispersed microparticles

Each color of the Qdots creates the intensity levels according to the amount of Qdots incorporated into the microparticles. The microparticles incorporated with calibrated amounts of Qdots to define the intensity levels are the external references. The intensity levels of encoded microparticles are identified by comparison with the external references. The number of barcodes increases exponentially by multiplexing multiple colors and their intensities. Only 3 colors can generate 999 (10^3-1) unique codes with 10 intensity levels. The number of barcodes, $C_{barcode}$, can be calculated as

$$C_{barcode} = M^N - 1 \quad (2.1)$$

where N is the number of Qdot colors and M is the number of intensity levels. However, because of the spectral overlapping and fluorescence intensity variations between the Qdots with different emission peaks, the actual coding capacity is substantial less than the theoretical calculation. Compared with using more intensity levels, using more colors is more effective to increase the coding capacity [35, 64]. To minimize the overlap between the emission spectra of Qdots, the interval between the emission peaks of the Qdots should be more than 80 nm. Only 3~4 colors are left in range from 450 nm to 700 nm for barcoding.

b. Barcodes formation in polydispersed microparticles

A ratiometric method defines the colorimetric barcode by the ratios of the fluorescence intensity of each color to the total fluorescence intensity of a microparticle. The total fluorescence intensity is the internal reference to scale the intensity level of each color, which is fixed as a constant. Therefore, if the intensity of a color is increased to create a new barcode, the intensities of other colors must be reduced to keep the internal reference of the total fluorescence intensity unchanged [71]. The combinations of colors and ratios can be calculated as

$$C_{barcode} = \sum_{i=0}^{N-1} (C_N^i \cdot C_{M-1}^{N-i-1}) = \sum_{i=0}^{N-1} \left(\frac{N!}{i! \cdot (N-i)!} \cdot \frac{(M-1)!}{(N-i-1)! \cdot (M-N+1)!} \right) \quad (2.2)$$

where N is the total number of colors, M is the total intensity levels and i is the number of colors which are not used for barcoding. Since the intensity-level reference of barcodes formed by this ratiometric approach is self-contained in each encoded microparticle, the instrumental error of the decoding system can be compensated. However, the coding capacity of this ratiometric algorithm is much lower than the using of external references to form the colorimetric barcodes in the monodispersed microparticles [64].

2.2.3 Spectrum-based decoding method

The Qdots are proposed for barcoding with a high coding capacity because of their narrow emission bands and high quantum efficiencies. The colorimetric barcodes of multiplexing colors and intensity levels are actually the combinations of multiplexing emission peaks and amounts of Qdots, which are the compositions of Qdot mixtures incorporated in the microparticles. However, the emission spectra of Qdots have overlaps between different colors. The fluorescence intensity at certain wavelength is attributed to the Qdots with different emission peaks. To identify the colorimetric barcodes defined by the compositions of Qdot mixtures, the fluorescent spectra of the encoded microparticles are captured by spectrometers or instruments with the spectral imaging function. A deconvolution algorithm is developed to separate the overlapped spectrum of a Qdot mixture into individual spectra for each color [64]. I made a demonstration of the deconvolution algorithm using Matlab (m-file: Peak Fitter, author: Tom O'Haver, <http://www.mathworks.com/matlabcentral/fileexchange/23611-peak-fitter>), as shown in Figure 2.5. The Qdot mixture contained the Qdots emitting at 500 nm, 550 nm and 600 nm, respectively. The overlapped spectrum of Qdot mixture

is separated into three Gaussian curves with centers at the three emission peaks. The composition of the Qdot mixture (barcode) can be implied from these separated individual spectra.

Figure 2.5 Deconvolution of the overlapped spectrum of a Qdot mixture.

2.3 Summary

Taking advantages of the narrow emission bands and high quantum efficiencies of Qdots, the colorimetric barcodes formed by incorporating Qdots in microparticles can be created with a much higher coding capacity than using conventional organic fluorophores. However, current existing Qdot barcodes all suffer from their intrinsic drawbacks. The barcodes formed by embedding and coating the Qdots over the outer surfaces of microparticles are unstable to ambient fluids. The Qdots at the outer surfaces of microparticles are sensitive to solvents and pH values of assay buffers and easily leaked to environments, which changes the fluorescent spectra of barcodes to

misidentify the probe biomolecules on the encode microparticles. Although the barcodes formed by doping the Qdots in the polymer matrix of microparticles can circumvent the problems of un-stability to ambient fluids and leakage, they meet another problem that the fluorescent spectra of Qdot-doped microparticles are different and unpredictable from the original Qdot mixtures. All the fluorescent spectra used for barcode identification require interrogations to check their specificities as unique barcodes; a specific Qdot mixture may not create a specific fluorescent spectrum as a barcode. Furthermore, due to FRET and reduction of quantum efficiencies of the Qdots in the solid polymer matrix, the fluorescent spectra of the Qdot-doped microparticles lack a necessary repeatability to form a barcode using an identical Qdot mixture. Actually, all these problems of existing Qdot barcodes can be summarized in one that these Qdot barcodes cannot be predefined from the formation to usage. A new Qdot barcode which can be predefined directly and only by the Qdot mixture is required to bring the Qdot barcode from current concept-and-proof stage to practical applications. In addition, the conventional decoding method of recording the fluorescent spectra is time-consuming and not suitable for high-throughput applications. A new express decoding method is need.

Chapter 3 Methodology

3.1 Introduction

Theoretically, the use of Qdots to form the colorimetric barcodes can promote the throughput capability of HTS by their high coding capability. However, the existing strategies fail to create stable and repeatable colorimetric Qdot barcodes. In this chapter, two new strategies of using the Qdots to form the colorimetric barcodes and the fluorescent-pattern barcodes in microcapsules are proposed, which are designed to form the predefinable barcodes with high coding capacities. These predefinable barcodes can maintain their emission profiles constantly from barcode formation to usage without any change and enable specific Qdot mixtures to form unique barcodes.

To form the predefinable barcodes, the barcode carriers should meet following basic requirements.

1. The Qdots incorporated in the carriers should be separated from ambient fluids to maintain stable emission profiles of the Qdot barcodes in different assay buffers.
2. The Qdots should be incorporated in high volume fractions to create as many intensity levels as possible.
3. The emission profiles of Qdot barcode should be determined directly and only

by the Qdot mixtures incorporated. The barcode formation process should avoid the high temperature and the phase change of Qdot suspension medium to maintain the original characteristics of the Qdots.

4. The barcode carriers should be monodispersed which can create much more barcodes than the polydispersed microspheres.
5. The barcode carriers should have sufficient mechanical stability to process the assay samples using vortex mixing or centrifuge.
6. The outer surfaces of the barcode carriers should be easily modified with various functional groups for optimal bioconjugation.

3.2 Predefinable barcodes I: colorimetric barcodes in microcapsules

The encoded microparticles are used as the solid supports to carry probe molecules for the multiplexed immunoassays. The barcodes should be stable in different bioassay buffers to identify the probe molecules on these encoded microparticles accurately. The fluorescent profiles of the microparticles incorporated with an identical barcode should be highly repeatable. The microparticles should be small enough down to tens of micrometers to conduct at least a 100-plex assay in a 1-mL tube. The monodispersed microparticles are preferred for barcoding with a high coding capacity. In addition, the monodispersed microparticles can offer quantitative measures of the target molecules, but not qualitative results from the encoded polydispersed microparticles.

Here, a totally new strategy is proposed that loads aqueous Qdot solutions as cores into polymer microcapsules to form the colorimetric barcodes. As illustrated in

Figure 3.1, the aqueous core contains the Qdot mixture for barcoding. A hard polymer shell encapsulates the core. The polymer shell is transparent to UV light to excite the Qdots and the fluorescence emitted from Qdots. With the protection of this polymer shell, the Qdot mixture in the aqueous core is separated from ambient fluids with constant fluorescence emissions in different bioassay buffers. The surface of polymer can be modified with functional groups easily for bioconjugation to probe molecules.

Figure 3.1 Microcapsule with a Qdot-loaded aqueous core.

Figure 3.2 Schematic diagram of the microcapsule formation to form colorimetric barcodes.

The microcapsules with the aqueous cores can be templated from water-in-oil-in-water (W/O/W) double emulsion droplets which are generated within narrow size distributions for both of the cores and the shells. As illustrated in Figure 3.2, the microcapsules are formed in a three-step process. Firstly, the water-in-oil (W/O)

emulsion is formed at the at a hydrophobic T-shape junction to generate W/O droplets of aqueous Qdot mixture in the flow of polymer precursor. Secondly, the W/O droplets are engulfed by polymer precursor globules to form the W/O/W double emulsion droplets at the hydrophilic flow-focusing junction in a flow of aqueous sodium dodecyl sulfate (SDS) solution. Finally, the polymer precursor globules are cured by UV light as the rigid polymer shells.

3.3 Predefinable barcode II: fluorescent-pattern barcodes in multi-component microcapsules

Taking advantage of the microfluidic techniques, the morphology of double emulsion droplets, including the sizes of droplets and the number of aqueous cores encapsulated in each outer globule, can be controlled precisely. Even the W/O droplets with different contents can be encapsulated in an outer globule to form multi-component double emulsion droplets [72]. By encapsulating the W/O droplets containing the Qdots with different emission peaks, discernible fluorescent-pattern barcodes can be formed in the multi-component microcapsules, as illustrated in Figure 3.3. Since the formation of the fluorescent patterns in the multi-component microcapsules does not use the photomask, these fluorescent-pattern barcodes can be made in small sizes without the dimensional limit of the minimum feature size of photomask. The manufacture of these fluorescent-pattern barcodes in the multi-component microcapsules is more cost-effective than previous photolithography methods to form the 2D-pattern barcodes.

The multi-component microcapsules are formed in a three-step process as illustrated in Figure 3.4. Firstly, the W/O droplets of the Qdot solutions are generated

at the T-shape junctions. The W/O droplets containing different Qdot contents queue in hydrophobic W/O microchannel alternately within the flow of the polymer precursor and go downstream. Secondly, the W/O droplets with different Qdot contents are encapsulated by a polymer precursor globule at the flow-focusing junction to form the multi-component double emulsion droplet. Finally, the polymer precursor globules are cured as solid shells by UV light to form the encoded microcapsules.

Figure 3.3 Schematic illustration of the fluorescent patterns formed in the multi-component microcapsules. The multi-component microcapsules can be formed with the aqueous cores containing the Qdots emitting in different colors.

Figure 3.4 Schematic diagram of the formation of the multi-component microcapsules to form the fluorescent-pattern barcodes.

3.4 Preliminary studies on microcapsule formation

The microcapsules templated from microfluidic double emulsion droplets are used as the carriers for the colorimetric Qdot barcodes and the maskless fluorescent-spot-pattern barcodes. Preliminary investigations were made on the microfluidic double emulsion and the monomer used to form the hard polymer shells as follows.

3.4.1 Microfluidic double emulsion

A double emulsion is a triple-phase dispersion in which droplets of inner-phase fluid are engulfed by a globule of middle-phase fluid and suspended in a continuing outer-phase fluid. The middle-phase fluid is immiscible to the inner- and outer-phase fluids. Two main types inclusive of water-in-oil-in-water (W/O/W) double emulsion and oil-in-water-in-oil (O/W/O) double emulsion are made for food, cosmetics and pharmaceuticals industry by a two-step emulsification method [73]. In the first step, the inner-phase fluid is dispersed in the middle-phase fluid as droplets by the turbulent shear force of mixing. In the second stage, the suspension of the inner-phase droplet in the middle-phase fluid is emulsified in the outer-phase fluid as the double emulsion droplets by mixing again. This traditional technique generates the inner-phase droplets and the middle-phase globules in broad size distributions.

Okushima *et al.* firstly reported the formation of the double emulsion droplets in highly uniform sizes by a microfluidic device made of glass substrates by wet etching [74]. The microfluidic device consists of two serial T-shape junctions with reverse wettability modification. As shown in Figure 3.5(a), a hydrophobic T-shape junction generates the aqueous inner-phase droplets in the middle-phase flow of corn oil. A hydrophilic T-junction generates the oil globules which engulf the aqueous inner-

Figure 3.5 Devices to form the microfluidic double emulsion (a) Glass device made of wet etching with two serial T-shape junctions [74]. (b) PU device made of soft lithography with a flow-focusing junction [75]. (c) Device assembled by glass capillaries with precision alignment [76]. (d) Functional modules assembled by glass capillaries [77].

phase droplets to form the W/O/W double emulsion. The number of inner-phase droplets in each middle-phase globule can be controlled by the ratio of breakup rates at the two T-shape junctions. However, this device cannot be used to form the microcapsules which are templated from the double emulsion droplets by UV-induced polymerization in chip, due to the high UV absorption of soda lime glass.

Nie *et al.* made more studies on the morphology of the double emulsion droplets which were generated by a flow-focusing junction [75, 78] as shown in Figure 3.5(b). Their device was made of polyurethane (PU) by soft lithography. The double emulsion droplets are generated from a liquid cylindrical jet in which an aqueous inner-phase

flow is surrounded by a middle-phase organic flow. Due to Rayleigh-Plateau hydrodynamic instability [79], the reduction of surface area under the action of interfacial tension breaks up the liquid jets into segments. The sizes of inner-phase droplets and middle-phase globules and the number of inner-phase droplets in individual middle-phase globules can be controlled by the flow rate of three phase of the double emulsion precisely and independently. Truncated microspheres, hemispheres and microcapsules were made by control the sizes of inner-phase droplets in the polymer precursor globules [80]. Since the device lacks a reversed wettability modification in its microchannel network, it has specific requirements for the three liquid phases of double emulsion to avoid the middle-phase liquid attaching at the microchannel. Therefore, the device is not suitable to form the microcapsules which may be formed by different monomers.

Utada *et al.* made a similar work on the double emulsion formation from a liquid jet in a device assembled simply by glass capillaries [76], as illustrated in Figure 3.5(c). Panizza *et al.* designed a flexible configuration to assemble the glass capillaries to form the double emulsion in complex structures [77] as shown in Figure 3.5(d). The devices assembled by the glass capillaries do not require any micromachining techniques to form the microchannels or the junctions. The restricted geometries of the glass capillaries are formed by using a pipette puller to taper the capillary tips. However, the drawbacks of the capillary-assembled devices are also rooted from the simple assembly without using any microfabrication techniques. For the capillary-assembled device shown in Figure 3.5(c), the concentricity of the tapered tip of injection tube to the narrowed tip of collection tube is quit important to enclose the inner-phase flow within the middle-phase flow. However, the necessary mechanical

tolerance ($\sim 3\ \mu\text{m}$) which allows the round capillaries being inserted into the square capillary may result an unacceptable deviation of concentricity, which causes the inner-phase flow immerse in to the outer-phase flow directly. In addition, the sizes of the tapered tips and narrowed tips cannot be made with repeatability by the pipette puller. The dimensions of the microfluidic junctions are made without control for the capillary-assembled device. Therefore, the capillary-assembled device is not suitable for mass production. For the capillary-assembled device shown in Figure 3.5(d), the microchannel cannot be made narrower than $100\ \mu\text{m}$ by commercially available glass capillaries, which cannot be used to create small microcapsules in the same scale.

3.4.2 Wettability patterning

The above microfluidic devices to form the double emulsion can be categorized into microfabricated devices and capillary-assembled devices. Besides the dimensions of microchannels, another critical point to form the double emulsion is to create a reverse wettability patterning in different sections of the microchannel network, which can turn the middle-phase fluid from a continuing fluid to a dispersing fluid [81, 82].

a. Wettability patterning in microfabricated devices

For the devices made by the microfabrication techniques, the geometry of the microchannel network can be defined precisely by photomask. The microchannel network can be made more easily by soft lithograph of PDMS rather than wet etching of the glass substrates. Due to the inert hydrophobic surface of PDMS, the main challenge is the wettability patterning in the PDMS microchannel network. Although the using of ionic wetting agents, oxygen plasma and chemical vapor deposition can turn the PDMS surface to be hydrophilic [83], these methods lack the ability to create

reversed wettability modifications at selected areas as patterning in the microchannel network. In the current state of the art, only three methods can pattern the wettability in the PDMS microchannels in the selected sections, including (1) layer-by-layer deposition of charged PE by flow confinement [84, 85], (2) photoreactive glass sol-gel coating [86, 87], and (3) UV-initiated graft polymerization of poly(acrylic acid) (PAA) [88].

The method of depositing the PE layers on the PDMS surface follows a straightforward protocol. The inert PDMS surface is firstly activated with hydroxyl groups by oxygen plasma or hydrogen dioxide solution. By using an inert flow of silicon oil to form a flow confinement, only the microchannels of being flushed by the reactive solutions of PSS and PAH, alternately, are modified to be hydrophilic. This method cannot form the wettability modification with accurate patterning, especially for the microchannels narrower than 50 μm . The alternative flow of the reactive solutions cannot form a clear boundary between hydrophobic region and hydrophilic region. In addition, since the PE molecules are attached on the activated PDMS surface by electrostatic force but not covalently anchored, the PE layers is not reliable for a long-time (~ 1 hour) aqueous flushing.

The method of sol-gel coating firstly fills the PDMS microchannels with a solution of photoreactive precursor and then cures the precursor by UV light to form a hydrophilic sol-gel layer at the walls of the PDMS microchannels. The hydrophilic sections can be defined precisely by a photomask. The hydrophilic sol-gel coating is robust against organic solvents. However, this hydrophilic sol-gel coating distorts the dimension of the microchannel due to the coating thickness of about 10 μm . In addition, the method of sol-gel coating is not accessible for general labs, due to its

complicated preparation of the photoreactive sol-gel precursor with explosive chemicals.

The method of UV-initiated graft polymerization of PAA also employs the photomask and UV light to pattern the wettability in the selected sections of PDMS microchannels as the method of sol-gel coating. Firstly, the PDMS microchannels are flushed by a photoinitiator solution of acetone to embed the photoinitiator molecules at the walls of the PDMS microchannels. Then the microchannels are filled with aqueous monomer solution of acrylic acid (AA). UV light is used to polymerize the AA monomers at the inner walls of microchannels which contain the photoinitiator molecules to form a thin layer of hydrophilic PAA. The method of UV-initiated graft polymerization of PAA not only achieves a robust hydrophilic coating, but also does not change the dimension of microchannel. The chemicals used for modification can be prepared easily without extra synthesis processes.

b. Wettability patterning in capillary-assembled devices

The glass capillaries can be easily modified to be hydrophilic and hydrophobic by 2-[methoxy(polyethylenoxy)propyl] trimethoxysilane and octadecyltrichlorosilane, respectively, respectively [89]. The wettability modifications made by the saline compounds are reliable on the glass surfaces. Before assembling the microfluidic devices, the glass capillaries are firstly modified with preferred wettabilities according the fluids they carry. Then the microchannel network with patterned wettability is assembled by these pre-modified glass capillaries.

3.4.3 Polymer precursor

The viscosity of the polymer precursor, μ , is related to *Reynolds number* (Re), *capillary number* (Ca) and *Weber number* (We), which have significant effects on the droplet generation process [90]. In addition, the polymer precursor should have a low shrinkage after polymerization to form complete microcapsules. Therefore, the monomer used to prepare the polymer precursor is another crucial part to form the microcapsules. Four monomers were investigated as the candidates of the middle-phase fluid, including 1,6-hexanediol dimethacrylate (HDDA), tripropyleneglycol diacrylate (TPGDA), trimethylolpropane triacrylate (TMPTA) and ethoxylated trimethylolpropane triacrylate (ETPTA). The molecule structures of these monomers are shown in Figure 3.6. Their molecule weights, viscosities and shrinkage upon polymerization are summarized in Table 3.1.

Figure 3.6 Molecules structures of the monomers to form the rigid polymer shells of the microcapsules

Table 3.1 *Molecular weights, viscosities and shrinkages of monomers [91-93].*

Monomer	Molecular weight (g/mol)	Viscosity (mPas, 25°C)	Shrinkage
HDDA	226	10	17 %
TPGDA	300	15	11~14 %
TMPTA	296	120	12 %
ETPTA	428	90	11 %

The ratio of viscosities of dispersing phase to continuing phase, μ_d/μ_c , characterizes the droplet breakup. Generally, the lower viscosity ratio results smaller droplets with higher generation frequency. According to previous research on the microcapsules formation [94], the viscous monomer is preferred to be used as the middle-phase fluid which can reduce the internal circulating flow in the double emulsion droplets to prevent the formation of leakage pores on the solidified polymer shells. The monomers with low molecular weights suffer from a drawback of significant shrinkage [92], which may cause the crack of the polymer shells after polymerization.

Taking account of the viscosities and the shrinkages of monomers, the low acid, low odor, and fast curing monomer ETPTA is the best choice to prepare the polymer precursor as the middle-phase fluid to form the microcapsules.

3.5 Summary

In this chapter, two new strategies to form the colorimetric barcodes and the fluorescent-pattern barcodes in the monodispersed microcapsules are proposed,

respectively. The microcapsules used for barcoding can be templated from the microfluidic double emulsion droplets with precisely morphology control. Preliminary studies on the microfluidic devices, including the device designs and the wettability patterning techniques, and the monomers to form the polymer shells by UV-induced polymerization give a guide to form the microcapsules for barcoding.

The microfluidic device to form the microcapsules should be fabricated with precisely controlled dimension by using soft lithography of PDMS. The method of UV-initiated polymerization of PAA can be used to pattern the wettability in the PDMS microchannel network without distortion of microchannels. The polymer precursor prepared by the monomer ETPTA with low shrinkage can be used as the middle-phase fluid to form the hard polymer shells.

Chapter 4 Hard-Shell Microcapsule Formation

4.1 Introduction

The microfluidic techniques provide precise morphology controls to form the double emulsions [74-76]. These monodispersed double emulsion droplets have high potentials to manufacture optical microelements and microsensors [95, 96]. However, the formation of fully closed transparent polymer microcapsules with the liquid cores is still a challenge. To stabilize the double emulsion droplets, surfactants are dissolved in the three phases of double emulsions in high concentrations. But the double emulsion droplets still easily break up after collection and before the solidification of the middle-phase globules to form the rigid shells. Instead of the surfactants, nanoparticles dispersed in the middle-phase polymer precursor is effective to stabilize the double emulsion droplets with a long time up to hours [97]. Unfortunately, these solidified polymer shells doped with the nanoparticles in a high volume fraction scatter and absorb light significantly, which are not suitable of the microcapsules containing the liquid cores with thermal-response materials or fluorescent dyes as optical microelements or biosensors.

In this chapter, a method is presented to form the transparent polymer microcapsules with aqueous cores by solidifying the middle-phase globules of polymer precursor immediately after generation of unstable double emulsion droplets

in a microfluidic device. The formation of these fully closed polymer microcapsules is the first step to form the stable and repeatable barcodes proposed in the Chapter 3.

4.2 Microfluidic device for hard-shell microcapsule formation

The microfluidic device for the microcapsule formation was made from soft lithography of PDMS, which provides a better dimension control than the device assembled by glass capillaries. The method of UV-initiated polymerization of PAA was used to pattern wettability in the hydrophobic PDMS microchannel network, which creates a reliable hydrophilic modification and does not change the microchannel dimensions.

4.2.1 Design of microfluidic device

The design of microchannel network of the microfluidic device is shown in Figure 4.1(a), which consists of four T-shape junctions (Figure 4.1(b)), a flow-focusing junction (Figure 4.1(c)) and a serpentine section. The four T-shape junctions are designed to generate the W/O droplets with four different contents to form the multi-component double emulsion droplets, in which the W/O droplets with maximum four different contents can be encapsulated in an ETPTA globule. The serpentine section offers a necessary length to cure the double emulsion droplets by UV light as the microcapsules. The microchannel downstream of the flow-focusing junction is higher than the other microchannels to suspend the double emulsion droplets spherically in the SDS solution flow. The dimensions of the microchannels are indicated in Figure 4.1(b, c).

Figure 4.1 (a) Design of the microchannel network. (b) Micrograph of the T-shape junction. (c) Micrograph of the flow-focusing junction. The dimensions of the microchannel network are labeled in (b) and (c).

4.2.2 Fabrication of microstructure molds

Figure 4.2 Photomasks for the mold fabrication. All black patterns are transparent areas. (a) Mask to form the alignment marks. (b) Mask to form the 1st-layer microstructure. (c) Mask to form the 2nd-layer microstructure.

Figure 4.3 Fabrication of the microstructure mold of the top cast. (a) Two alignment marks were made by DRIE. (b) Formation of 1st-layer microstructure of the mold (c) Formation of 2nd-layer microstructure of the mold (d) Development of SU-8 microstructure.

A mold with a two-and-half dimensional (2.5D) microstructure was made on a piece of 4-inch silicon wafer by photolithography of SU-8 photoresists (MicroChem, USA). Figure 4.2 shows the three photomasks to fabricate the mold. Firstly, two alignment marks were made on the silicon wafer by deep reactive-ion etching (DRIE) (Figure 4.3(a)). A layer of positive photoresist AZ9260 (MicroChem, USA) in thickness of 10 μm was spin-coated on the silicon wafer. The two alignment marks were transferred on the silicon wafer from a mask shown in Figure 4.2(a) by a mask aligner (MA6, Karl Suss, Germany). After DRIE, the layer of AZ9260 was removed by acetone and the wafer was cleaned by piranha acid washing. Then, a layer of SU-8 2015 was spin-coated on the silicon wafer in thickness of 25 μm . After soft bake of the

SU-8 layer, the pattern of the microchannel network defined by a mask shown in Figure 4.2(b) was transferred to the layer of SU-8 2015 with the two alignment marks as the first layer of the mold (Figure. 4.3(b)). Another layer of SU-8 2050 was spin coated above the first layer of SU-8 2015 in thickness of 175 μm . The pattern of the serpentine section and the microchannel carrying the outer-phase flow, as shown in Figure 4.2(c), were transferred above the first layer of the mold with the two alignment marks to form the second layer of the mold (Figure 4.3(c)). Finally, the cured SU-8 photoresist were removed by SU-8 developer (Microchem, USA) (Figure 4.3(d)). The mold was washed by isopropyl alcohol (IPA) and deionized water. The speeds for spin coating were set according to the manuals offered by the photoresist manufacturers. As shown in Figure 4.4, an acrylic (PMMA) frame with a square hollow in thickness of 5 mm was adhered on the wafer by epoxy, which defines the size and thickness of the PDMS cast peeled off from the mold.

Figure 4.4 Photo of the mold for soft lithography of PDMS.

4.2.3 Microfluidic device assembly and wettability patterning

Figure 4.5 Microfluidic device assembly and wettability patterning of the microchannel network (a) Soft lithography of PDMS (b) The cure PDMS cast was bonded covalently to a PDMS film on a PMMA substrate by oxygen plasma. (c) The microchannel network was flushed by PI solution for 10 min. (d) The microchannels were filled with AA monomer solution. (e) The serpentine section was exposed to UV light to graft PAA on the PDMS inner wall of the microchannel. The region above the other microchannels was covered by a piece of aluminum foil to avoid the UV exposure. (f) The microchannel network was flushed by ethanol for 30 min and water (pH 10) for another 30 min.

The microfluidic device was made by soft lithography of PDMS. Firstly, PDMS base was mixed with its curing agent in a ratio of 10:1 (w/w) and degassed before being poured on the SU-8 mold. After curing at 80 °C for 4 hours in an oven, the cured PDMS cast was peeled off from the mold (Figure 4.5(a)). Holes were punched as the inlets and the outlets on the PDMS cast. The cured PDMS cast was bonded covalently to a PDMS film coated on a PMMA substrate by oxygen plasma to assemble the microfluidic device (Figure 4.5(b)). The microfluidic device was placed in the oven at 80 °C for three days to form a reliable bonding and reverse the original hydrophobic surface of PDMS.

To form the W/O/W double emulsion, the serpentine section downstream of the flow-focusing junction was modified to be hydrophilic by UV-initiated graft polymerization of (PAA) [88]. A photoinitiator (PI) solution of 10 % (v/v) 2-hydroxy-2-methylpropiophenone (Daracure 1173) in acetone flushed through the microchannel network (Figure 4.5(c)). The PI molecules were embedded at the wall surfaces of the PDMS microchannels. After 10 min, the PI solution in the microchannels was removed by air and the microchannels were dried in a vacuum chamber for 10 min. Then the microchannels were filled with a monomer solution of 10 % (v/v) acrylic acid (AA) in deionized water (Figure 4.5(d)). To graft hydrophilic PAA on the walls of PDMS microchannels, the serpentine section of the microfluidic device was placed under a microscope (LV-100, Nikon, Japan) with UV exposure from a mercury lamp. The region above the other microchannels was covered by a piece of aluminum foil to avoid the UV exposure (Figure 4.5(e)). After 5 min UV exposure, the microchannel network was flushed by ethanol for 30 min and water (pH 10, adjusted by 1M NaOH solution) for another 30 min to remove the uncured AA (Figure 4.5(f)). After the

wettability patterning, the contact angle of the hydrophobic PDMS surface turned from 92° to 64° as shown in Figure 4.6. Figure 4.7 is the photo of the microfluidic device.

Figure 4.6 (a) Micrograph of the PDMS microchannel before modification. (b) Micrograph of the microchannel after grafting PAA.

Figure 4.7 Photo of the microfluidic device. The microchannels are filled with blue ink for illustration.

4.2.4 Fluid components

The inner-phase fluid was prepared by adding red or blue dye in deionized water. If the microcapsules are formed with the fully closed shells, their dyed aqueous cores can be observed in their bright-field micrographs. The middle-phase fluid was the polymer precursor of ETPTA added with 4 % (v/v) photoinitiator of Daracur 1173 and 2 % (w/v) surfactant of sorbitan monooleate (Span80). The outer-phase fluid was deionized water added with 2 % (w/v) SDS. Poly(vinyl alcohol) (PVA) was added in the outer-phase SDS solution to adjust the viscosity of the outer-phase fluid.

4.2.5 Experimental setup

Figure 4.8 shows the experimental setup for the microcapsule formation. The three fluids of the W/O/W double emulsion were injected into the microfluidic device by three syringe pumps (F100, Chemyx, USA) independently. Teflon tubings were used to connect the syringes and the microfluidic device. The UV-light (340~380 nm) spot emitted upwards from the 4X objective lens of an inverted microscope (Eclipse Ti, Nikon, Japan) covered the serpentine section to cure the ETPTA globules as the polymer shells. The using of PMMA substrate but not glass substrate to assemble the microfluidic device is due to PMMA has a higher transmittance (> 85%) for UV light than glass to cure the ETPTA globules. The microcapsules were collected in a petri dish at the outlet of the microfluidic device. The droplet generation processes at the T-shape junction and the flow-focusing junction were observed and recorded by a high speed camera (Fastcam APX-RS, Photron, Japan).

Figure 4.8 Experimental setup.

4.3 Microfluidic operation

Previous research on the microfluidic double emulsion presents a powerful capability to control the morphology of double emulsion droplets. Both of the inner-phase droplet and the middle-phase globules can be created in narrow size distributions. Furthermore, the number of inner-phase droplets in each middle-phase globule can be controlled precisely. The mechanisms of droplet generations at the T-shape junction and the flow-focusing junction are mainly governed by the capillary number ($Ca = \eta_c Q_c / \sigma S$) that describes the competition between viscous force and interfacial tension [98-102], in which η_c is the viscosity of the continuing phase fluid, Q_c is the flow rate of the continuing phase fluid, σ is the interfacial tension between the immiscible fluids, and S is the cross-section area of the microchannel. To control

the morphology of the microcapsules templated from the double emulsion droplets, I firstly investigated the W/O emulsion at the T-shape junction and the W/O/W double emulsion at the flow-focusing junction before the solidification of the ETPTA globules.

4.3.1 Effect of middle-phase flow rate on volume of W/O droplet

At the hydrophobic T-shape junction, the continuing phase is the ETPTA polymer precursor and the dispersing phase is the dyed water. As shown in Figure 4.9(a), the volumes of the W/O droplets can be controlled by the flow rate of the middle-phase ETPTA polymer precursor, Q_m , in three distinct regimes, including a squeezing regime, a dripping regime and a jetting regime. The flow rate of the inner-phase dyed water, Q_i , is set at 0.02 mL h^{-1} , constantly. When the Q_m is 0.01 mL h^{-1} , the W/O droplets are generated in the squeezing regime as shown in Figure 4.9(b1). The dyed water fills the microchannel as slugs till the pressure of the ETPTA polymer precursor at the T-shape junction is high enough to push the aqueous slugs forward. The volumes of the W/O droplets are only determined by the geometry of the microchannel and the ratio of Q_m to Q_i . Increasing Q_m to 0.03 mL h^{-1} , the W/O emulsion begins to transfer from the squeezing regime to the dripping regime, in which the shear stress at the interface of the two immiscible fluids becomes important gradually for the W/O emulsion process. The volumes of the W/O droplets are changed slightly in a relatively large range of Q_m ($0.03\sim 0.06 \text{ mL h}^{-1}$), while the intervals between them extend apparently with Q_m as shown in Figure 4.9(b2~b5). Further increasing Q_m to 0.08 mL h^{-1} , the W/O emulsion transferred from the dripping regime to the jetting regime. A jet of the dyed water downstream of the T-shape junction produces

spherical W/O droplets suspended in the ETPTA flow as shown in Figure 4.9 (b6 and b7).

Figure 4.9 (a) The volume of the W/O droplet controlled by Q_m . (b1) The W/O emulsion in the squeezing regime. (b2~b5) The W/O emission in the dripping regime. (b6~b7) The W/O emulsion in the jetting regime. The inner-phase fluid is the red-dyed water. Q_i is 0.02 mL h^{-1} , constantly.

4.3.2 Effect of interfacial tension on volume of W/O droplet

The volumes of the W/O droplets can be also be controlled effectively by a little addition of SDS in the aqueous inner-phase fluid, as shown in Figure 4.10. Using the synergistic interaction between the nonionic polymeric surfactant of Span 80 in the middle-phase polymer precursor and the anionic surfactant of SDS in the inner-phase aqueous fluid at the ‘oil-water’ interface [103], the interfacial tension between the two immiscible fluids can be reduced significantly by adding 0.01 % (w/w) SDS in the aqueous inner-phase fluid. Here, Q_i and Q_m are set at 0.02 mL h^{-1} and 0.06 mL h^{-1} , respectively. The five inner-phase fluids of the dyed water are prepared by adding SDS in concentrations from 0 to 0.04 % in an increment of 0.01 %. Firstly, the W/O droplets containing no SDS are formed in the dripping regime, as shown in Figure 4.10 (b1). Increasing the SDS concentration to 0.01 %, the W/O emulsion at the T-shape junction begins to transfer from the dripping regime to the jetting regime. A short jet of the dyed water emerges downstream of the T-shape junction as shown in Figure 4.10 (b2). The jet of the dyed water produces smaller W/O droplet by adding more SDS as shown in Figure 4.10 (b3). Further increasing the SDS concentration, the jet of the dyed water goes downstream and generates much smaller W/O droplets, as shown in Figure 4.10(b4). However, 0.04 % SDS in the inner-phase fluid causes the failure to form the W/O emulsion. The jet of inner-phase fluid goes gradually to the flow-focusing junction and forms a parallel flow of the dyed water with the ETPTA polymer precursor as shown in Figure 4.10(c). The addition of SDS in the aqueous inner-phase fluid not only reduces the interfacial tension between the two immiscible fluids but also decreases the contact angle of the aqueous inner-phase fluid on the

hydrophobic wall of PDMS microchannel. The dyed water added with 0.04 % SDS is no longer a dispersing phase but one more continuing phase.

Figure 4.10 (a) The volume of W/O droplet controlled by the concentration of SDS in the inner-phase dyed water. Q_i is 0.02 mL h^{-1} and Q_m is 0.06 mL h^{-1} . The SDS concentrations of the red-dyed water increase from 0 to 0.04 % in an increment of 0.01% in (b1~c). (b1) The W/O emulsion in the dripping regime. (b2) The W/O emulsion starts to transfer from the dripping regime to the jetting regime. (b3, b4) The W/O emulsions in the jetting regime. (c) A parallel flow of the red-dyed water with the ETPTA polymer precursor.

For the W/O/W double emulsion droplets, the size ratio of the aqueous cores to the ETPTA globule, d_{inner}/d_{outer} , can be represented by $(\frac{Q_i}{Q_i + Q_m})^{-1/3}$ [94]. If only Q_m is used to control d_{inner}/d_{outer} , a smaller aqueous core must be encapsulated in a relatively larger ETPTA globule. Combining the two approaches of altering Q_m and the adjusting the SDS concentration in the aqueous inner-phase fluid, d_{inner}/d_{outer} of the double emulsion droplets can be adjusted with a high flexibility.

4.3.3 Controlling number of aqueous cores in each ETPTA globule

At the hydrophilic flow-focusing junction, the ETPTA polymer precursor turns to be the dispersing phase and the continuing phase is the outer-phase SDS solution. As shown in Figure 4.11, the double emulsion droplets can be formed with multiple aqueous cores. The generation frequency of the double emulsion droplets can be controlled by the flow rates of the outer-phase fluid, Q_o . The number of the aqueous cores in each ETPTA globule is the ratio of the W/O droplet generation frequency at the T-shape junction to the double emulsion generation frequency at the flow-focusing junction. By adjusting Q_o to obtain the ratio of generation frequencies in an integer, the double emulsion droplets are generated with an integer number of aqueous cores as shown in Figure 4.11 (b1, b3~b5). If the ratio of generation frequencies is not an integer, as shown in Figure 4.11(b2), a theoretical calculation on the number distribution of aqueous cores in each ETPTA globule is given as follows. Supposing the ratio of generation frequencies is N ($1 < N < 2$), $(2-N) \times 100\%$ microcapsules contain one aqueous core, and $(N-1) \times 100\%$ microcapsules contain two aqueous cores.

Figure 4.11 Number of the aqueous core in individual ETPTA globules controlled by Q_o . The outer-phase fluid of 2 % SDS solution is added with 1 % PVA. Q_i is 0.02 mL h⁻¹ and Q_m is 0.08 mL h⁻¹. Q_o are 3.9 mL h⁻¹, 3.4 mL h⁻¹, 2.8 mL h⁻¹, 2.2 mL h⁻¹ and 1.8 mL h⁻¹ in (b1~b5), respectively.

4.3.4 Effect of outer-phase viscosity on double emulsion generation

As shown in Figure 4.12, the interval between the double emulsion droplets in the serpentine can be shorted by adding PVA in the aqueous outer-phase fluid. It should be noted that the double emulsion droplet generation frequency is the same in Figure 4.12 (a and b). The addition of the PVA increases the viscosity of the aqueous outer-phase fluid and shear stress at the interface between the middle-phase ETPTA and outer-phase SDS solution. The double emulsion droplets can be generated with lower Q_o by adding higher concentration of PVA. Compared with the double emulsion droplets in Figure 4.13(a and b), the d_{inner}/d_{outer} of the double emulsion droplets, which is only determined Q_i and Q_m , are the same.

Figure 4.12 (a) Double emulsion formation by the outer-phase fluid added with 2% SDS and 1% PVA. Q_o is 4.5 mL h^{-1} . (b) Double emulsion formation by the outer-phase fluid added with 2% SDS and 2% PVA. Q_o is 3 mL h^{-1} . Q_i and Q_m are 0.02 mL h^{-1} and 0.08 mL h^{-1} , respectively.

4.4 Formation of fully closed microcapsules

To lock the Qdot-loaded aqueous cores inside the microcapsules without leakage, the microcapsules should be formed with fully closed polymer shells. The morphology of the microcapsules is attributed to the size ratio of the cores to the shells (core-to-shell size ratio) and the relative position of the cores in the shells (core-in-shell position). Both of them are investigated to form the fully closed microcapsules.

4.4.1 Solidification of ETPTA globules in the serpentine section

Figure 4.13 (a) Bright-field micrograph of the double emulsion droplets queuing in the serpentine section. (b) The double emulsion droplets are cured to form the microcapsules under UV exposure in the serpentine section.

The double emulsion droplets queue in a line in the serpentine section downstream of the flow-focusing junction as shown in Figure 4.13(a). Under UV exposure, the ETPTA globules are cured as the solid polymer shells. Due the relatively low shrinkage of ETPTA (~11 %) [91], no obvious size difference between the microcapsules and their double emulsion templates was observed in Figure 4.13(b).

The core-to-shell size ratio can be represented by d_{inner}/d_{outer} . The microcapsules collected in the petri dish were completely cured in a UV crosslinker (XL-1000, Spectrolinker, USA) with UV exposure for 5 min. Due to the serpentine section made with a square cross section, the microcapsules templated from the double emulsion droplets have spherical shapes, as shown in Figure 4.14. The microcapsules in Figure 4.14 are templated from the double emulsion droplets in Figure 4.11.

Figure 4.14 Microcapsules with different numbers of aqueous cores that are templated from the double emulsion droplets in Figure 4.11.

4.4.2 Monodispersity

To investigate the monodispersity of microcapsules, 20 single-core microcapsules were chosen randomly from Figure 4.14 as measurement samples. As shown in Figure 4.15, both of the sizes of cores and shells distribute within $\sim 5 \mu\text{m}$. The average size of cores and shells are $27.52 \mu\text{m}$ and $51.84 \mu\text{m}$, respectively. The coefficients of variation (CV) of the core sizes and shell sizes are 4.1 % and 3.3 %, respectively. The microcapsules made by the microfluidic method have narrow size distributions for

both the cores and shells. The colorimetric barcodes formed in these monodispersed microcapsules can be encoded with external references to create more barcodes than the polydispersed microspheres.

Figure 4.15 Size distributions of cores and shells. 20 single-core microcapsules in Figure 4.14 were chosen randomly as measurement samples. The coefficients of variation for core sizes and shell sizes are 4.1 % and 3.3 %, respectively.

4.4.3 Effect of core-to-shell size ratio on microcapsule formation

Four batches of microcapsules were made with increasing $d_{\text{inner}}/d_{\text{outer}}$ to investigate the effect of $d_{\text{inner}}/d_{\text{outer}}$ on the formation of the fully closed polymer shells. As shown in Figure 4.16 (a), no determined relation between $d_{\text{inner}}/d_{\text{outer}}$ and the formation of fully closed shells was observed; $d_{\text{inner}}/d_{\text{outer}}$ is not a major factor on the formation of the fully closed shells.

Figure 4.16 (a) percentages of the fully close shells templated from the double emulsion droplets in increasing $d_{\text{inner}}/d_{\text{outer}}$. (b1~b4) Micrographs of the microcapsules made with increasing $d_{\text{inner}}/d_{\text{outer}}$. The broken polymer shells leak their dyed aqueous cores. All scaler bars are 50 μm in length.

Figure 4.17 SEM micrographs of the broken microcapsules. (a) The cracked microcapsule with irregular surface damage. (b) A part of a batch of the microcapsules made with pores. The microcapsules were templated from the double emulsion droplets generated in the dripping regime. (c) The microcapsule with a circular pore due to the inhabitation of oxygen in the aqueous fluids to the UV-induced polymerization. (d) The edge of the pore in (c). The thinnest shell is about 0.6 μm in thickness.

If the double emulsion droplets are generated with a large $d_{\text{inner}}/d_{\text{outer}}$, the cured thin polymer shells crack due to the shrinkage of polymerization [94], as shown in Figure 4.16(b4) and Figure 4.17(a). Theoretically, the higher $d_{\text{inner}}/d_{\text{outer}}$ results the higher possibility of the breakup of microcapsules. Previous research indicates that the relative position of a droplet to a second immiscible droplet in a third immiscible fluid in equilibrium state is determined by the interfacial tensions between the them [104] (see Appendix A). Here, the relative positions of the aqueous cores in the ETPTA globules maintain constantly after the double emulsion generation because of the

relatively high viscosity of ETPTA ($\sim 90 \text{ mPa}\cdot\text{s}$). If the aqueous cores are at the periphery of ETPTA globules, the thin layer of ETPTA cannot be cured due to the inhibition effect of oxygen in the aqueous fluids to the UV-induced polymerization [50]. Pores are left at the surfaces of the cured polymer shells as shown in Figure 4.16(b2) and Figure 4.17(c). The thinnest shell formed is about $0.6 \mu\text{m}$ as indicated in Figure 4.17(d).

4.4.4 Controlling core-in-shell position

I found that the core-in-shell position plays a significant role in the formation of fully closed shells, which can be controlled by the mechanism of double emulsion generation. As shown in Figure 4.18(a), the double emulsion is formed in the dripping regime when Q_m and Q_o are 0.06 mL h^{-1} and 4.5 mL h^{-1} , respectively. The W/O droplets come to the flow-focusing junction and slow down at the end of the ETPTA flow region. Each ETPTA globule develops gradually and detaches from the ETPTA stream to form a complete double emulsion droplet. Due to the velocity difference between the emerging ETPTA globule and the flow of the outer-phase SDS solution, the shear stress at their interface causes an internal circulating flow inside the ETPTA globule, which forces the aqueous core towards the periphery of the ETPTA globule. The ETPTA globules with the off-centered aqueous cores are cured as the polymer shells with the pores. The unstable double emulsion droplets even break up immediately at the flow-focusing junction, if the aqueous core is located extremely close to the interface between the ETPTA and the outer-phase SDS solution. In a word, the formation of the fully closed shell is a random incident if the microcapsules

Figure 4.18 Image sequences of the double emulsion generation. (a) Double emulsion in the dripping regime. Q_i is 0.02 mL h^{-1} , Q_m is 0.06 mL h^{-1} , and Q_o is 4.5 mL h^{-1} . (b) Double emulsion in the jetting regime. Q_i is 0.02 mL h^{-1} , Q_m is 0.08 mL h^{-1} , Q_o is 6.2 mL h^{-1} . The time interval between images is 1.6 ms .

are templated from the double emulsion droplets generated in the dripping regime. Further increasing Q_m and Q_o to 0.08 mL h^{-1} and 6.2 mL h^{-1} , respectively, the double emulsion is formed in the jetting regime as shown in Figure 4.18(b). The double emulsion in the jetting regime generates the droplet emulsion droplets due to the Rayleigh-Plateau hydrodynamic instability [79] of the ETPTA jet in the flow of SDS solution. A jet of ETPTA stream breaks up into segments containing the inner droplets individually. The segments of the ETPTA jet in the same velocity of the flow of SDS solution confine the W/O droplets centrally as cores and turn to the spherical ETPTA

globules by reducing their surface areas. The aqueous cores are fully encapsulated by the polymer shells without leakage of the dyed aqueous cores as shown in Figure 4.19.

To verify the analysis that the core-in-shell position can be controlled by the mechanism of double emulsion generation, two batches of the microcapsules in Figure 4.20 were made with the same d_{inner}/d_{outer} as the fully closed microcapsules in Figure 4.16 (b1). PVA was dissolved in the outer-phase fluid of 2 % SDS solution to increase the outer-phase viscosity and reduce Q_o to form the double emulsion in dripping regime. The percentage of broken microcapsules increases with the PVA concentration added in the outer-phase SDS solution; the higher PVA concentration the more microcapsules are leaked. As shown in Figure 4.20 (b), the microcapsules templated from the double emulsion droplets in Figure 4.12 (b) all have deep pores on their surfaces like concaves, which have a potential to be used as microlenses.

Figure 4.19 Micrograph of the fully closed microcapsules. Two batches of the microcapsules containing the red and blue aqueous cores were templated from the double emulsion droplets generated in the jetting regime.

Figure 4.20 (a) Microcapsules templated from the double emulsion droplets generated in the dripping regime in Figure 4.12(a). The outer-phase fluid is 2 % (w/w) SDS solution added with 1 % (w/w) PVA. (b) Microcapsules templated from the double emulsion droplets generated in the dripping regime in Figure 4.12 (b). The outer-phase fluid is 2 % (w/w) SDS solution added with 2 % (w/w) PVA. The scale bars are 50 μm in length.

4.5 Failure cases

Two failure cases of the microcapsule formation using the microfluidic device are presented in Figure 4.21. Figure 4.21 (a) shows the blocking of serpentine section. Under UV exposure, the ETPTA globules in liquid state are solidified as the rigid polymer shells. If the double emulsion droplets are generated as an intensive stream in the slow flow of SDS solution ($Q_o < 1.5 \text{ mL h}^{-1}$), the partially cured polymer shells may crowd together in the serpentine section till big enough to block the microchannel. What is worse, the ETPTA globules following them will fill the spare room among the crowded polymer shells and cured by UV light to damage the microfluidic device beyond retrieve. Figure 4.21 (b) shows the overflow of the middle-phase ETPTA at the flow-focusing junction which fails to form controllable double emulsions. When the

double emulsion generation is conducted with high Q_m ($>0.09 \text{ mL h}^{-1}$) and Q_o , the middle-phase ETPTA flow may go over the flow-focusing junction without droplet breakup. Although the double emulsion is still formed, the double emulsion droplets are generated without morphology control. The problem is due to the vertically unsymmetrical 2.5D geometry at the flow-focusing junction, which causes the ETPTA flow is pressed by the flow of SDS solution at the bottom of microchannel.

Figure 4.21 (a) Blocking of the serpentine section (b) Overflow of the middle-phase ETPTA at the flow-focusing junction.

4.6 Summary

In this chapter, a method is presented to form the transparent polymer microcapsules by UV-induced polymerization of the ETPTA globules of unstable W/O/W double emulsion droplets in a microfluidic device. The microfluidic device was made by soft lithography of PDMS with reliable wettability patterning to form the microcapsules. As a complementary approach to control the size of aqueous cores, a little addition of SDS in the aqueous inner-phase fluid can also control the size of

aqueous cores effectively in the dripping and jetting regimes. Particularly, I found an effective approach was used to control the core-shell positions by using different mechanisms of double emulsion generation. The double emulsion droplets generated in the jetting regime can confine the aqueous cores centrally in the polymer precursor globules and produce the fully closed polymer shells. In contrast, the internal circulating flow in the emerging double emulsion droplets generated in the dripping regime forces the aqueous cores off centered and produces pores on the resultant microcapsules. The fully closed microcapsules with the aqueous cores made in a small size of $\sim 30\text{ }\mu\text{m}$ will be a perfect vehicle to carry the Qdot barcodes as proposed in Chapter 3.

Chapter 5 Colorimetric Barcodes

5.1 Introduction

The aqueous core of transparent polymer microcapsule is an excellent choice as the vehicle to form the colorimetric Qdot barcode. With the protection of the rigid polymer shells, the Qdots loaded in the aqueous cores are separated from the ambient fluids with stable fluorescent emissions. No matter before or after the formation of the microcapsules, the Qdot suspension medium remains the same as the aqueous solution without any phase change. The strategy to form the colorimetric barcodes in the microcapsules can avoid the unpredictable changes of fluorescent profiles caused by FRET and the shrinkage of Qdot-doped polymer microparticles. In this chapter, detailed investigations are made to form the colorimetric Qdot barcodes in the single-component microcapsules.

5.2 Experimental

5.2.1 Qdot concentration measurement and calibration

Some previous works on the formation of Qdot-encoded microparticles used mass weight to scale the amount of Qdots [105]. Since the highly bright Qdots are usually used in scale of ~1 mg for preparation of the Qdot-mixed polymer precursor, using a

regular digital balance cannot make accurate measurements and calibrations of the amounts of Qdots. Here, molar concentration (M: molar L⁻¹) is used to scale the amounts of Qdots. The inner-phase fluid of the W/O/W double emulsion was prepared by dispersing carboxyl-group modified CdTe Qdots (PlasmaChem, Germany) in deionized water to form the barcodes. The Qdot emission peaks are at 500 nm (Qdot500), 550 nm (Qdot550), 600 nm (Qdot600) and 650 nm (Qdot650), respectively. The molar concentrations of the Qdots were measured and calibrated based on Lambert-Beer's law with the absorbances and extinction coefficients of the Qdots at their first excitons [106]. The first excitonic absorption peaks of the Qdots, ω_{1st} , and absorbances at the first exciton absorption peaks, A , were measured by a UV-VIS spectrophotometer (UV-2450, SHIMADZU, Japan). According to ω_{1st} , measured, the size of CdTe Qdots, D , can be calculated by an empirical equation [106].

$$D = (9.8127 \times 10^{-7})\omega_{1st}^3 - (1.7147 \times 10^{-3})\omega_{1st}^2 + (1.0064)\omega_{1st} - 194.84 \quad (5.1)$$

It should be noted that Equation 5.1 is invalid if ω_{1st} is out of the range from 550 nm to 750 nm, and this empirical equation is just valid for CdTe Qdots. The CdTe Qdot's extinction coefficient per mole (L mol⁻¹ cm⁻¹), ϵ , is also calculated by an empirical equation.

$$\epsilon = 10043 D^{2.12} \quad (5.2)$$

The first excitonic absorption peaks, sizes and extinction coefficients of the Qdots are listed in Table 5.1.

Table 5.1 First absorption peaks, sizes and extinction coefficients of the CdTe Qdots.

Name	Emission Peaks (nm)	ω_{1st}	Size (nm)	$\epsilon (\omega_{1st}, \text{M}^{-1} \text{cm}^{-1})$
Qdot500	500±5	459	2.0	37575
Qdot550	550±5	511	2.5	63410
Qdot600	600±5	567	3.4	154166
Qdot650	650±5	596	3.6	154383

The molar concentrations of the Qdots emitting at different peaks are measured by the according to Lambert-Beer's Law

$$T = 10^{-\epsilon lc} \quad (5.3)$$

T is the transmission of light through a liquid. c is the molar concentration of the Qdots. And l is the path length (cm) of the radiation beam used for recording the absorption spectrum. The transmission is expressed in term of absorbance.

$$A = -\log_{10} \left(\frac{I}{I_0} \right) = -\log_{10}(T) \quad (5.4)$$

I_0 and I are the intensities of incident light and transmitted light, respectively.

$$A = \epsilon lc \quad (5.5)$$

According to the Qdot extinction coefficients in Table 5.1, the molar concentrations of Qdots dispersed in deionized water were calculated. The molar concentrations of the Qdots were calibrated to 20 μM or 100 μM for the storage and preparation of the Qdot mixtures to form the colorimetric barcodes.

5.2.2 Preparation of colorimetrically encoded microcapsules

The inner-phase fluids were prepared by mixing the Qdot solutions with calibrated molar concentrations. 0.02 % (w/w) SDS was added in the Qdot solutions. The middle-phase fluid of the ETPTA polymer precursor and the outer-phase fluid of SDS solution were prepared as described in Chapter 4. The microcapsules loaded with Qdot aqueous cores were templated from the double emulsion droplets generated in the jetting regime.

5.2.3 Image capturing

The grey-scale fluorescent micrographs at the Qdot emission peaks were captured by the monochromatic CCD camera equipped to the microscope with narrow bandpass optical filters (Thorlabs, USA). UV light (340~380 nm) from the mercury lamp was used to excite the Qdots. The camera exposure time was set at 500 ms. The fluorescence intensities of Qdot-loaded aqueous cores were measured according to the grey-scale fluorescent micrographs. The bright-field micrographs of the microcapsules were also captured by the monochromatic camera.

5.2.4 Spectrum capturing

The emission spectra of Qdot solutions were recorded by a fluorescence reader (Infinite M200, TECAN, USA). The fluorescent spectra of Qdot-encoded microcapsules were captured by a laser scanning confocal microscope (LSM710, Carl Zeiss, Germany) with its hyperspectral imaging function under UV laser excitation (405 nm). The wavelength interval of spectral imaging was set at 3 nm.

5.3 Optical properties

5.3.1 Transmittance of cured ETPTA polymer

The cured ETPTA polymer should be transparent to UV light to excite the Qdots in the aqueous cores and the emissions from the Qdots for barcode identification. A piece of ETPTA thin film was prepared by curing the ETPTA polymer precursor between two glass substrates in thickness of 200 μm . The transmittances of the cured ETPTA

Figure 5.1 Transmission spectrum of the cured ETPTA film.

film were measured by the UV-VIS spectrometer in range from 300 nm to 800 nm. The transmission spectrum of the cured ETPTA polymer film is shown in Figure 5.1. The transmittance of ETPTA polymer is as high as 92.5 % from 800 nm to 400 nm and decreases sharply from 375 nm (transmittance: 80%) to 300 nm. The UV light and visible emissions from the Qdots can go through the ETPTA polymer shells without any significant distortion to the optical signals of barcodes.

5.3.2 Comparison of Qdot emissions and ETPTA autofluorescence

Figure 5.2 Comparisons of the Qdot emissions and the cured ETPTA autofluorescence. (a) Fluorescent spectra of the Qdot500 and cured ETPTA film. (b) Fluorescent spectra of the Qdot550 and cured ETPTA film (c) Fluorescent spectra of the Qdot600 and cured ETPTA film (d) Fluorescent spectra of the cured ETPTA film. The concentrations of Qdots are label beside their respective emission spectra. The cured ETPTA film is 200 μm in thickness.

The autofluorescence of the ETPTA polymer should be low and do not interfere to the optical signals of colorimetric barcodes. The fluorescent spectra of the Qdot500, Qdot550 and Qdot600 were recorded under UV excitation at 365 nm as shown in Figure 5.2. The fluorescent spectrum of the cured ETPTA thin film was also recorded as a reference. The concentrations of Qdot500 decrease from 10 μM to 0.3125 μM by half for six serial dilutions. The concentrations of Qdot550 and Qdot600 decrease from 1 μM to 0.03125 μM by half for six serial dilutions. As shown in Figure 5.2(a~d), the autofluorescence from the ETPTA polymer is quite low, 0.625 μM Qdot500, 0.125 μM Qdot550 and 0.125 μM Qdot600 can create bright emissions to differentiate from the ETPTA polymer, respectively.

5.3.3 Aqueous core loaded with single-color Qdots

Figure 5.3(a) shows a single-core microcapsule in size of 32 μm . The aqueous core of this microcapsule was loaded with the Qdot600 in concentration of 0.5 μM . As shown in Figure 5.3(b), the fluorescent micrograph of this microcapsule shows a highly contrasted bright spot under UV illumination. The fluorescence intensities along line A-A' across the microcapsule follows an intense parabola profile with a maximum at the center of the Qdot-loaded aqueous core. The cured polymer shell has a low autofluorescence. Taking the fluorescent micrograph of the aqueous core without Qdot (Figure 5.3(c)) as a reference, the autofluorescence of the cured polymer shell is lower than 4 % of the fluorescence intensity of the Qdot-loaded aqueous core in Figure 5.3(b).

Figure 5.3 (a) Bright-field micrograph of a microcapsule with an aqueous core containing Qdot600 in concentration of $0.5 \mu\text{M}$. (b) Fluorescent micrograph of the microcapsule in (a). (c) Fluorescent micrograph of a microcapsule with an aqueous core containing no Qdot. The scale bars in (a), (b) and (c) are $10 \mu\text{m}$ in length. (d) The emission intensities across the microcapsule along line AA' shown in (b).

Figure 5.4 (a) The normalized emission spectra of the aqueous cores containing the Qdot550, Qdot600 and Qdot650 (solid curves) and the Qdot aqueous solutions (dashed curves), respectively. (b) The emission intensities of the aqueous cores loaded with the Qdot600 in a function of Qdot concentration. The fluorescence intensity of the aqueous core is linear to the Qdot concentration.

After encapsulation, the Qdots in the aqueous cores maintain the same emission peaks (Figure 5.4(a)) and a linear relation between the emission intensity and concentration as those from the same Qdots before encapsulation (Figure 5.4(b)). The formation of the microcapsules by UV-induced polymerization avoids the long-time heating required in doping the Qdots into the solid-phase polymer microparticles [68-70], which I found that the heating at 80°C for two hours decreases the Qdot emission intensities significantly. Although the UV-induced polymerization of the ETPTA polymer precursor is an exothermal reaction, the high thermal capacity of the aqueous outer-phase fluid can cool down the microcapsules immediately and prevents the Qdots in the aqueous cores from a significant temperature change.

5.3.4 Aqueous core loaded with multi-color Qdots

It should be noted that the possibility of distance-depended FRET between the Qdots emitting at different wavelengths increases with the total concentration of the Qdots in the aqueous cores. Five batches of microcapsules were made to investigate this issue, the aqueous cores of which contained five Qdot mixtures prepared by mixing the Qdot550 and Qdot600. The Qdot550 concentrations increased from 1 μM to 5 μM in an increment of 1 μM for the five Qdot mixtures. The Qdot600 concentrations were 0.5 μM constantly. The fluorescence intensities of the aqueous cores at 550 nm and 600 nm were recorded as a bar chart in Figure 5.5. The linear model fits the fluorescence intensities of the aqueous cores at 550 nm and 600 nm with adjusted coefficients of determination (adjusted R^2) of 0.98 and 0.99, respectively, which indicates that the fluorescence intensities of the aqueous cores have a linear relation with the Qdot concentrations in the aqueous cores. The slightly linear

Figure 5.5 Bar chart of the fluorescence intensities of the aqueous cores containing five different Qdot mixtures which are prepared by mixing the Qdot550 and Qdot600. The concentrations of Qdot550 increase from 1 μM to 5 μM with an increment of 1 μM for the five Qdot mixtures. The concentrations of Qdot600 are 0.5 μM constantly. The dash line and solid line are the linear fitting lines of the fluorescence intensities of liquid cores at 550 nm and 600 nm, respectively. No interference of photoluminescence emissions between the Qdots with these two different emission peaks is observed when their total concentration is less than 5.5 μM .

increments of the fluorescence intensities of the aqueous cores at 600 nm were attributed to the increasing concentrations of the Qdot550 which has the spectral overlapping with the Qdot600. No obvious interference of photoluminescence emission between the Qdots with different two emission peaks was observed when the total Qdot concentration was less than 5.5 μM . The fluorescence emissions used to identify the barcodes have a one-to-one correspondent relation to the compositions of Qdot mixtures in the aqueous cores. This allows the barcoding to be highly simplified

as a multiplexing process for the Qdot emission peaks and concentrations in the aqueous cores. The fluorescence profiles of the Qdot-loaded aqueous cores can be predefined by the compositions of the Qdot mixtures.

5.3.5 Stability of Qdot barcodes to ambient fluids

Protected by the rigid polymer shell, the emission intensities of the Qdot-loaded aqueous cores are constant regardless of the ambient fluids. The microcapsules with aqueous cores containing 0.5 μM Qdot600 were dispersed in deionized water, ethanol, phosphate buffered saline (PBS buffer, pH 7.0) and 0.1 M 2-(morpholino) ethanesulfonic acid buffer (MSE buffer, pH 4.7) and stored in a fridge at 4 °C overnight, respectively. The emission intensities of these Qdot-loaded aqueous cores were the same as each other. The Qdot barcodes formed within the microcapsules can keep the stable emission profiles for identifying the biomolecules on the encoded microcapsules in different bioassay buffers.

5.3.6 Thermal stability of Qdot barcodes

Usually, the immunoassays are conducted at the room temperature (~ 20 °C) at which the Qdot barcodes maintain stable emission profiles. To perform the biological reactions optimally, the reaction temperature may be increased above the room temperature. To investigate the thermal stability of Qdot barcodes, the microcapsules loaded with 0.5 μM Qdot600 were suspended in the deionized water at 40 °C and 80 °C on two hotplates, respectively. Their emission intensities of Qdot-loaded aqueous cores were recorded in every 5 min. As indicated in Figure 5.6, the emission intensity of Qdot600 in the aqueous core kept constant at 40 °C. While, the emission

intensities of Qdot600 in the aqueous core decreased gradually at 80 °C. The CdTe Qdots have a low thermal stability at 80 °C. The decrease of Qdot emission intensity is irreversible. To maintain the constant emission profiles of the Qdot barcodes formed in the microcapsules, the reaction temperature to conduct the multiplexed assays should not be higher than 40 °C.

Figure 5.6 Thermal stability of Qdot barcodes. The emission intensity of Qdot600 in the aqueous core kept constant at 40 °C. The emission intensity of Qdot600 in the aqueous core decreased gradually at 80 °C.

5.4 Colorimetric barcode identification

Previous methods to identify the colorimetric barcodes use spectrometers [35] or flow cytometers [107] to record the fluorescent spectra or intensities of the Qdot-incorporated microparticles. I propose a simple image-based decoding method for the aqueous-core barcodes by capturing the fluorescent micrographs of the microcapsules at the respective Qdot emission peaks.

5.4.1 Decoding algorithm

Since the fluorescent emission of the Qdot-loaded aqueous core at certain wavelength is attributed to the Qdots emitting at this wavelength, the fluorescence intensity of a Qdot mixture prepared by the Qdots emitting at 550 nm, 600 nm and 650 nm respectively, $F(\omega)$, at certain Qdot emission peak, ω , can be expressed as

$$F(\omega) = a \cdot f_{550}(\omega) + b \cdot f_{600}(\omega) + c \cdot f_{650}(\omega) \quad (5.6)$$

where $f_{550}(\omega)$, $f_{600}(\omega)$ and $f_{650}(\omega)$ are the fluorescence intensities of the aqueous cores at certain emission peak, ω , containing the Qdot550, Qdot600 and Qdot650, respectively, in concentrations of 1 μ M. a , b and c are positive real numbers representing the relative composition of this Qdot mixture. The fluorescence intensities of these microcapsules containing the single-color Qdots at the three Qdot emission peaks are used to compose an *intensity reference matrix*, D .

$$D = \begin{bmatrix} f_{550}(550) & f_{550}(600) & f_{550}(650) \\ f_{600}(550) & f_{600}(600) & f_{600}(650) \\ f_{650}(550) & f_{650}(600) & f_{650}(650) \end{bmatrix} \quad (5.7)$$

The fluorescence intensities of the aqueous core containing the Qdot mixture at these three Qdot emission peaks can be expressed by a *barcode emission matrix*, F .

$$F = [F(550) \quad F(600) \quad F(650)] \quad (5.8)$$

where $F(550)$, $F(600)$ and $F(650)$ are obtained from Equation 5.6. The matrix $[a, b, c]$ is the relative composition of the Qdot mixture and used as the barcode of the microcapsule. It can be calculated after acquiring F and D are from the fluorescent micrographs of the Qdot-encoded microcapsules at the Qdot emission peaks.

$$[a, b, c] = F \cdot D^{-1} \quad (5.9)$$

5.4.2 Case study

To demonstrate the image-based decoding method, a batch of Qdot-barcoded microcapsules was made, in which the Qdot mixture in the aqueous cores consisted of the Qdot550, Qdot600 and Qdot650 in concentration of 0.3 μM , 0.2 μM and 0.1 μM , respectively. Another three batches of microcapsules containing the single-color Qdots emitting at these three wavelengths in concentration of 1 μM were also made as the references of fluorescence intensities. If the previous spectrum-based decoding method is used to identify this barcode, the fluorescent spectrum of this Qdot-barcoded microcapsule is recorded as shown in Figure 5.7(a). The fluorescent spectrum of the Qdot-barcoded microcapsule was recorded by the hyperspectral imaging function of the laser confocal microscope in a time of 7~8 min. For the image-based decoding method, the fluorescence intensities of the aqueous cores loaded with the single-color Qdots and the Qdot mixture at the three Qdot emission peaks were recorded from their fluorescent micrographs as shown in Figure 5.7 (b). According to the composition of this Qdot mixture, $[a, b, c]$ should be $[0.3 \ 0.2 \ 0.1]$ which is an ideal barcode. Based on the fluorescence intensities recorded in Figure 5.7 (b) to compose F and D ,

respectively, the calculated barcode is [0.31 0.18 0.14]. The difference between the ideal barcode and the calculated barcode come from the Qdot emission intensity variations between the Qdots with different emission peaks, the error in formulating the Qdot mixture, and the instrumental error of the decoding system [35]. The absolute error of barcode is [0.03 0.1 0.4], respectively. Particularly, the relative error for Qdot650 is unacceptable up to 40%.

Figure 5.7 Case study 1. (a) Fluorescent spectrum of the encoded microcapsule with the liquid core containing a Qdot mixture prepared by $0.3 \mu\text{M}$ Qdot550, $0.2 \mu\text{M}$ Qdot600 and $0.1 \mu\text{M}$ Qdot650. (b) Bar chart of fluorescent intensity of the encoded microcapsule and the intensity-reference microcapsules. The intensity references contain Qdot550, Qdot600 and Qdot650 in concentration of $1 \mu\text{M}$, respectively.

Figure 5.8 Case study 2. (a) Fluorescent spectrum of the encoded microcapsule with the liquid core containing a Qdot mixture prepared by 5 μM Qdot550, 0.5 μM Qdot600 and 0.3 μM Qdot650. (b) Bar chart of fluorescent intensity of the encoded microcapsule and the intensity-reference microcapsules. The intensity references contain Qdot550, Qdot600 and Qdot650 in concentration of 5 μM , 0.1 μM and 0.1 μM , respectively.

To reduce the adverse effects of spectral overlapping and Qdot emission intensity variations on decoding accuracy, another three intensity-reference microcapsules were made by 5 μM Qdot550, 0.1 μM Qdot600 and 0.1 μM Qdot650, respectively, whose emission intensity variations were less than an order of magnitude. Another batch of

Qdot-encoded microcapsules was made, the liquid cores of which contain Qdot550, Qdot600 and Qdot650 in concentrations of 5 μM , 0.5 μM and 0.3 μM , respectively. According to the compositions of this Qdot mixture and the intensity references, the ideal result of barcode identification should be [1 5 3]. The fluorescent spectrum of the Qdot-encoded microcapsules is shown in Figure 5.8(a). The emission intensities of the Qdot-encoded microcapsule and the intensity-reference microcapsules at the Qdot emission peaks were also recorded as a bar chart in Figure 5.8(b). The calculated was [1.02 4.86 2.94]. The readout error was [0.02 0.14 0.06]. The relative errors for Qdot550, Qdot600 and Qdot650 are 2%, 2.8% and 2%, respectively. The readout errors of barcodes can be reduced by using the intensity references with similar emission intensities but not the same Qdot concentrations.

5.4.3 Coding capacity

Han *et al.* gave a simple estimation on the coding capacity of Qdot barcodes, $m^n - 1$, in which n is the number of colors and m is the number of intensity levels [35]. Since the colors and intensity levels of the colorimetric barcodes formed in the microcapsules are determined directly by the Qdot emission peaks and concentrations in the liquid cores, respectively. n can be represented by the number of Qdot emission peaks and m can be represented by the number of Qdot concentrations gradients. For the monochromatic CCD camera used, 0.1 μM Qdot550, 0.05 μM Qdot600 and 0.02 μM Qdot650 can create discernible intensity gradients, respectively. If the maximum total Qdot concentration without FRET is set at 5.5 μM , the maximum number of concentration gradients is 32 ($0.1m + 0.05m + 0.02m \leq 5.5$, $m=32$) and 32767 ($32^3 - 1 = 32767$) barcodes can be created by mixing Qdot550, Qdot600 and Qdot650,

theoretically. However, the actual number of discernable intensity gradients and the actual coding capacity are substantial less than the above estimations. Taking above the case study 2 for example, the relative concentration gradients for Qdot550, Qdot600 and Qdot650 are 0.02 ($0.1/5=0.02$), 0.5 ($0.05/0.1=0.5$) and 0.2 ($0.02/0.1=0.2$), respectively. The calculation result is recognized mistakenly as the ideal barcode of [1.02 5 3] but not [1 5 3]. The Qdot550 concentration gradient should be increased more than 0.2 μM to avoid the mistaken identification. Compared with using more intensity levels, the approach of using more colors is much more effective to promote the coding capacity practically. Suppose Qdot500 is also used for barcoding and the maximum number of discernable intensity levels is only 15. 50624 (15^4-1) barcodes can be created. Although the number of intensity levels is only the half of previous estimation, using one more color can promote the coding capacity significantly.

5.4.4 Graphical information for barcoding

Due to the variations of Qdot photoluminescence intensities and instrumental errors, the actual number of discernable colorimetric barcodes is substantial less than the theoretical estimation. Here, the image-based decoding method shows its advantage to enhance the coding capacity. Because the microcapsules can be made with the aqueous cores in different sizes and multiple aqueous cores, as shown in Figure 5.9, the graphical information of the core size and number can also be used to differentiate the microcapsules from each other. Besides the fluorescence profiles of the Qdot-loaded aqueous cores, the fluorescent image of the microcapsules can also provide the graphical information of the sizes and numbers of the Qdot-loaded aqueous cores in individual microcapsules, as shown in Figure 5.10. The graphical information of the

aqueous cores offers another dimension to form the barcodes, which can increase the coding capacity at least one order of magnitude.

Figure 5.9 (a) Bright-field micrograph of the microcapsules with the aqueous cores in different sizes, in which the bigger cores are 8 μm in diameter and the small cores are 5 μm in diameter. (b) Bright-field micrograph of the microcapsule with two aqueous cores. The scale bar is in length of 10 μm .

Figure 5.10 (a) Fluorescent micrograph of the microcapsules. The aqueous cores contain the Qdot600. Microcapsules α contain one small core, individually. Microcapsule β contains one big core. Microcapsule γ contains two cores. The scale bar is 10 μm in length.

5.5 Summary

In this chapter, the colorimetric barcodes are formed by loading the Qdots in the aqueous cores of the monodisperse microcapsules. The Qdots loaded in the aqueous cores maintain their original characteristics, especially the linear relation between the Qdot emission intensity and concentration. The emission intensities from the Qdots emitting at different wavelengths have no interference in the aqueous cores. The barcoding process can be simplified as a multiplexing process for the Qdot emission peaks and concentrations in the aqueous cores. The fluorescent profiles of the Qdot-loaded aqueous cores can be predefined before the formation of the barcodes directly by the compositions of Qdot mixtures. In addition, the graphical information from the fluorescent micrographs of the microcapsules about the sizes and numbers of the Qdot-loaded aqueous cores further enhances the coding capacity. The barcodes of microcapsules are formed by combinations of the optical signals of fluorescence emissions and the graphical information of the sizes and numbers of the aqueous cores, which can be identified easily in the fluorescent micrographs of these encoded microcapsules.

Chapter 6 Fluorescent-Pattern Barcodes

6.1 Introduction

Compared with the existing optical barcodes, especially the fluorescent colorimetric barcodes, the graphical-pattern barcodes are much more stable, repeatable and reliable. However, these graphical-pattern barcodes [27, 29] are hard to be synthesized in a small size down to tens of micrometers, which is more preferred for multiplexed assay in small-volume samples. The flat microparticles should be big enough to carry the patterns consisted of several spots in the minimum feature size of photomask and an extra area to carry the probe biomolecules. The using of the photomasks and precise alignment instruments is a huge cost hurdle to create millions of these graphical-pattern barcodes.

In this chapter, I present a new strategy that loads Qdot aqueous solutions as aqueous cores into multi-component microcapsules to form florescent-pattern barcodes, as proposed in Chapter 3. The fluorescent-pattern barcodes are made in a small size down to tens of micrometers by the microfluidic techniques without the using of photomask. The fluorescent-spot patterns formed by the Qdot-loaded aqueous cores in the microcapsules can be deciphered easily by their fluorescent micrographs. The coding capacity of the fluorescent-pattern barcodes is quite high up to millions.

6.2 Multi-component double emulsion

The microcapsules used to form the fluorescent-pattern barcodes are templated from the multi-component double emulsion droplets. Before the discussion on the formation of the fluorescent-pattern barcodes in the microcapsules, I firstly demonstrate the formation of the multi-component double emulsion droplets. Because the Qdot solutions used for barcoding are colorless under bright-field illumination, I use aqueous dye solutions in different colors to demonstrate the formation of the multi-component double emulsion droplets.

6.2.1 Flow components

The two inner-phase fluids were prepared by adding red and blue dyes in deionized water, respectively. These inner-phase fluids were added with 0.02 % (w/w) SDS. The inner-phase fluids in different colors were injected in the microfluidic device by a syringe pump in an identical flow rate. The middle-phase fluid was the polymer precursor of ETPTA added with 4% (v/v) Daracur 1173 and 2 % (w/v) Span 80. The outer-phase fluid was deionized water added with 2 % (w/w) SDS.

6.2.2 Multiple W/O emulsions at T-shape junctions

The red and blue inner-phase fluids were injected into the microfluidic device from two inner-phase inlet in the same flow rates of 0.02 mL h^{-1} . The W/O emulsions at two T-shape junctions are generated in the jetting regime when Q_m is set at 0.04 mL h^{-1} as shown in Figure 6.1 (a). The red and blue W/O droplets are generated alternately in the same size and queue in the microchannel. Increasing Q_m to 0.06 mL h^{-1} , the jets of

the inner-phase fluids are narrowed and go downstream to generate smaller W/O droplets as shown in Figure 6.1(b).

Figure 6.1 Multiple W/O emulsions at the T-shape junctions. $Q_i = 0.02 \text{ mL h}^{-1}$ for both of the red and blue aqueous fluids (a) Multi W/O emulsions in the jetting regime at the T-shape junctions. $Q_m = 0.04 \text{ mL h}^{-1}$ (b) Multi W/O emulsions in the jetting regime near the flow-focusing junction. $Q_m = 0.06 \text{ mL h}^{-1}$.

Figure 6.2 Image sequences of the coalescence of different W/O droplets (indicated by black arrows). The time interval between images is 0.33 ms.

A main challenge to form the multi-component double emulsion is to avoid the coalescence of the W/O droplets with different contents before encapsulation. As

shown in Figure 6.2, two contacted W/O droplets in different colors merge into one droplet. The addition of surfactants in the inner-phase fluids to reduce the interfacial tension cannot solve this problem, which may cause the failure to form the W/O emulsion at the T-shape junctions (see Section 4.3.2). For my empirical experience, the addition of 5 % (w/w) PS nanoparticles (~500 nm) in the inner-phase fluids to form Pickering emulsion [108-110] can reduce the possibility of the W/O droplet coalescence. A layer of these nanoparticles locates at the interface between the W/O droplets and the polymer precursor globule spontaneously and stabilizes the core droplets.

6.2.3 Multi-component double emulsion at flow-focusing junction

At the flow-focusing junction, the alternately queued red and blue W/O droplets are engulfed by the ETPTA globules. Q_o was adjusted to form the double emulsion droplets with two aqueous cores in different colors. As shown in Figure 6.3, each double emulsion droplets contains one red core and one blue core. The coalescence of the aqueous cores in the double emulsion droplets may occurs in the downstream of the serpentine microchannel. An effective approach to avoid this problem is to form relative small W/O droplets as aqueous cores in the ETPTA globules.

Figure 6.3 Formation of the two-component double emulsion droplets. $Q_o = 5 \text{ mL h}^{-1}$.

6.3 Fluorescent-pattern barcodes formed in multi-component microcapsules

The single-color Qdot solutions added with 0.02 % (w/w) SDS and 5 % (w/w) PS nanoparticles were prepared as the inner-phase fluids to form the fluorescent-pattern barcodes in the multi-component microcapsules.

6.3.1 Fluorescent-spot pattern formation

Figure 6.4 (a) Fluorescent pattern formation. The fluorescent micrographs of a microcapsule captured at 550 nm (a1) and 600 nm (a2). The overlay (a3) of the two fluorescent micrographs composes the fluorescent-spot pattern. The letters beside the bright spots indicate their colors (G: green, R: red). (b) Overlay fluorescent patterns. The Qdot contents and numbers of aqueous cores in individual microcapsules can be controlled by flow rates of double emulsion. (c) Color fluorescent micrographs of the fluorescent-spot patterns. Red arrows indicate the blurring spots. The blue arrow indicates overlapped spots. The scale bars are 10 μm in length.

As shown in Figure 6.4(a), the fluorescent-spot pattern is formed by the bright spots of the Qdot-loaded aqueous cores in the fluorescent micrograph at the Qdot emission peaks. At least one aqueous core in a microcapsule contains the Qdots to differentiate it from background (Figure 6.4(b1)). The single-color Qdots can be loaded in the aqueous cores at high concentrations without upper limit to create the intensity levels as many as possible regardless of FRET. The choices of color and concentration of the Qdots loaded in the aqueous cores are independent to each other. Different aqueous cores in a microcapsule may contain the Qdots in an identical color (Figure 6.4(b3, c3, c4)). The patterns formed by these randomly located aqueous cores are identified by their numbers, fluorescent colors and intensity levels, but not coordinate locations as the traditional graphical-pattern barcodes. There is no order among the aqueous cores in a microcapsule, since the aqueous cores are located randomly in the microcapsules.

6.3.2 Pattern orientation by centrifuge

As shown in Figure 6.5(a), the overlapped fluorescent spots may make the patterns un-discernible. To decipher the pattern correctly, the randomly located aqueous cores should be placed in a plane without overlap. Due to the slight density difference between the Qdot aqueous cores (density: 1g/mL) and the cured ETPTA shell (density: 1.1g/mL), centrifuge was used to position the light aqueous cores in a plane at the top of the microcapsules. The fluorescent-pattern barcodes consisted of 3 cores were suspended in water in a petri dish and centrifuged in speed of 2000 rpm for 1 min. Then the centrifuge slowed down gradually in 5 min to avoid intensive turbulence. The centrifuge-orientated patterns in the multi-component microcapsules are shown in Figure 6.5(b). Theoretically, a maximum of 3 cores in a microcapsule can be

orientated in a plane. The pattern overlap is inevitable when the 4 or more cores in a microcapsule.

Figure 6.5 Pattern orientation by centrifuge. (a) Overlapped pattern (b) Centrifuge-orientated pattern

6.3.3 Fluorescent-pattern barcode identification

The fluorescent-pattern barcodes in the multi-component microcapsules can be identified simply from the fluorescent micrographs at the Qdot emission peaks. I made five batches of the two-component microcapsules that contain two aqueous cores to create the fluorescent-pattern barcodes as listed in Table 6.1 to demonstrate the barcode generation and identification. To minimize the interference of autofluorescence from the cured ETPTA shells, the minimum concentrations of the Qdots loaded into the aqueous cores were $1\ \mu\text{M}$ for both green Qdot550 and red Qdot600. Due to the linear relation between the Qdot emission intensity and concentration, the increment of Qdot concentration in the aqueous core enhances the fluorescence intensity of the aqueous cores linearly. The concentration increments were $0.2\ \mu\text{M}$ for the Qdots of these two colors to define the intensity levels. Each barcode in the Table 6.1 is given a symbol to indicate the pattern formation. *a* and *b*

indicate the Qdot emission peaks at 550 nm and 600 nm, respectively. The numbers after them are the corresponding intensity levels controlled by Qdot concentrations. *Code0* and *Code1* are used to set the baselines and define the intensity levels for the green (Qdot550) and red (Qdot600) fluorescence emissions. As mentioned above, there is no order among the aqueous cores in a multi-component microcapsule; the barcode *a6b2* is the same as *b2a6*. The Qdots loaded in two aqueous cores can be in an identical color as *Code3*. Not all the aqueous cores need to load with the Qdots for barcoding as *Code4*.

Table 6.1 The Qdot contents of the aqueous cores in the two-component microcapsules

Code	Qdot concentration (μM)		Symbol
Code0	<div><div></div>1.0</div>	<div><div></div>1.0</div>	a0 b0
Code1	<div><div></div>1.2</div>	<div><div></div>1.2</div>	a1 b1
Code2	<div><div></div>2.2</div>	<div><div></div>1.4</div>	a6 b2
Code3	<div><div></div>1.6</div>	<div><div></div>2</div>	b3 b5
Code4	<div><div></div>2</div>		b5
<div><div></div>Qdot550<div></div>Qdot600</div>			

The fluorescence intensities of the aqueous cores at 550 nm and 600 nm were measured and recorded as a bar chart in Figure 6.6. After setting the baselines and defining the intensity levels by *Code0* and *Code1*, the other barcodes in Table 6.1 can be identified easily from their fluorescence micrographs at the Qdot emission peaks as their symbols. The relatively large errors of intensity levels for *Code2*, as shown in Figure 6.6, are partially from the imaging blurring due to the low focus depth of

microscope. As shown in Figure 6.4(b4, c2), the aqueous cores located at two different heights in a microcapsule cannot be imaged clearly at the same time on a focusing plane. Fortunately, using a lens with a smaller aperture to increase depth of focus can solve this problem. In addition, many of these fluorescent-pattern barcodes can be identified simultaneously to perform the multiplexed immunoassays by a simple and cost-effective optical system assembled by a CCD camera, narrow bandpass filters and a UV light source.

Figure 6.6 Decoding bar chart of the fluorescent-pattern barcodes in Table 6.1. Code0 and Code1 are used to set the baselines and define the intensity levels for the fluorescence emissions at 550 nm and 600 nm, respectively. The other barcodes can be recognized with reference of the baselines and intensity levels.

6.3.4 Coding capacity

The coding capacity of fluorescent-pattern barcodes in the multi-component microcapsules is determined by the number of Qdot colors, p , the fluorescence intensity levels of the Qdot-loaded aqueous cores, n , and the maximum number of the aqueous cores in a microcapsule k . For each aqueous core, the number of combinations of multiplexing colors and intensity levels are pn . Since the aqueous cores are located randomly in the individual microcapsules, there is no order among them. If i ($i < k$) aqueous cores are loaded with Qdots for barcoding and the other $(k-i)$ aqueous cores are blank, the total combinations of fluorescent-spot patterns consisted of i aqueous cores without repeat can be given by

$$\frac{(pn + i - 1)!}{(pn - 1)! \cdot i!} \quad (6.1)$$

To form fluorescent-spot pattern that can be differentiated from the background, at least one of these i aqueous cores in a microcapsule is loaded with Qdots. The total combinations of these spot patterns from 1 to k aqueous cores is expressed as

$$\sum_{i=1}^k \frac{(pn + i - 1)!}{(pn - 1)! \cdot i!} = \frac{(pn + k)!}{(pn)! \cdot k!} - 1 \quad (6.2)$$

For example, if the Qdots of 5 colors in 50 intensity levels ($p=5$ and $n=50$) are used for barcoding in three-component microcapsules ($k=3$), the total number of fluorescent-pattern barcodes is 2,667,125.

6.4 Summary

The fluorescent-pattern barcodes are formed without using the photomask by loading single-color Qdot solutions as the aqueous cores in multi-component microcapsules. These fluorescent-pattern barcodes formed by the randomly located aqueous cores are identified by their fluorescence colors, intensity levels and numbers of the Qdot-loaded aqueous cores in each microcapsule, which can create a high coding capacity up to millions. The microfluidic method allows the formation of the fluorescent-pattern barcoded microcapsules in small sizes down to tens of micrometers and a high production rate up to hundreds per second.

Chapter 7 Suitability of Quantum-dot Barcodes for Biological Assays

7.1 Introduction

Due to the low mechanical stability and the difficulty in surface functionalization, many existing Qdot barcodes are not suitable for conducting biological assays practically. For example, the Qdots coated over the outer surfaces of microparticles by layer-by-layer self assembly can be easily released to ambient buffers, especially if vortex mixing is used to speed up the binding efficiency between the targets and probes. In addition, the polyelectrolyte layers coated the Qdots over the microparticles are difficult to be tailored with different functional groups for biomolecule immobilization. In this chapter, the mechanical stability of the Qdot barcodes formed in the microcapsules and their functionalization methods are investigated.

7.2 Mechanical stability of Qdot barcodes

Mechanical responses of the microcapsules to vortex mixing, centrifuge and glass-bead grinding are examined, respectively, which are mostly used for processing biological samples.

7.2.1 Suitability for vortex mixing and centrifuge

Vortex mixing and centrifuge are two mostly used approaches to process assay samples. The Qdot-barcoded microcapsules should have a sufficient mechanical stability to suffer from vortex mixing and centrifuge without breakup. Firstly, 100 mg of the microcapsules were dispersed in deionized water for vortex mixing by a shaker (REAX top, Heidolph, Germany) in speed of ~2500 rpm for 10 min. All of the microcapsules maintained their fully closed shells without breakup. Secondly, this microcapsule suspension was centrifuged in speed of 3000 rpm for another 10 min. The microcapsules at the bottom of the tube were still fully closed without any breakup.

The microcapsules made with rigid polymer shells can resist to the abrasion during vortex mixing and centrifuge. The Qdot barcodes in the aqueous cores of the microcapsules are protected by the polymer shells without any leakage upon 2500-rpm vortex mixing or 3000-rpm centrifuge.

7.2.2 Suitability for cell lysis by glass-bead grinding

A potential application of the Qdot barcodes is to capture specific cells and then analyze their contents, such as proteins and genes, after lysis. Grinding of using glass beads is an effective approach for cell lysis. For this application, the Qdot-encoded microcapsules which capture the cells should undergo the grinding by glass beads without breakup. The 100 mg of glass beads (105~150 μm , Polyscience, USA) was mixed with 100 mg of the microcapsules in 1 mL water as shown in Figure 7.1(a). 10 % (w/w) of glass beads is high enough for cell lysis in a sample. After grinding by vortex mixing in speed of ~2500 rpm for 10 min, all of the microcapsules were intact as

shown in Figure 7.1(b). The addition of glass beads to 30 % (w/w) also did not break the microcapsules as shown in Figure 7.1(b). The rigid polymer shells can resist the intensive abrasion from the grinding without breakup. The toxic Qdots can be locked inside the polymer shells without leakage after grinding.

Figure 7.1 Grinding test (a) Mixture of microcapsules and glass beads before grinding (b) Mixture of microcapsules and 10 % glass beads after grinding for 10 min (c) Mixture of microcapsules and 30 % glass beads after grinding for 10 min.

7.3 Functionalization of barcodes

A main advantage of the microparticle-based suspension bioassays is the surfaces of the encoded microparticles can be tailored with various functional groups for optimal bioconjugation to different biomolecules. After the formation of encoded microcapsules, the outer surfaces of ETPTA hard shells are hydrophobic. The

modification of microcapsules with the functional groups is the initial step to immobilize the biomolecules on the encoded microcapsules specifically for the multiplexed assays. The selective function groups, as shown in Figure 7.2, provide reliable and specific conjugations between the biomolecules and the microcapsules.

Figure 7.2 Common functional groups on microparticles for bioconjugation [111].

The methods inclusive of plasma treatment [112], chemical vapor deposition, bioinspired catecholic chemistry [113] and the using of silane compounds and copolymerization [114] are employed to modify the polymer surfaces with the functional groups. The methods of using the silane compounds and copolymerization are cost-effective and follow relatively simple protocols, which are suitable to functionalize the polymer microparticles. These two methods are introduced with the immobilization of the amine groups (-NH_3) and the carboxyl groups (-COOH) which

are the widely used for immobilizing proteins and oligonucleotides on the polymer microparticles.

a. Using silane compounds

As shown in Figure 7.3(a), the general molecule structure of silane compounds consists of four constituents. A functional or reactive group at the end of an organic alkyl chain is linked to the central silicon atom which has up to three hydrolysable groups. Since the polymer surface has abounded hydroxyl groups (-OH) groups, the silane compound can be immobilized on the polymer surface covalently through the formation of hydrogen bonding. To activate the polymer surface with the -OH groups,

Figure 7.3 (a) General molecule structure of silane compounds [111]. (b) 3-aminopropyltriethoxysilane (APTS) for amine functionalization. (c) Carboxyethylsilanetriol, disodium salt (CES) for carboxyl functionalization.

the polymer surface can be treated by an oxidation mixture of H_2O , HCl and H_2O_2 in ratio of 5:1:1. Figure 7.3 (b and c) are the molecule structures of 3-aminopropyltriethoxysilane (APTS) and carboxyethylsilanetriol disodium salt (CES) which are used to functionalize the polymer surfaces with $-\text{NH}_3$ and $-\text{COOH}$, respectively. The spherical microcapsules can be modified with the functional groups at their outer surfaces uniformly by suspending them in the silane compound solutions.

b. Copolymerization

The copolymerization method does not require any extra procedure after the formation of microcapsules. The polymer surfaces of microcapsules can be created with the functional groups by building the monomers with the functional groups into backbones of the polymer matrix. The polymer precursor is prepared by mixing the functional monomers within the host monomer. The addition of functional monomers, which are water-dissolvable, may changes the surface tension or viscosity of the polymer precursor [114]. To avoid the significant changes to the fluidic properties of the polymer precursor, the addition of functional monomers should be less than 5 % in the polymer precursor. Figure 7.4 is two molecule structures of tri (2-aminoethyl)

Figure 7.4 Functional monomers. Left is tri(2-aminoethyl)amine used for amine functionalization. Right is the acrylic acid for carboxyl functionalization.

amine and acrylic acid, which can be used for preparation of polymer precursors to form -NH_3 and -COOH on the cured polymer surfaces, respectively. For certain functional group, such as -NH_3 , a lot of commercially available monomers [115] can be selected according to particular conditions.

c. Determination of functional groups

X-ray photoelectron spectroscopy (XPS) and Fourier transform infrared spectroscopy (FTIR) are two main techniques to analyse thin surface layers. However, these two techniques are not suitable to determine the functional groups on polymer microcapsules. The elemental composition of a surface with accurate relative ratios can be measured by XPS, and FTIR can investigate the chemical bonds on a surface. However, neither the elemental composition nor the chemical bonds can give a direct evidence of the presences of the organic functional groups on a polymer surface.

A reliable technique to determine the functional groups on the spherical polymer microcapsules is the using of selective fluorescence labeling [116]. Similar to the specific bioconjugation between the function groups and the biomolecules, fluorophores can also form specific covalent bindings to the functional groups and indicate the presences of the functional groups.

7.3.2 Experimental and protocols

Two batches of the microcapsules were made to demonstrate the functionalization of their surfaces with -NH_3 and -COOH by the methods of using the silane compounds and copolymerization, respectively.

a. Preparation of amino-functional microcapsules

The microcapsules are modified with -NH_3 at their outer surfaces by using APTS. The abounded -OH groups on the polymer surfaces can react spontaneously with the silanols after hydrolysis of APTS, as illustrated in Figure 7.5. The experimental procedure followed the protocol described below.

1. Wash 100 mg of the polymer microcapsules by ethanol to remove the uncured ETPTA monomer. Then, rinse the microcapsules by deionized water for three times with vortex mixing.
2. Suspend the microcapsules in a 5 mL ethanol solution (pH 4.5~5.5, adjusted by 1M HCl solution) which contained 5 % (v/v) of water and 5 % (v/v) of APTS with gentle mixing for 2 hours.
3. Rinse the microcapsules with deionized water for three times.

Figure 7.5 Deposition of APTS on the surfaces of polymer microcapsules.

b. Determination of amine groups on microcapsules

The -NH_3 on the microcapsules are determined by the specific labeling of fluorescamine to the -NH_3 , as illustrated in Figure 7.6. The amine-functional microcapsules were immersed in a solution of 5 mg fluorescamine in 20 mL acetone for 5 min. Then, the microcapsules were washed once by ethanol and twice by deionized water. Finally, the microcapsules were suspended in PBS buffer for fluorescence imaging as shown in Figure 7.7. The fluorescamine was also added in a suspension of the microcapsules without amine functionalization as the negative reference, whose fluorescent micrograph is totally dark. The excitation and emission wavelengths of fluorescamine are 420 nm and 472 nm, respectively.

Figure 7.6 Specific labeling of fluorescamine to the amine-functional microcapsules.

Figure 7.7 (a) Bright-field micrograph of the amino-functional microcapsules labeled with fluorescamine. (b) Fluorescent micrograph of the amino-functional microcapsules labeled with fluorescamine.

c. Preparation of carboxyl-functional microcapsules

The carboxyl-functional microcapsules were made by copolymerization of the acrylic acid (AA) monomer and the ETPTA polymer precursor, as illustrated in Figure 7.8. The ETPTA polymer precursor added with 1 % (v/v) acrylic acid was used as the middle-phase fluid to form the polymer microcapsules.

Figure 7.8 Formation of the carboxyl-functional polymer microcapsules by copolymerization of 1% AA monomer and ETPTA monomer.

d. Determination of carboxyl groups on microcapsules

The -COOH groups on the microcapsules were determined by the specific coupling of dansylcadaverine with the -NH₃ groups through carbodiimide reaction, as illustrated in Figure 7.9. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was used as catalysis to form the covalent bonding between the dansylcadaverine and the carboxyl-functional microcapsule. The experimental procedure followed the protocol described below.

1. Wash 100 mg of the carboxyl-functional microcapsules once with ethanol and three times with deionized water with vortex mixing to remove the uncured monomers. Then, wash the microcapsules by MSE buffer once, and suspend the

microcapsules in 5 mL MES buffer.

2. Dissolve 10 mg dansylcadaverine in the microcapsule suspension and mix thoroughly.
3. Add 20 mg EDC in the microcapsule suspension and mix to dissolve.
4. React at room temperature for 3 hours with gentle mixing.
5. Wash the microcapsules with deionized water three times

Figure 7.9 Specific labeling of dansylcadaverine on the carboxyl-functional microcapsules.

Figure 7.10(a)Bright-field micrograph of the carboxyl-functional microcapsules that are labeled by dansylcadaverine (b) Fluorescent micrograph of the carboxyl-functional microcapsules that are labeled by dansylcadaverine.

The microcapsules were suspended in PBS buffer for fluorescent imaging as shown in Figure 7.10. The dansylcadaverine was also added in a suspension of the microcapsules without carboxyl functionalization as the negative reference, whose

fluorescent micrograph is totally dark. The excitation and emission wavelengths of dansylcadaverine are 350 nm and 518 nm, respectively.

7.4 Summary

In this chapter, the Qdot-encoded microcapsules are examined for the suitability of conducting biological assays. Both of the mechanical stability of the microcapsules and the methods to functionalize the microcapsules are investigated. The rigid polymer shells of the microcapsules can protect the Qdot-loaded aqueous cores without leakage, when vortex mixing, centrifuge and glass-bead grinding are employed for processing assay samples. Therefore, the Qdot barcodes formed in the microcapsules can not only be used for interrogating biomolecules, but also for capturing specific cells for lysis and analysis of their contents. The functionalization of microcapsules is the first and critical step to immobilize the probe biomolecules on the microcapsule specifically and covalently for the multiplexed immunoassays. The methods of using saline compounds and copolymerization are presented to modify the polymer microcapsules with the functional groups. These two methods follow simple procedures and can make the uniform functionalization on the microcapsules. The functional groups on the microcapsules were determined reliably by specific fluorophore labeling. The Qdot barcodes formed within the microcapsules can be used as a general tool for various screening applications due to their high mechanical stability and easily tailored polymer surfaces.

Chapter 8 Conclusions and Future Work

8.1 Conclusions

The use of encoded microparticles to conduct the suspension multiplexed assays may serve as a next-generation platform for high-throughput screening applications, in which each microparticle has its own specific labeling as a barcode to identify the probe biomolecule immobilized on the microparticle. By tracking the barcodes, the assays on different microparticles can be interrogated simultaneously. Compared with the microtiter plate and the microarray, the encoded microparticle has obvious advantages over them to conduct the multiplexed assays with less consumption of biological reagents, optimized bioconjugation between solid supports and biomolecules and a higher throughput capability.

This work focuses on the development of predefinable, stable and repeatable barcodes in the microcapsules with high coding capacities. The microcapsules with the aqueous cores and the transparent polymer hard shells were templated from the microfluidic double emulsion droplets. A microfluidic device made of soft lithography of PDMS was developed to form the microcapsules. The microfluidic device consists of four T-shape junctions and a flow-focusing junction to generate the aqueous cores and the W/O/W double emulsions, respectively. The wettability of the flow-focusing junction and serpentine microchannel downstream of it was modified to be hydrophilic

by UV-initiated polymerization of PAA, which creates reliable and reversed wettability pattern in the microchannel network. By controlling the flow rates of the three fluids of W/O/W double emulsion and the interfacial tension between the immiscible fluids, the sizes of the aqueous cores and the double emulsion droplets were controlled precisely and independently. Furthermore, the number of aqueous cores in each microcapsule can be controlled by the ratio of droplet generation frequencies at the T-shape junction to the flow-focusing junction. Under UV exposure, the middle-phase ETPTA globules of the double emulsion droplets were cured as the hard polymer shells to form the microcapsules in the serpentine microchannel of the microfluidic device. To form the fully closed hard polymer microcapsules without leakage of the aqueous core contents, the double emulsion droplets were generated in a jetting regime to confine the aqueous cores centrally in the ETPTA globules.

The fully closed microcapsules with the aqueous cores and the transparent hard polymer shells were used as the barcode carriers. By using the aqueous Qdot mixtures as the inner-phase fluid to form the microcapsules, colorimetric barcodes were formed in the microcapsules. Because of the protection of the hard polymer shells, the Qdot aqueous cores are separated from ambient fluids with stable fluorescent emissions in different assay buffers. The Qdots in the aqueous cores maintain their original characteristics including their emission peaks and the linear relation between emission intensity and concentration. The colorimetric barcodes can be formed simply by multiplexing the Qdot emission peaks and concentrations in the inner-phase fluids without any change of fluorescent profiles after the microcapsule formation. An image-based decoding method was demonstrated to identify the colorimetric barcodes in the microcapsules. The fluorescent profiles of the Qdot-encoded microcapsules that

were pre-defined by the compositions of the Qdot mixtures in the aqueous cores were recorded in their fluorescent micrographs at the Qdot emission peaks. Without using any complicated deconvolution algorithm for the traditional spectrum-based decoding method, the Qdot mixture compositions in the aqueous cores were calculated by a simple matrix algorithm according to the fluorescence intensities of Qdot-loaded aqueous cores at the Qdot emission peaks. In addition, the coding capacity can be promoted by using the graphical information of the size and number of the Qdot-loaded aqueous cores in each microcapsule, which are also recorded in the fluorescent micrographs of the Qdot-encoded microcapsules.

Besides the colorimetric barcodes, fluorescent-pattern barcodes were also formed in microcapsules, in which the several aqueous cores contain the Qdots in different colors. This strategy to form the fluorescent-pattern barcodes in multi-component microcapsules does not require photomasks. The Qdot aqueous cores emitting in different colors were generated at different T-shape junctions and encapsulated in the microcapsules to form the fluorescent patterns as barcodes. Different from previous coordinate-orientated graphical patterns made by lithography techniques, the fluorescent patterns in the microcapsules were identified by the combinations of numbers, colors and intensities of the Qdot aqueous cores. This fluorescent-pattern barcode formed in the multi-component microcapsule has a quite high coding capacity up to millions.

The mechanical stability and the methods to modify the polymer microcapsules with functional groups for optimal bioconjugation were examined. The rigid polymer shells can protect the Qdot barcodes in the aqueous core when vortex mixing, centrifuge and glass-bead grinding are used to process the assay samples with the

encoded microcapsules. The using of saline compounds and the formation of copolymerization are two suitable methods to functionalize the spherical microcapsules. The saline compounds with various functional groups can be anchored on the microcapsules easily. By adding the monomers with the functional groups in the host polymer precursor, the functional groups can be built in the backbones of the polymer matrix and presented at the surfaces of microcapsules. These functional groups on the microcapsules were determined reliably by specific labeling of fluorophores. The Qdot-barcoded microcapsules can be tailored with various functional groups for optimal immobilization of different biomolecules. The stable Qdot barcodes formed within the microcapsules can be used as a general tool for various screening applications.

The thesis contains interdisciplinary works on microfabrication, microfluidics, surface chemistry and optics. To clarify the originality, the main novelties of this thesis are summarized in follows.

1. Formation of fully closed polymer microcapsules with liquid cores by double emulsion in jetting regime.
2. Effective size controlling for aqueous core by synergistic interaction of surfactants at the interface between two immiscible phases.
3. Barcoding strategy of loading Qdots in aqueous-core microcapsules to form predefinable colorimetric barcodes.
4. Express decoding algorithm for the predefinable colorimetric barcodes
5. Image-based decoding method which records both colorimetric and graphical information to enhance the coding capacity.
6. Formation of small multi-component microcapsules down to 50 um using PDMS-based microfluidic device.
7. Maskless pattern barcoding strategy of using multi-component microcapsules.

8.2 Future work

Further work on conducting multiplexed assays on the encoded microcapsules is required to bring the barcode technology to practical applications. Significant aspects and some preliminary studies are summarized as follows.

8.2.1 Colorimetric barcode identification by flow cytometry

Taking advantage of the simplicity of the decoding algorithm described in Section 5.4.1, it is promising to use a flow cytometry to identify the colorimetric barcode and interrogate the assay result on each microcapsule with a high throughput. To avoid blocking the flow channel of cytometer in diameter of $\sim 50\text{ }\mu\text{m}$, the barcoded microcapsules will be made in a smaller size down to $20\text{ }\mu\text{m}$. The Qdots in the aqueous cores can be excited with high emissions by commonly used 355 nm or 488 nm laser of the cytometer, and the colorimetric signals can be read out in a high speed up to 2000~40000 per second. As illustrated in Figure 8.1(a), the targets in a sample are firstly labeled with fluorophores. Then the colorimetric-barcoded microcapsules carrying specific probes are pooled together within the sample. After incubation, the barcoded microcapsules with assay results are analyzed by the cytometer. Both of the signals of barcodes and the targets can be recorded simultaneously. Compared with the *xMAP technology* from *Luminex* which requires specially designed cytometer containing two or more excitation light sources, the Qdot-encoded microcapsules with higher coding capacity can be decoded by the common cytometers. The using of the Qdot-encoded microcapsules not only promotes the throughput capability for multiplexed assays but also reduces the cost on instruments.

Figure 8.1 (a) Schematic illustration of using a flow cytometry to conduct multiplexed immunoassays by the colorimetric-barcoded microcapsules. (b) Schematic diagram of the encoded microcapsule with probe biomolecules.

8.2.2 Immobilization of biomolecules on encoded microcapsules

The work on the hybridization DNA molecules on the polymer microcapsules has been done. 5'-amine-modified oligonucleotides (oligos) were immobilized on the carboxyl-functional microcapsules through carbodiimide reaction as the probes. The complementary oligos were modified with 6-carboxyfluorescein (FAM) at 5' end as the targets. The preliminary results indicated that the probe oligos on the

microcapsules can capture the target oligos. However, the signal-to-noise ratio of the result was too low. To improve the sensitivity and the accuracy of the multiplexed assays, systematical study is required on the surface density of the carboxyl groups, specificities of biomolecules (genes or proteins), the immobilization approach, the hybridization types (direct hybridization or sandwiched hybridization) and effects of the reaction temperature and the concentrations of the oligos. The surface chemistry of the polymer microcapsules needs further and deeper investigation for practical biological applications.

8.2.3 Pneumatic flow control system

Three syringe pumps were used to inject three fluids independently into the microfluidic device to form the microcapsules. However, the syringe pumps are not suitable to control the flow rates down to 0.05 mL h^{-1} . When the syringe pump was used to drive the inner-phase fluids stored in a 1-mL syringe (BD, USA) in 0.01 mL h^{-1} , the respond time to reach a stable flow rate was longer than 30 min. The flow rate control by syringe pump is extremely time-consuming and intolerable. What is worse, the syringe pump has an inherent drawback of flow pulse due to the using of stepper motor. The unstable flow rates must produce the droplets with relatively larger size distributions.

To circumvent the drawbacks of syringe pump, a pneumatic flow control system was set up according to a reference [51]. The working principle of this system is to use stable and adjustable compressed-air pressures to drive the fluids. As illustrated in Figure 8.2(a), a pump is used to compress air as a high pressure source. An air filter (Festo, USA) is linked to the pump for dust removal and desiccation. A low-pressure

regulator (0~0.7 bar outlet range, Festo) protects the downstream of the system and stabilize the pressure primarily. The compressed air is stored in a cylinder tanker (volume: 0.25 L, Festo) which stabilizes the compressed-air pressure further. Using a quadruple distributors (QSQ-6-4, Festo), four control channels are branched off from the main channel. Each control channel has two air flow valves (GRO-QS-4, Festo) and a gauge (MAP-40-1-1/8-EN, Festo) connected in series. The pressure used to control the liquid flow rate is leaded from the point monitored by the gauge which is controlled by the two air flow valves. As illustrated in Figure 8.2(b), the controllable pressure injects the reagent stored in the tube to the microfluidic device. The ends of control channels are linked together and opened to atmosphere.

Figure 8.2 (a) Schematic illustration of the pneumatic flow control system. (b) Schematic illustration of the fluid reservoir.

Figure 8.3 shows the experimental setup of this pneumatic flow control system. The pressures were adjusted by the pneumatic control channels independently; the flow rates of the fluids stored in the tubes were also controlled individually. In addition, the response time of flow rate controlled by this pneumatic system was quite short in one second. The pressures used to control the flow rates of the inner-, middle- and outer-

phase fluids were 0.4~0.6 bar, 0.2~0.5 bar and 0.1~0.3 bar, respectively. Since the minimum scale of the gauge used in this prototyping system is 0.02 bar, the precision of this system is 0.01 bar (1 kPa). A more precise control will be achieved by using digital pressure gauges which have a resolution of 1 Pa.

Figure 8.3 *Experimental setup. The fluids are injected into the microfluidic device by the pneumatic flow control system.*

8.2.4 Three-dimensional PDMS microfluidic device

The 2.5D microfluidic device was made by bonding a PDMS cast with the microchannels in two heights to a plain substrate as illustrated in Figure 8.4(a). A three-dimensional (3D) microfluidic device was made by bonding a 2.5D PDMS top cast to a 2D PDMS bottom cast as illustrated in Figure 8.4(b), which provides a symmetrical confinement geometry in both horizontal and vertical directions [117, 118] at the flow-focusing junction to form the double emulsion. The ETPTA flow can be

suspended in the aqueous outer-phase flow without attachment to the inner walls of the microchannels. Figure 8.5 shows the dimensions of the 3D microchannel networks.

Figure 8.4 (a) Illustration of the 2.5D device (b) Illustration of the 3D device

Figure 8.5 (a) Micrograph of the T-shape junction. (b) Micrograph of the flow-focusing junction. (c) Cross-section view of the microchannel at the T-shape junction along line AA'. (d) Cross-section view of the flow-focusing junction along line BB'. (e) Cross-section view of the serpentine section along line CC'. The dimensions of the microchannels are labeled in (a~e).

Figure 8.6 (a) The top cast with 2.5D structure. The grey part is $25\ \mu\text{m}$ in thickness. The black part is $100\ \mu\text{m}$ in thickness. (b) The bottom cast with 2D structure in thickness of $100\ \mu\text{m}$

Firstly, the molds of the top and bottom casts were made on two silicon wafers by photolithography of SU-8 photoresists (MicroChem, USA), as designed in Figure 8.6(a, b). To make the mold of the 2.5D top cast, two alignment marks were made on the silicon wafer by DRIE. Then, a SU-8 structure of the microchannel network in thickness of $25\ \mu\text{m}$ was made on the silicon wafer with the two alignment marks as the first layer of the mold. Another SU-8 structure of the serpentine section and the microchannel carrying the outer-phase fluid was made in thickness of $75\ \mu\text{m}$ above the first layer structure with the two alignment marks to form the second layer of the mold. The mold of the 2D bottom cast was made in thickness of $100\ \mu\text{m}$. It should be noted that the serpentine section in the bottom cast is mirror-symmetrical to the serpentine section in the top cast, which can match with each other face-to-face during the assembly of the 3D device.

Figure 8.7 Schematic illustration of the 3D microfluidic device fabrication

Secondly, PDMS base was mixed with its curing agent in a ratio of 10:1 (w/w) and degassed before being poured on the molds. After curing at 80 °C for 2 hours in an oven, the cured PDMS top cast and bottom cast were peeled off from the molds as shown in Figure 8.7(a1, a2). After punching the inlets and outlet on the top cast, both of the top and bottom casts were treated with oxygen plasma. For the bottom cast, the surface with the microchannels was faced up. The bottom cast faced up was covered by the top cast faced down with alignment under a microscope to form a complete microchannel network as illustrated in Figure 8.7(b). Then, the microfluidic device was placed in the oven at 80 °C for two days to form reliable bonding and reverse the original hydrophobic surface of PDMS. Thirdly, the serpentine section downstream of the flow-focusing junction was patterned to be hydrophilic by UV-initiated graft polymerization of PAA as described in Chapter 4.

Figure 8.8 shows the advantage of the 3D device over the 2.5D device to form the controllable double emulsion at the flow-focusing junction in high flow rates. As shown in Figure 8.8(a1, a2), the flow of the outer-phase SDS solution tends to press the flow of ETPTA polymer precursor at the bottom of the microchannel at the 2.5D flow-focusing junction. When Q_m was set higher than at 0.1 mL h⁻¹, the ETPTA flow with the aqueous droplets went over the 2.5D flow-focusing junction and failed to form the controllable double emulsion. In contrast, the flow of outer-phase SDS at the 3D flow-focusing junction was not only focused horizontally but also vertically as shown in Figure 8.8(b1, b2). The jet of ETPTA flow with the aqueous droplets was suspended in the flow of SDS solution without attachment to the inner walls of the microchannels. The 3D device can be operated in high Q_m and Q_o to form the double

emulsion droplets in the jetting regime. The microcapsules will be made with much smaller sizes and a higher production rate by this 3D microfluidic device.

Figure 8.8 (a1) Double emulsion at the flow-focusing junction of the 2.5D device. (a2) Schematic cross-section view at the flow-focusing junction of the 2.5D device. (b1) Double emulsion at the flow-focusing junction of the 3D device. (b2) Schematic cross-section view at the flow-focusing junction of the 3D device. $Q_i = 0.02 \text{ mL h}^{-1}$, $Q_m = 0.1 \text{ mL h}^{-1}$ and $Q_o = 6.2 \text{ mL h}^{-1}$.

Appendix

A. Immiscible liquids in equilibrium state

Figure A1 Equilibrium state of three immiscible phase of double emulsion.

The spreading coefficients of three liquid phases can give an analysis of the equilibrium state of droplets with the three phases [104]. The spreading coefficient, S , describes the spontaneous spreading of an oil droplet on a water film in air.

$$S = \gamma_{rw} - \gamma_{ro} - \gamma_{ow} \quad (\text{A.1})$$

γ_{rw} is the air-water surface tension, γ_{ro} is the air-oil surface tension and γ_{ow} is the oil-water surface tension. If $S > 0$, the oil droplet spreads as film. If $S < 0$, it remains an oil droplet. For the three liquid phases of double emulsion, three conditions may occur.

$$S_i < 0 \quad S_m > 0 \quad S_o < 0 \quad (\text{A.2})$$

$$S_i < 0 \quad S_m < 0 \quad S_o < 0 \quad (\text{A.3})$$

$$S_i < 0 \quad S_m < 0 \quad S_o > 0 \quad (\text{A.4})$$

S_i , S_m and S_o are the spreading coefficients of the inner phase, middle phase and outer phase, respectively. The first condition is that the inner-phase droplet is completely engulfed by the middle-phase globule to form a stable double emulsion droplet. The second condition is that the inner-phase droplet is partially engulfed by the middle-phase globules which can be used as templates to form Janus particles and nonspherical particles. The third condition is that the inner-phase droplet and middle-phase droplet are suspended independently in the outer phase.

For the W/O/W double emulsion, the inner phase and outer phase are miscible. The interfacial tension between them is zero. The spreading coefficient of middle phase is less than zero. Reducing the interfacial tension between the middle phase and the outer phase can improve the stability of the double emulsion droplets. However, the W/O/W double emulsion is still thermodynamically unstable. The aqueous inner-phase may immerse into the outer phase flow. The middle globule should be cured as solid shell to form the microcapsule immediately in the microfluidic device after the double emulsion droplet generation. And the middle phase and outer phase should be added with sufficient surfactants to stabilize the double emulsion as long as possible.

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