Fabrication of Microfluidic Devices and their Applications in Chemical and Cellular Researches

by

Yizhe Zheng

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This is to certify that I have examined the above PhD thesis and have found that it is complete and satisfactory in all respects, and that any and all revisions required by the thesis examination committee have been made.

Dr. Hongkai Wu  Thesis Supervisor

Prof. Zhenyang Lin  Head of Department

Department of Chemistry

9 May 2011
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Fabrication of Microfluidic Devices and their Applications in Chemical and Cellular Researches

by

ZHENG Yizhe

Department of Chemistry
The Hong Kong University of Science and Technology

Abstract

Microfluidics has grown into a flourish field that holds the promise to offer better solutions for various areas such as bioanalysis and biomedical sciences with a number of advantages: small quantities of samples and reagents; high resolution and sensitivity of separation and detection; low cost; short times for analysis; and small size for portable devices. However, there is no universal fabrication method for all microfluidic applications. After two decades of fast growth in microfluidics, there are still great interests in the development of new fabrication methods of microfluidic devices for wider applications, lower cost and higher efficiency.

In this work, I have solved three practical problems in the microfluidic fabrications. The first, I developed a screw-actuated system that obviates the use of compressed air to control the valve; this improvement greatly reduces the size of the whole device and thus makes it more portable and less expensive as well. The second is the establishment of a general fabrication method for hydrogel microchannels; a mixed gel with two hydrogel ingredients
can not only strongly bond gel slabs together but also facilitate the bonding of the hydrogel to other materials such as glass and polymers. In the third, I invented a convenient method to fabricate microperforated membranes in photocurable materials.

I have also demonstrated the application of microfluidic techniques in chemical and biological researches with construction of stepwise concentration gradient in arrays. I used three microfluidic methods that represent three different strategies to form varying concentrations: (1) dilution of the same concentrated solution with different dilution factors; (2) diffusion of molecules from a concentrated solution reservoir; (3) stepwise dilution from a concentrated solution.

This research invented several microfluidic fabrication techniques and several microfluidic designs for establishment of concentration gradient. These inventions and new designs help expand the potential applications of microfluidic devices.
Chapter 1

Introduction

In 1979, a miniaturized gas-chromatography chip was fabricated in silicon at Stanford University using standard microfabrication techniques from the micro-electronics industry [1]. This work inspired more explorations in the miniaturization and integration of fluidic components together with the ultimate goal of fitting an entire “lab on a chip”, in much the same way that a microelectronic circuit is an entire computer on a chip. The whole field started to boom in the mid 1990s with tremendous interest in harnessing the full potential of this approach [2]. It has currently become one of the most exciting areas in the research frontier with the development of countless microfluidic devices and fabrication methods. Now the term of Microfluidics is often regarded as “the science and technology of systems that process or manipulate small (10^{-9} to 10^{-18} liters) amounts of fluids, using channels with dimensions of tens to hundreds of micrometers” [3]. As numerous investigators have pointed out, scaling down fluidic systems and processes to the microscale provides a number of significant advantages: low consumptions of samples and reagents; high resolution and sensitivity of separation and detection; low cost; short times for analysis; and small size for portable devices [2-7]. In addition, the low thermal mass and large surface to volume ratio of small components facilitates rapid heat transfer, enabling quick temperature changes and precise temperature control [8]. Because diffusion length scales with the square of diffusion time, diffusive mixing is fast at the microscale, thus increasing the speed and accuracy of reactions [9, 10]. Some particular properties of fluids at the microscale such as the laminar nature of flow in microchannels permit new methods for studying biological cells [11, 12], offering convenient tools for immunoassays [13-15], and performing solvent exchange and two-phase reactions [16, 17]. These properties give the microfluidic technique great potentials in wide areas such as bioanalysis and biomedical sciences, especially potable and inexpensive
diagnostic equipments and research platform for the study in which samples are too little to be analyzed by traditional macroscale equipment [6, 18-21].

Albeit all the advantages, microfluidics is different from microelectronics because there are still no corresponding microfluidic components for fluid control as the microelectronics components such as diodes and transistors that can conveniently control electron flows. No standards (if they exist) in fabrication of microfluidic devices have been established in this field as in microelectronics. Often a new microfluidic task requires a new microchip with a different design that has to be fabricated from beginning. Therefore, research laboratories still need to make the microfluidic chips with different purposes one by one. One needs to design their chip layout, choose the proper materials, fabricate the microchannels and make their own microchips for different applications.

1.1 Fabrication of Microfluidic Chips by Rapid Prototype

Nowadays, the most popular strategy in microfluidic chip fabrication is based on a rapid prototyping method [22, 23] (Figure 1.1). First, the desired patterns are transferred to a computer file by painter software such as Adobe Illustrator and AutoCAD; second, these patterns are printed on a transparency film; third, this film with patterns can be used in photolithography as mask that transfers the patterns from the mask to a photosensitive material (photoresist) by photolithography (See 1.1.2). Finally, the photoresist patterns are used as master structures for molding them into wanted microfluidic materials.

1.1.1 Materials in microfluidics

In the beginning of the development of microfluidics, silicon and glass are the common materials for the construction of microfluidic chips [1, 24-29]. However, their fabrication is rather complicated with sophisticated equipment and dangerous chemicals. Also, they are not suitable for long-term on-chip cell culture and study because they are not gas-permeable [3].
As a result, other materials such as polydimethylsiloxane (PDMS) [9, 30, 31], Poly(methyl methacrylate) (PMMA) [32-34], hydrogel [35, 36], SU-8 [37-39] and Norland Optical Adhesive (NOA) [40, 41] have been introduced to build microfluidic chips. Among them, PDMS has become the most popular and its introduction into this field is regarded as one of the most important contributions to the development of microfluidics [3].

Figure 1.1. The process of rapid prototyping for fabrication of microfluidic structures.

Figure 1.2. The chemical structure and picture of PDMS. (A) The chemical structure of PDMS. (B) The picture of cured PDMS slab, showing that the cured PDMS is elastic and transparent.
PDMS is a series of silicone with similar chemical formulas (Figure 1.2A). According to different degrees of polymerization, PDMS is ranging from a liquid to a rubbery solid. The commercial PDMS which is used in microfluidics consists of two parts: a liquid PDMS with low degree of polymerization and a liquid cross-linking agent. Under heating, the mixture of the two parts is crosslinked into a rubbery solid (Figure 1.3). The solidified PDMS (Figure 1.2B) is transparent, gas permeable and biocompatible; so this material is highly suitable for optical detection and biological samples (e.g., biomolecules and cells) [22, 23, 31, 42]. In addition, the elasticity of PDMS facilitates the fabrication of a pneumatic valve (See 1.2) that has gained wide popularity. At last, the fabrication of PDMS chip is fast (generally less than 1h) with low cost, which in turn reduces the total time and cost for the experiments on such microchips [43].

![Chemical formulas of PDMS](image)

**Figure 1.3.** Crosslink of PDMS. In the presence of Pt-based catalyst, the vinyl group can react with Si-H group to crosslink.
1.1.2 Fabrication techniques in microfluidics

To fabricate micropatterns onto surface, there are several methods which are suitable for different materials. Although methods such as laser writing and e-beam lithography can generate microstructures with high resolutions, they are normally serial processing and require sophisticated equipment with high cost. Common techniques for forming microstructures in microfluidics are categorized below. (1) Photolithography [44-46]. Photolithography is a technique using light mask to transfer patterns onto a photosensitive substrate. According to different properties of the photosensitive materials [47], the exposed area can stay or be washed away after development. Although photolithographic techniques are limited in the fabrication of structures in photosensitive materials, it acts as the basic technique in current microfabrication because of its role in the rapid prototyping (Figure 1.1). The microstructures on photosensitive materials can be used as mask in etching or master in hot embossing and replica molding as described below. (2) Etching [48-51]. Etching is the process using certain agent to cut into unprotected parts of material surface. Etching is a basic fabrication method for glass, silicon and metal microstructures. (3) Hot embossing [34, 52-54]. Hot embossing is the process using master mold to imprint structures onto heat-soft substrate. This technique is generally used in microfabrication on PMMA, Teflon, etc. (4) Replica molding [31, 55-57]. Replica molding is the process using master to transfer structure into another material by solidifying a liquid when in contact with the master. This method is always used in the microstructure fabrication in PDMS, NOA, hydrogel, etc. Take PDMS as an example, the master is fabricated by rapid prototyping (See above); after placing some posts to define the positions of inlets and outlets, mixed prepolymer of PDMS is poured onto the master; the solid PDMS replica can be peeled off from the master after curing (Figure 1.4).

Once the microchannel structures are form onto a surface, this surface is bonded to a flat surface to obtain an enclosed microchannel system. Thermal bonding, which is irreversible, is
commonly used for the bonding of glass, silicon and thermoplastics such as PMMA. For comparison, PDMS can form conformal and reversible seal with flat surfaces of most solids because of its low surface tension. After plasma treatment, PDMS surface changes from hydrophobic to hydrophilic and can form irreversible sealing to surfaces of plasma-treated PDMS, glass and silicon [31] (Figure 1.4).

**Figure 1.4.** Schematic illustration of the fabrication of the enclosed microchannel in PDMS [31].

1.2 Microcomponents on Microfluidic Chips

A complex microfluidic chip is often required to perform different functions; therefore
microfluidic components with particular functions are important. For example, microvalves are used for controlling the flow in the channel [58-63], micromixers for mixing different solutions [64-67], and micropumps for driving the fluid flow [27, 68-71]. After the development for three decades, there are many certain designs and techniques for the microcomponents.

Among them, microvalve is probably the most important component that regulates and controls the flow of liquids in the microchannels. Microvalves can be roughly classified into two groups: active microvalves and passive valves. Examples of active valves include thermally actuated gels [72, 73]; paraffin [74]; liquid itself via rapid cooling and thawing [60]; magnetically actuated ferrofluid [59] or monoliths [58]; pneumatically, controlled membranes [63]; electronically or electrochemically controlled bubbles [68], and controlled surface wetting [75]. Passive valves provide a more affordable method to control the liquid flow because they do not need any moving parts. They include capillary valves [61], polymer check valves [76], elastomer valves [77], and hydrophobic valves [78].

Among all kind of valves, the pneumatically activated valves on the basis of soft-lithographic procedures by Quake, et al. [63] have become the most widely used. This valve is fabricated in PDMS with two perpendicular microchannels in different layers that are separated by a thin PDMS membrane. The bottom layer is the fluidic channel. The top is the control channel that is connected to a pressure regulator; the pressure applied to the top channel can press the in-between membrane down into the bottom channel and close it. Thus, the valve can control the fluid flow by changing the pressure in the top channel (Figure 1.5). Compared to other types of valves that have also been developed [73, 77, 79-83], this monolithic, pneumatic valve offers a number of advantages: simple to fabricate; easy to be integrated; fast in response time and easy to reach high density. In addition, this valve does not generate dead volume and its operations can be programmed under computer control [63, 84-86].
Figure 1.5. Schematic illustration of pneumatic valve. (A) Process flow of pneumatic valve fabrication. (B) Scheme of valve closing for square and rounded channels [63].

1.3 Construction of Concentration Gradients in Microfluidics

One of the important applications of microfluidic is in the construction of concentration gradients for biological studies. Concentration gradient plays an important role in many areas such as crystallization, drug screening [87-89] and chemotaxis study [90, 91]. There are two different kinds of concentration gradient. (1) Concentration gradient in continuous media.
This kind of gradient is suitable for study of chemotaxis [90, 92-94]. Traditionally, people use the Dunn Chamber (Figure 1.6A) for the generation of stable chemical gradients with linear profiles [92-94]. As shown in Figure 1.6, the wall between the two wells is slightly lower so that a gap will be formed after covering a glass slide. When the solution concentrations in the two wells are different, molecules will diffuse through the gap and form a linear gradient in-between. This design is borrowed to establish linear gradients of chemicals in microchips [90]. With an improved design on a microfluidic chip, arbitrary gradients of chemicals can be obtained by assembling arbitrary microchannel under a piece of hydrogel where a linear gradient has been established [95]. (2) Step-wise concentration gradients in separated wells. Compared to the continuous gradient in bulk solutions [87, 96-99], the concentration in each separated well is fixed without interference from neighbor wells in this case. The most widely used example in research is the 96-well plate (Figure 1.6B). The biggest advantages of microfluidic methods to establish such gradient are low sample consumption, large number of microwells in parallel and the ease of establish the gradients. Several methods have been demonstrated to establish such concentration gradients in microwells. Zheng, et al. used vacuum treated PDMS to suck the different solutions from Teflon tubes to microwells [100]. In this method, one should prepare the solutions which have different concentrations one by one. The “Christmas tree-shaped” microfluidic design [101] has also been used to generate concentration gradients in micowell arrays [102]. Although it is easier to form the gradient, the design of the whole chip is rather complicated. “Slip chip” [103, 104] is another potential method where one can slip a row of wells to meet another rows of wells on another chip in sequence. Mixing happens every time when the well row meets the rows in the well array. An exponential gradient can be formed in the well array.
Figure 1.6. Traditional methods for fabrication of concentration gradients in continuous media and separated wells. (A) The design of Dunn Chamber which is used for establishing gradient in continuous media. There is a gap between the coverslip and the bridge; the diffusion between the solutions in the inner and outer wells establishes a continuous gradient across the gap. (B) 96-well plate which is used for establishing gradient in separated wells.

1.4 Thesis Objectives

The design of the PDMS pneumatic valve (Figure 1.5) is the most widely used nowadays in microfluidics because of a number of unique advantages including ease of fabrication and control, small size, fast response, low dead volume and ready for integration. However, it also has two major limitations — it requires bulky and expensive external hardware (usually gas cylinders with pressure regulators) for controlling pressure, and the off-mode of the valve
requires constant pressure [63, 84-86]. These limitations hinder the applications of this valve in portable and disposable microfluidic devices. To overcome these problems, I improved the Quake’s valve by using a convenient and portable screw-actuated system (Chapter 2).

PDMS is useful and popular in microfluidics and some other research areas such as soft lithography and nanoscience, but it has its own problems so that PDMS is not always the first choice in many cases. Its disadvantages include absorption of small, hydrophobic molecules from solution and swelling up when it absorb hydrocarbon solvent [43]. So under some situations, other materials and corresponding fabrication methods are needed. In Chapter 3 and Chapter 4, I will discuss two such situations.

In Chapter 3, I focus on hydrogel materials for making microfluidic devices. Major advantage of hydrogel in microfluidics devices is that they can mimic extracellular matrix in real tissues and simulate physiological circumstances for the cells which are cultured in gel block. Such cell culturing in hydrogel is termed three-dimensional (3D) cell culture, which provides cells a more close-to-real environments than common two-dimensional (2D) cell culture in dishes [105-109]. However, 3D cell culture in block hydrogel encounters an issue of mass exchange: the cells that are cultured in the center of the gel block cannot get sufficient culture media and remove metabolized waste effectively because of long diffusion distance. Such situation becomes more and more severe when the gel block becomes bigger and bigger [105, 106]. The microchannel system in hydrogel can overcome this problem by acting as vascular system in real tissue. Unfortunately it is very difficult to fabricate well-defined microstructures in hydrogel and to fabricate microchannel systems in a hydrogel piece. There are a few existing methods that have been demonstrated, but each has major limitations such as low bonding strength, complicated process or being limited to only a specific hydrogel. In this chapter, I will describe a universal method to fabricate microstructures into general hydrogels.

The fabrication of microperforated membrane is another important example where
PDMS is not the best. Microperforated membranes have wide applications in many fields such as surface patterning, particle filtering and constructing 3D microfluidic devices [110-112]. The simplest and most popular way to make PDMS microperforated membrane is replica molding. A liquid PDMS prepolymer is first spincoated onto a master which has microposts. After solidify the PDMS, a microperforated membrane can be peeled off from the master [113]. Although PDMS is widely used, it has two problems in making microperforated membranes: (1) Because of the surface tension, liquid PDMS prepolymer will form meniscus surface around microposts so that one surface of the resulting PDMS membrane is not flat; this hinders its usage in constructing 3D microfluidic devices in which a microperforated membrane with two flat surfaces is required; (2) Because PDMS is soft and easy to stick to itself; it is hard to handle very thin PDMS membrane (e.g. < 50 μm). As a result, when we want to make small holes into PDMS membrane, we need high aspect ratio microposts to get a membrane thick enough. However, peeling a membrane off from a high aspect ratio structure is very challenge and not easy to complete without damage. So PDMS is not suitable for making perforated membrane with very small holes (e.g. <30 μm). In Chapter 4, I invented a strategy which is universal to all photosensitive materials to make microperforated membranes that can overcome these problems.

In Chapter 2 to Chapter 4, I focus on the problems in microfluidic fabrication techniques; while in Chapter 5 to Chapter 7, I mainly focus on the development of microchip platforms with our microfluidic techniques to establish concentration gradients in separated microcontainers and use the gradients for chemical analysis and study of cellular behavior.

In Chapter 5, I use valves to control the fluid in microchannels so that two solutions can mix in different microchambers with different volume ratios; these different volume ratios give different solution concentrations in chambers. In this application, I use the valve design which is described in Chapter 2 so that the device is diminished and gives easier handling properties in normal biological labs. I finally demonstrate a screening of anti-cancer drug on
this platform.

The platform described in Chapter 6 is more focused on how to generate duplicate gradient profile in multiple microwell arrays in parallel. I make a hydrogel slab which contains two parallel reservoirs; then two solutions with different concentrations in the reservoirs can form a gradient in between by diffusion. This gel slab with gradient is brought in contact with a gel-filled microwell array to print the gradient. After the gel slab is removed, a microwell array that requires gradient is aligned to this gel-filled one to transfer the gradient. For each gel slab which have existed gradient, it can be used to print multiple well arrays; and the gradient can be transferred from one array to another. Therefore this method is useful not only in making parallel concentration gradients but also combinational gradient of two substances.

In Chapter 7, I introduce a method that can form molecular gradients with wide concentration ranges, various steepness, and extremely large number of data points at varying concentrations. I pattern an array of hydrophilic microspots on a hydrophobic substrate that is aligned to a PDMS microchannel cover with a microperforated membrane. When an aqueous buffer-in-oil plug is flowed through the microchannel, the hydrophilic spots will attract a small amount of the buffer and form an array of buffer microdroplets along the channel. Subsequent flow of an aqueous solution plug that contains wanted solute through the microchannel partitions the solute molecules into the microdroplets—the droplets close to the inlet will have higher concentration of the solute than those close to the outlet. That is, a concentration gradient has been established in the array of microdroplets.

Although these three chapters are all concerned with concentration gradients, they are distinct from each other: the technique developed in Chapter 5 is good for generating very precise concentration gradients (however, the concentration range formed is narrow); the technique in Chapter 6 is suitable for the formation of parallel gradients and combinational 2D gradients of two solutions; and the method in Chapter 7 is appropriate for creating
gradients with wide concentration ranges, various steepness, and extremely large number of data points at varying concentrations.
Chapter 2.

A Screw-Actuated Pneumatic Valve for Portable, Disposable Microfluidics

This chapter describes a simple and inexpensive approach for controlling the pneumatic valves that are invented in Quake’s group to miniaturize the whole system for portable and disposable microfluidic devices. The valves are assembled from two parts. One is the polydimethylsiloxane (PDMS) part that includes multilayer structures of control channels and microfluidic channels formed by multilayer soft lithography. The other is a polymethylmethacrylate (PMMA) frame for pressure control. Turning the screws into the control channel inlet (filled with water and covered with a thin PDMS membrane) actuates the valve by creating pressure in the control channel. With this PMMA frame, the valve avoids the bulky and expensive external pressure-control facilities that are used in a normal pneumatic valve without losing most merits of Quake’s valve (simple to fabricate; easy to be integrated; no dead volume; and ready to gain high density). They can also be easily integrated into portable, disposable microfluidic devices for doing various reactions.

2.1 Introduction

Microfluidics has grown into a flourish field that holds the promise to offer better solutions for various areas such as bioanalysis and biomedical sciences with a number of advantages: small quantities of samples and reagents; high resolution and sensitivity of separation and detection; low cost; short times for analysis; and small size for portable devices [2, 3, 5-7]. There have been two contributions that are considered particularly important to the development of this field [3]. One is the development of soft lithography in polydimethylsiloxane (PDMS) as a method for fabricating prototype devices. The use of PDMS greatly reduces the cost and time for fabricating a microfluidic device; this material also offers a number of unique properties that are compatible with optical detection and
biological samples (e.g., biomolecules and cells) [22, 23, 31, 42]. The other is the development of a simple method of fabricating pneumatically activated valves on the basis of soft-lithographic procedures by Quake, et al [63]. This valve is fabricated in PDMS with two perpendicular microchannels in different layers that are separated by a thin PDMS membrane. The bottom layer is the fluidic channel. The top is the control channel that is connected to a pressure regulator; the pressure applied to the top channel can press the in-between membrane down into the bottom channel and close it. Thus, the valve can control the fluid flow by changing the pressure in the top channel. Although many other types of valves have also been developed [73, 77, 79-83], this monolithic, pneumatic valve has become the most widely used and offers a number of advantages: simple to fabricate; easy to be integrated; fast in response time and easy to reach high density. In addition, this valve does not generate dead volume and its operations can be programmed under computer control [63, 84-86]. However, it also has two major limitations: it requires bulky and expensive external hardware (usually gas cylinders with pressure regulators) for controlling pressure; and the off-mode of the valve requires constant pressure [63, 85, 86]. These limitations hinder the applications of this valve in portable and disposable microfluidic devices. Alternatively, Whitesides, et al demonstrated a torque-actuated valve that directly uses small machine screws to press on PDMS microchannels for controlling fluid flows [114]. Although the whole device is convenient with portable size, the large size of screws (~1 mm) makes it impossible to fabricate valves in high density. Also, each valve on a microchip needs to be treated individually during the fabrication. What’s more, Takayama, et al showed a computer-controlled Braille display can be used to operate the microvalves [115]. But it still takes a relative large space and needs an expensive Braille display. Here we describe the fabrication of miniaturized pneumatic microvalves that are conveniently actuated with small machine screws. While the whole valves are very small in size and only require machine screws and polymethylmethacrylate (PMMA) plates, we demonstrate their use in portable and disposable microfluidic devices.
2.2 Experimental Section

2.2.1 Fabrication of the PDMS part of microfluidic chips

We fabricated the device in PDMS (GE Silicones RTV 615, Wilton, CT, USA) with multilayer soft photolithography [22, 23, 63]. The silicon masters for control channels and fluidic channels were generated with SU-8 2025 (MicroChem, Newton, MA) and AZ 4620 (Clariant Corp.), respectively, by standard photolithography. AZ 4620 patterns were reflowed at 120 °C for 10 min to be rounded.

PDMS prepolymer (A:B = 10:1) was used to mold replica of the control channels in a 70 °C oven for 1 h. Cork borer (3 mm diameter) was used to make inlets of the control channels. PDMS prepolymer was spin-coated (500 rpm for 48 s) onto the mold with fluidic channels and partially cured at 70 °C for 10 min. The PDMS mold of control channels was placed onto this membrane and permanently bonded at 70 °C for 1 h. A syringe needle (~1 mm diameter) was used to punch holes in this bonded PDMS piece as the inlets and outlets for the fluidic channels. This PDMS piece was finally enclosed by bonding with a glass slide after plasma oxidization.

A separate PDMS thin membrane was prepared by spin-coating a layer of PDMS prepolymer on a glass slide at 500 rpm for 18 s and cured at 70 °C for 1 h. On top of this membrane, another layer of PDMS prepolymer was spin-coated at 500 rpm for 9 s plus 2000 rpm for 30 s and partially cured at 70 °C for 10 min. After the control channels were completely filled with a 5% SDS solution, this PDMS membrane was placed onto the control channels and permanently sealed the solution in the control channels within a enclosed Petri dish (with a few water drops inside) at 70 °C for 20 min.

2.2.2 Fabrication of control part for screw-assisted valve

The control part of screw-assisted valve was made up of two PMMA plates and ten steel screws. Ten screw holes were drilled equally spaced in a row; the last two screws at each side
were used for fixing chip and the central six screws are used for controlling the valves. For each controlling screw, we added a small drop of epoxy to the bottom so that the bottom of the screw became rounded; after solidifying the epoxy in air for 1 h, we applied a layer of grease on the epoxy.

2.2.3 Performance test of screw-assisted valve

We added some water on the surface of the chip. Then the chip was placed in a petri dish with some drops of water. After 10 days, this chip was used to test the valve property. We closed a valve on the chip and examined whether the channels were blocked. When the screw was twisted up again, the chip was placed under a stereoscope to examine whether the valve opened immediately. We repeated this close/open cycle for 15 times. Then we closed the valve again and waited for a relative long time before opening it. We tried 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 12 h, successively.

2.2.4 Demonstration of chemical reaction in screw-assisted valve-controlled chip

The reaction between crystal violet and sulfate acid was used to demonstrate the simplest usage of the valve. We first closed the valves at the center of the reaction channels, followed by injecting two solutions, 0.1 M crystal violet (Merck) in water and 0.5 M sulfate acid into two sides of the valves, respectively. Then other two valves at the ends of the reaction channels were closed to keep the solutions away from the solutions out of the reaction channels. At last, the valve at the center was opened to allow the two solutions to react with each other.

2.3 Results and Discussion

2.3.1 Fabrication of the microfluidic devices with screw-assisted valve.

Figure 2.1 schematically illustrates the fabrication process of the screw-actuated
pneumatic valve. The fabrication of the two layers of channels and their bonding are similar to those of the monolithic, pneumatic valves with multilayer soft lithography [63]. The completion of the valve control layer is different. In a normal pneumatic valve, the control channel is connected to an external pressure source (e.g., gas tank and pressure regulator). In our approach, after the control channels are completely filled with water by adding water directly into the inlet and waiting for several minutes to let water fill whole control channels, their inlets are permanently sealed with a thin (~300 μm) PDMS membrane to enclose the water. Adding a small amount of surfactant such as sodium dodecyl sulfate (SDS) in the water makes it much easier to fill into the channel. To prevent water evaporation from the control channel, the whole chip is placed in an environment saturated with water vapor during the sealing process (70 °C in an oven). After this sealing, the PDMS chip is completed and needs to be assembled with the pressure-control part. A machine screw is used to generate the pressure to close the underlying microfluidic channel by twisting it into the inlet of the control channel.

In order to hold the screws in position that is right above each inlet of the control channels, the microfluidic chip is placed between two parallel PMMA plates. For convenience of operations, the chip pattern is arranged into three grouped areas: control channel inlets are on one side; fluidic channel inlets on the other side and the channels in the middle (Figure 2.2). A set of threaded holes are drilled through the plates; the positions of these holes correspond to that of the control channel inlets. Two screws at the sides (screws 0 & 9) are used to hold the two plates together and adjust the space between them. After the PDMS chip is placed between these two plates with the inlet of each control channel right under each threaded hole, screws 1 & 8 are used to clamp the chip tightly in position. Other screws (# 2-7) can be twisted clockwise into the holes and press into the inlets. The whole device is now assembled and ready for use.
Figure 2.1. Schematic illustration of the fabrication process of the screw-assisted pneumatic valve. This schematic starts with a multilayer microfluidic structure that is created with soft-lithography. The dashed line in the third drawing shows the bonding interface of the control channel and the top membrane.

Figure 2.2. Assembly of the PDMS chip with the valve control frame. (A) Typical design of a microfluidic chip with pneumatic valves. The inlets of control channels and fluidic channels are intentionally separated into two sides of the design. (B) Photograph of the formed PDMS chip from the design shown in A. The inlets of control channel are numbered (2-7) to match
with the screws shown in C. (C) Photograph of PMMA frame with screws. The screws are numbered (0-9). (D) Assembly of the PDMS chip in B and the frame in C. The numbered inlets are placed into the positioned right under the screws with the same number.

2.3.2 Important values and tips in fabrication

In our experiments, the control channel inlets are generated with a 3-mm-diameter cork borer, thus 3-mm-diameter screws are used accordingly. The critical depth of the screws pressing into the inlets for complete valve close is dependent on (1) the thickness of the PDMS membrane that separates the two channel layers - we found 100 - 200 μm is the proper thickness (thinner membranes are easy to collapse and stick to the bottom surface of the fluid channels and thicker ones require high pressure to close the valve), and (2) the volume of the control channel inlet – an inlet (dia. ~3 mm) with height ~6 mm is optimal. Although the thickness of the PDMS membrane covering the control channel inlets does not affect this critical depth, 300 μm is the optimal thickness for our experiments: thinner membranes are easy to break and thicker ones are not elastic enough. To avoid the possible damage of this membrane from the rough surface of the screw, small amount of epoxy is applied to the bottom of each screw and cured to form a rounded surface; a thin layer of grease on the epoxy surface facilitates the turning of the screws.

2.3.3 Performance test of screw-assisted pneumatic valve

Figure 2.3 shows the photographs of three pneumatic valves that are controlled with screws on the same microchip. For comparison, only the middle valve is shown in both the open and the close states. The screw closes the valve completely with a depth of 2 mm into the control channel inlet; smaller penetration depth partially closes the valve. This capability of partially closing valves with easy control may be useful in some applications (e.g., filtering particles and trapping cells).
Figure 2.3. Photographs of functioning valves. Three screws (A) control three valves (B), and each valve controls three channels with the design shown in Figure 2.2. The screws and the valves are numbered according to their relation. When the middle screw (#2) is twisted into the control channel inlet for ~2 mm (C), the corresponding valve (#2) is closed completely (D). The dashed lines in B and D indicate the positions of corresponding valves.

We evaluate this valve in four aspects. The first is the effectiveness of closed valve. A basic solution containing a pH indicator (1 mM NaOH, with 0.1 M crystal violet) and an acidic solution (0.5 M sulfuric acid) are introduced into the two sides of a closed valve. No color change is observed after 12 h, which indicates that the solutions remain isolated in the two sides of the closed valve without leakage for at least 12 hours. The valve can be used without any impairment after this long-time closing state. The second is its repeatability. The valve can be opened and closed for over dozens of times without failing. The third is its response time. When the control channel is filled with water (viscosity ~1 mPa s at room temperature), the valve responds immediately to the pressure from the screws. Liquids with higher viscosity (e.g., silicone oil and NOA optical adhesives) can largely slow the response of the valve. A valve with silicone oil of a viscosity ~350 mPa s increases the response time to over 1 min to the close/open of the valve; this slow response is unwanted in most applications.
We fill the control channel with water for all our experiments. The response time is dependent on the rate of the actuation of the screws. Currently, we actuate the screws manually and it takes a couple of seconds to reach ~2 mm depth. The fourth is the storage of the chip. It is possible that the water in the control channel can evaporate during the storage and therefore degrade the function of the valve. The chips can be stored in a closed Petri dish that contains a few drops of water to create a humid environment. Our results show no obvious evaporation of water from the control channels after storage for at least 10 days in this Petri dish. The valves on the storage chip can still be closed and re-opened immediately under the control of screws.

2.3.4 Demonstration of chemical reaction in screw-assisted valve-controlled chip

To demonstrate the capability of this valve, we perform a base-acid reaction in a closed chamber on a PDMS chip. We choose this reaction because the performance of this reaction includes the basic steps for other types of reactions, i.e., metering and isolating reagents, mixing the reagents and monitoring the reaction. The base-acid reaction can be easily monitored by color with proper pH indicators. In Figure 2.4, the device (with total size of ~3 cm wide and 8 cm long) includes two microfluidic systems with the same design; each system has three microreactors synchronized by three groups of valves, which are controlled independently by three screws. The fluidic flow channels at the valve region have a cross section of a curved profile (150 μm wide and 10 μm tall), and the control channels are 150 μm wide and 50 μm tall. All the fluids are perfused into the fluidic channel by syringe. We deliver three aliquots of sodium hydroxide solution with volume of ~400 pL into the top microchambers by closing the center valves (twist the middle screw down) and opening the other two valves. After the top chamber is completely filled, the top valves are closed with screw 5. Sulfuric acid solution is delivered into the bottom microchambers with similar procedure by using screws 6&7. The middle valve is finally opened to mix the base and acid.
The pH indicator changes color and the images are collected.

**Figure 2.4.** Performing a base-acid reaction on a portable chip with screw-assisted valves. We use the chip design with two identical parts (each having three microreactors controlled by valves), as shown in Figure 2.2. The left column gives the schematics of the device; the middle is the photographs of the device and the right is the microphotographs of the valves on the right part of the device. The dashed lines in the right column indicate the position of the valves. (A) The assembled device with all six valves open. (B) For performing the reaction, the middle valves are closed by twisting down the screws 3 & 6. (C) NaOH solution (1 mM, with 0.1 M Crystal violet) and sulfuric acid solution (0.5 M) are injected into the top and
bottom channels, respectively. (D) All valves in the right design are closed and the valves in the left design are left untouched for comparison. (E) The middle valve in the right design is opened to allow the solutions to mix. The solutions in all three microchambers change color from purple to green, indicating the undergoing of the reaction. The valve (#3) in the left design shows no leakage of solutions. Valves with dark and light colors in the schematics on the left column indicate close and open states, respectively.

2.4 Conclusion

In this chapter, we have demonstrated convenient pneumatic valves for microfluidic devices that are actuated mechanically by machine screws. The method uses machine screws to replace the normally bulky external pressure source to mechanically actuate the pneumatic valves fabricated with multilayer soft lithography. We apply pressure on the water enclosed in the control channel by turning the screws to collapse the underlying microfluidic channels.

This screw-actuated pneumatic valve has a major disadvantage: it is relatively slow because of manual operation in current design (it takes a couple of seconds to rotate the screw to close or open a valve).

Compared to the normal actuation with external pressure source, this actuation method does not change the way how the pneumatic valve functions. Thus, it retains most merits of this valve: simple to fabricate; easy to be integrated; no dead volume; and ready to gain high density. In addition, this method greatly miniaturizes the whole device and makes the devices to be portable (small size) and disposable (cheap materials) without external facilities. This valve also has a power-off mode by turning the screws at the right position. Because the PMMA frame can be disassembled from the PDMS chip, it can be recycled for different chips to further lower the cost. This valve should be of interest to the scientists who work in the general areas of microfluidics, biotechnology and diagnostics.
Chapter 3

A Strategy of Mixed Gel to Make Microstructures in Hydrogel-Based Microchip

This chapter describes a strategy of using two-ingredient pre-gel solution to fabricate microfluidic channels in hydrogel or hydrogel dominant device by a two-step gelation, in which the first gelation is used to mold pattern and the second to bond patterned slabs together. We demonstrate this strategy in two mixed gel systems: agarose-alginate mixed gel and agarose-polyacrylamide mixed gel, which exploit different bonding methods. We show that the concentration of the second ingredient and the pore size of the first gel will affect the bonding efficiency according to different gel system. We think this strategy is universal and useful in making microstructures in hydrogel; and researchers can choose particular mixed gel system which is suitable for their own requirement.

3.1 Introduction

Hydrogels, as a substitute for the extracellular matrix, play an important role in biomedical applications [116-119]. The microfabrication of hydrogels is an important challenge in tissue engineering, cell biology, and physiology [36, 120-124]. For example, microchannels can be used to modulate the concentration of chemicals in bulk hydrogel and mimic the function of microvascular system in real tissue [35, 125]. There are two approaches to fabricate microchannels in hydrogels: (1) gelling a pre-gel around a network of channels followed by removing the network [126] and (2) layer-by-layer stacking of gel slabs which have been molded with microstructures [35, 127-130]. The first approach is limited by the inconvenience in handling the micron-size channel network. In the second approach, the weak adhesion between hydrogel slabs or hydrogel and other materials (e.g. glass) requires special bonding materials or mechanical pressure to seal, for example, microfluidic devices. Khademhosseini et al. [129] and Stroock et al. [127] used a bonding strategy in which
alginate/agarose gel on the surface of two slabs was dissolved/melted before the slabs were assembled and then gelled again. Tien and coworkers demonstrated a strategy of bonding two assembled gel slabs using reversible perturbation, which was caused by low molecular weight solutes [130]. Here, we demonstrate a strategy to fabricate sealed microfluidic structures that contain channels in devices constructed using hydrogel. We mix two kinds of pre-gel solutions which have different gelling conditions. When one of these solutions gels, the mixed gel becomes solid. Thus, we can mold microfluidic structures using mixed gels and only gelling one component in the mixture. We assemble two partially gelled mixed hydrogel slabs (or a mixed gel slab and a slab with another material) and gel the second component of the mixture. The second material gels at the interface between both slabs and crosslinks to neighboring monomers from the other slab; if the second slab is a homogenous material, the second component reacts with functional groups that are at the surface of the second slab (Figure 3.1).

**Figure 3.1.** Schematic illustration of constructing microchannel into mixed gel.
3.2 Experimental Section

3.2.1 Preparing of PDMS master

We fabricated the PDMS (GE Silicones RTV 615, Wilton, CT, USA) masters using soft photolithography [22, 23]. The silicon master which had the same pattern with the final PDMS master was generated with SU-8 (MicroChem, Newton, MA, USA), by standard photolithography. PDMS prepolymer (A:B = 10:1) was used to mold a replica of the silicon master in a 70 °C oven for 1 h. After peeled off, the PDMS slab was plasma oxidized (Plasma Cleaner/Sterilizer PDC-32G, Harrick Scientific Products, Pleasantville, NY, USA) and placed into an atmosphere of trichloro(1H, 1H, 2H, 2H-perfluorooctyl) silane (Sigma) overnight. PDMS prepolymer (A:B = 10:1) was used again to mold a replica of the first PDMS slab in a 70 °C oven for 3 h. This secondary PDMS master was plasma oxidized for 1 min right before molding mixed gel.

3.2.2 Fabrication of agarose-alginate mixed gel device

We prepared the agarose-alginate mixed pre-gel solution by dissolving sodium alginate powder (Fuchen Chemical Reagents Factory, Tianjin, China) and UltraPure™ agarose (Invitrogen) in de-ionized water. The mixture was heated on a 140 °C hotplate with stirring (300 rpm). After the mixed gel became homogeneous, we poured it onto the PDMS master and let it cool down to room temperature. We drilled holes with diameter of 1.0 mm in the gel slab. We prepared flat mixed gel slab by pouring mixed pre-gel solution directly onto a glass slide followed by cooling down the gel at room temperature. After using a paper to absorb the water on the surfaces, we assemble the two gel slabs together. We plugged a polyvinyl chloride (PVC) tube (outer diameter: 1.5 mm; inner diameter: 0.6 mm) into one inlet on the device and provided a gentle pressure to prevent leakage between the plastic tube and the inlet in the gel slab. We connected the PVC tube to a test tube with 0.1 M calcium chloride inside to inject the solution into the channel at 0.4 kPa by siphon effect. The solution was kept...
flowing for 1.5 h. Then the device was put into 0.1 M calcium chloride for 0.5h.

3.2.3 Fabrication of agarose-polyacrylamide mixed gel device

We first added 1-Hydroxyclohexyl phenyl ketone (Sigma) in hot water (70 °C) to produce a saturated photoinitiator solution. 13 mL such saturated solution was used to dilute 2 mL 30% acrylamide (Richjoint Chemical, Shanghai, China), 0.8% bisacrylamide (Damao Chemical, Tianjin, China) solution to make the final concentration of acrylamide to 4%. Then we added 0.6 g agarose powder to this solution and heated it on a hotplate with stirring to make a homogenous mixed gel solution. Then we poured this mixed gel into a PDMS mold (or a flat glass slide) to make a mixed gel slab with channel patterns (or a flat gel slab). After drilling holes at certain locations, the two slabs were put into a water-saturated nitrogen atmosphere and assembled together directly. Finally, the polyacrylamide was formed under exposure of 254 nm UV (from a 12 W UV lamp for thin layer chromatography detection) for 1 h. In three-layer structure fabrication, 10 mL saturated photoinitiator solution was used to dilute 5 mL 30% acrylamide, 0.8% bisacrylamide solution. After adding a few drops of pre-gel solution onto a PDMS mold, a glass slide was used to press onto the PDMS mold to yield a replica with through holes as the central layer. The other steps are the same as previous described.

3.2.4 Fabrication of gel-glass and gel-polyurethane device

We made 4% agarose-6% polyacrylamide mixed gel by the method described above in which 12 mL saturated photoinitiator solution was used to dilute 3 mL 30% acrylamide, 0.8% bisacrylamide solution. Polyurethane surface was obtained by spin-coating a layer of prepolymer (NOA 73, Norland Products Inc.) onto a glass slide followed by 365-nm-UV exposure. Polyurethane microperforated membrane was prepared using the method mentioned in Chapter 4. Polyurethane coated glass slide, polyurethane microperforated membrane and
bare glass slide were pre-treated by oxygen plasma for 50 s, followed by assembling with partially gelled agarose-polyacrylamide mixed gel slabs. At last, we exposed the device under 254 nm UV (from a 12 W UV lamp for thin layer chromatography detection) for 1 h.

3.2.5 Fluorescent microsphere leakage experiment
The first, we fabricated a bonded chip by our method with 1% agarose and 2% alginate. For control, we used another chip which was fabricated by the same process flow except any calcium chloride solution treatment. 1 μm fluorescent microsphere solution (Polysciences, Inc. CAT # 17154, Warrington, PA, USA) was filled into the channel of these two chips. Both chips were stored in a humid environment to prevent drying for 36 h.

3.2.6 Bursting pressure measurement
First, we filled a straight channel (2 mm in width and 2 mm in height) in gel-chip with dye by siphon effect, using a plastic tube connected to a dye-filled test tube. Then we blocked the plastic tube connecting to the other inlet. After that, we slowly lifted the test tube until the dye came out from the interface of the two gel slabs. We marked the final lever height (h) and calculated the bursting pressure by $P = \rho gh$, where $\rho$ represents the density of the dye solution, $g$ is gravity constant. In order to strengthen the connection between the plastic tube and the hydrogel, we used a plastic tube with diameter of 1.5 mm and took holes of 1.0 mm on the gel slab.

3.3 Results and Discussion
3.3.1 Fabrication of hydrogel microfluidic chip
We use two mixed gel systems to demonstrate our strategy: agarose-alginate mixed gel and agarose-polyacrylamide mixed gel. To make a mixed gel, we dissolve agarose powder in sodium alginate solution or polyacrylamide pre-gel solution (containing acrylamide,
bisacrylamide and photoinitiator) and we heat it to produce a homogeneous solution. This mixed pre-gel solution is poured on to a polydimethylsiloxane (PDMS) mold, which is fabricated using soft lithography [23]. The PDMS mold is pretreated with oxygen plasma; we add a few drops of water (optional) to avoid the adhesion of air bubbles. Upon cooling, agarose solidifies the mixed gel. We peel this mixed gel slab from the substrate and we drill holes in the slab to connect the channel to a syringe pump. We assemble the partially gelled slabs; we gel the second component in the mixed gel. For agarose-alginate mixed gels, we flow a solution of calcium chloride (0.1 M) which is able to crosslink the alginate monomers into the channel using a low pressure (~0.4 kPa, 1.5 h). We place the device into a solution of calcium chloride for 0.5 h to complete gelation. In agarose-polyacrylamide mixed gel system, the assembled slabs are exposed to UV in N₂ atmosphere. The N₂ is saturated with water to prevent gel drying. Figure 3.2 shows the channels which we fabricate in these two mixed gel systems. In agarose-alginate system, only the area within ~3 mm around the channels bond together. It seems not ideal but such an area is enough for most of the experiments, because even a small molecule will take hours to diffuse across such a distance. Besides, elongating the calcium treated time can strengthen the bonding. We find that our method can achieve a bursting pressure of 21.5 kPa when we elongate the calcium chloride flow time from 1.5 h to 18 h; while the device made up of two partially gelled slabs without calcium treatment can only afford a pressure of 1.1 kPa. In agarose-polyacrylamide system, Figure 3.2D shows a clear dark circle appears around the cross section of the channel. Such dark area can also be found at the surfaces of the gel device. When we directly polymerize the mixed gel slabs in the air, the radius of the dark circle is bigger (data not shown). So we conclude that this is caused by the trace oxygen in the nitrogen atmosphere.

During the assembling of partially gelled slabs, the water adsorbed on the slab surfaces is easy to partially fill the microchannel, especially when the channel size becomes small. The pre-gel molecules will diffuse from the mixed gel into this aqueous solution and gel in the
channel when we set the second gelling condition, thus block the channel. Two methods are exploited to overcome this problem in our experiments: (1) Use higher agarose concentration which can make the gel surface less wet; (2) Use a paper to cover the surface of gel slabs before assembling to absorb the extra water.

![Figure 3.2](image_url)

**Figure 3.2.** Photographs of channels which are fabricated in mixed gel. (A) A 100 μm wide, 80 μm deep channel in 2% agarose, 2% alginate mixed gel. (B) A 300 μm wide, 200 μm deep channel in 2% agarose, 1% alginate mixed gel. The size of the device is 3 inch long and 1 inch wide. (C) A 30 μm wide, 25 μm deep channel. (D) A channel with 70 μm height and 30 μm depth, fabricated in 4% agarose, 4% polyacrylamide mixed gel. The fluorescent light in (A) and (C) is from 1 μm fluorescent microspheres.

### 3.3.2 The difference between bonded and non-bonded hydrogel slabs

In order to demonstrate the bonding effect of our method, we carry out two experiments. In the first experiment, fluorescent microsphere solution is filled into the channel of bonded and non-bonded chips, respectively. After 36 h, both chips are examined under fluorescent...
microscope (Figure 3.3). From Figure 3.3 we can notice that our method can seal the channel very well and prevent fluorescent microspheres spreading out.

**Figure 3.3.** The images of fluorescent microspheres filled channels. Both images were taken at 36 h after we filled the channel with 1 μm fluorescent microspheres. (A) The image of a channel which was fabricated by our method. (B) The image of a channel which is formed by simply assembling two agarose-sodium alginate mixed gel slabs together without calcium ion crosslink. The scale bar represents 200 μm.

**Figure 3.4.** The relationship between the gel concentration and the bursting pressure of agarose-alginate mixed gel. (A) The bursting pressure of several mixed gel with same alginate concentration (2%). (B) The bursting pressure of several mixed gel with same agarose concentration (1%).
In the second experiment, we compare the bursting pressure of the bonded and non-bonded agarose-alginate mixed gel chips. We fabricate two chips for control by simply assembling gel slabs together: one is a chip made up of two agarose-sodium alginate mixed gel slabs without calcium crosslink; the other is a chip made up of two agarose-calcium alginate mixed gel slabs which immersed into the 0.1 M calcium chloride solution separately for 2 h before assembling. The bursting pressure of the former chip is 1.1 kPa; the latter one, 0.8 kPa (The agarose and alginate concentration of both chips are 1% and 2%, respectively). In contrast, the chips which are fabricated by our method have a bursting pressure ranging from 6 kPa to 12 kPa, depending on the gel concentration (Figure 3.4).

3.3.3 The roles and relationship of two ingredients in mixed gel system

The gelation process of the second ingredient at the interface of slabs is the most critical step in bonding. Generally, the bonding strength mainly relies on the concentration of the second pre-gel ingredient. For macromolecule pre-gel ingredients such as alginate, the molecules from each of the two slabs should diffuse into the other to ensure the bonding. In this case, the network of the first gel may behave as a diffusion obstacle. As a result, the concentration of the first gel will also affect the bonding strength (Figure 3.4). For small molecule pre-gel ingredient such as acrylamide, the pore size of the agarose network is too large to block the diffusion (around 100 nm for 2% w/w agarose [131]). So we can use agarose with much higher concentration in agarose-polyacrylamide system than it in agarose-alginate system. The high concentration of the agarose not only reduces the possibility of water to partially filling the channel, but also provides better mechanical property of the partially gelation gel. Therefore, smaller channels can be fabricated in a mixed gel system with small molecules as the second pre-gel ingredient. We successfully fabricate a channel with 30 μm width and 25 μm height in 4% agarose, 4% polyacrylamide mixed gel.
(Figure 3.3C. Unless stated, all the percentage of concentration in this chapter is weight/volume ratio).

3.3.4 The criteria in choosing ingredients for mixed hydrogel

Depending on different requirements for the application of hydrogel device, one can choose different hydrogels according to their intrinsic chemistry, surface property, gelling condition and so on. For the second gelation ingredient, different gelling requirement gives different application potential of the gel device. For example, in agarose-polyacrylamide mixed gel system, UV exposure is a contactless treatment to bond the slabs. It provides the probability to fabricate very complicated 3D structures by assembling over two hydrogel slabs (Figure 3.5A-B). However, UV exposure is a violent treatment for cell culture at the same time. It makes it impossible to encapsulate living cells in this UV-cured gel structure. In contrast, the gelation requirement of alginate is much gentler compare to polyacrylamide. The treatment using calcium solution will not obviously affect the cell viability [35]. As a result, the gels, such as alginate and collagen, with biocompatible gelling condition give the gel device a potential for applications related to cell culture. The proper gelling sequence of the two ingredients is also important because it could make the fabrication easy to handle. For example, if we gel alginate before agarose in agarose-alginate mixed gel system, the first step needs to be completed under a high temperature to prevent the gelation of agarose. In addition, we suggest the first-solidified gel should have a large network pore size compare to the second pre-gel molecules; otherwise it prevents the diffusion of the second ingredient at the interface of slabs. In agarose-polyacrylamide system, the acrylamide molecule is small enough (less than 1 nm) to freely diffuse in agarose network (with the pore size over tens of nm [131]). As a result, the agarose is suggested to be gelled first.
Figure 3.5. Images of channels in agarose-polyacrylamide mixed gel system. (A) Cross section of a structure composed of three gel slabs. The big black circle is an air bubble. (B) Photograph focusing on the bottom layer of network. The network on the top layer is not clear because it is out of focus. (C, D) A double-helix channel structure which is composed of two gel slabs and a polyurethane microperforated membrane in between. The channels are 200 μm wide. Diffusion of dye molecules make them seem wider.

3.3.5 Fabrication of microfluidic chip made up of hydrogel and other materials

Beside two mixed gel slabs, the bonding can be achieved between gel and other materials when the pre-gel chemical can link to the functional group on the surface of the other materials. We demonstrate such bonding between agarose-polyacrylamide mixed gel and other materials such as glass and polyurethane. Partially gelled 4% agarose, 6% polyacrylamide gel (with microchannel pattern) is covered onto oxygen plasma treated glass/polyurethane surface. After polymerization in water-saturated N₂ atmosphere, the
bonding is observed. We successfully establish 100 μL/min flow in 300 μm wide, 200 μm high and 4.5 cm long channels fabricated in both gel-glass devices and gel-polyurethane devices. Such a high flow rate demonstrates that the bonding is strong enough for most applications. Moreover, materials other than hydrogel can provide advantages in some cases.

In the device made of over two layers, a microperforated membrane is always needed in the central. The polyurethane microperforated membrane, advantageous in its better mechanical property, facilitates more complicated fabrication (Figure 3.5C-D).

### 3.4 Conclusion

In this chapter, we demonstrate a novel strategy of bonding distinct gel slabs (or gel slabs and other materials) to form sealed microfluidic structures. The key point of our method is that we mix two pre-gel solutions which have different gelling conditions. We demonstrate this strategy with two mixed gel systems: agarose-alginate and agarose-polyacrylamide mixed gel system. Bonding of the partially gelled slabs is the most critical step in fabricating hydrogel microchips. The crosslinking efficiency at the interface determines the bonding strength. As a result, the concentration of the second ingredient as well as the pore size of the first gel will affect the bonding performance.

To choose a proper mixed gel system for different applications, both intrinsic gel property and fabrication requirements should be considered. Materials which can bond to hydrogel also provide extra advantages depending on certain requirement. By using the principles mentioned above, we successfully fabricated a complicated 3D channel structure in hydrogel. We think other applications such as tissue engineering, drug screening and diffusion-based studies can also be realized by using this strategy in different mixed gel systems.
Chapter 4

Fabrication of Free-standing, Microperforated Membranes and their Applications in Microfluidics

This chapter describes a convenient method for the fabrication of freestanding, microperforated membranes in photocurable polymers using only one step of photolithography. We used photosensitive prepolymers to make the membranes and photolithography to define the micropatterns. We demonstrated the fabrication of single- and multilayer microperforated membranes in SU-8 photoresist and Norland Optical Adhesive prepolymer. These membranes can be used to pattern surfaces in various materials and to fabricate complex three-dimensional microfluidic channel structures.

4.1 Introduction

This chapter describes a convenient method for the generation of thin, perforated polymer membranes that contain single- or multi-layer structures and their applications in microfluidics. Free-standing, perforated membranes (or stencils) have three main functions in microfluidics. First, the membrane can be used as a filter to separate particles with different sizes. Second, the membranes can act as a shadow mask for surface patterning [111, 113, 132-141] (e.g., using chemical vapor deposition). This application simplifies the patterning of materials that are incompatible with conventional photolithography, because it does not require the use of photoresists or organic solvents. Third, the membranes can be used as components in the fabrication of three-dimensional (3D) microfluidic devices: the perforations in the membrane can be used as a filter or can interconnect channels that are positioned above and below the membrane to form networks of 3D channels [110, 112, 142-144].
Several methods are available that fabricate free-standing, perforated membranes containing ordered patterns of microstructures. Direct photolithography can pattern well-defined perforated membranes in photoresists, but it is limited to photoresists and most photoresists cannot generate free-standing membranes. Similarly, reactive ion etching (RIE) can fabricate free-standing, perforated membranes in more materials, e.g., parylene [134-136, 140, 141] and parylene-SU-8 bilayer membranes [137]. Such membranes are reversibly sealable and can pattern cells, lipid bilayers and proteins, however, three steps are required to produce the membranes (vapor deposition, photolithography, and RIE). In contrast, phase separation micromolding (PSµM) uses a substrate that contains microposts and a polymer solution that contracts during phase separation to fabricate perforated membranes [145, 146]. The contraction of the polymer along the vertical axis creates perforations in the polymer film, however, contraction along the horizontal axes deforms the perforations [146].

Alternatively, soft lithography provides a simple way that involves spin-coating a thin layer of liquid prepolymer on a substrate that contains microposts; the prepolymer, when cured, is peeled off from the substrate to produce a membrane that contains holes defined by the microposts. This method is used predominantly to fabricate perforated polydimethylsiloxane (PDMS) membranes [111, 113, 132, 139], however, the meniscus of the liquid prepolymer at the microposts produces irregular features at the surface of the membrane. In addition, thin PDMS membranes tear easily and are difficult to manipulate (they are very easy to stick to other materials and to itself). To produce smooth surfaces, a perforated membrane can be obtained by squeezing a solution of polymer between a flat substrate and a master patterned with microposts, however, because it is difficult to completely remove the polymer liquid thin layer between the substrate and microposts, non-through holes are often observed. The pressure required by the method can also easily damage the microposts [133, 145]. Overall, all the soft lithographic methods require a master that contains microposts that is generated with conventional photolithography.
Here, we introduce a photolithographic method that fabricates high-quality perforated membranes that are characterized by flat surfaces, well-defined thicknesses, and that can be detached easily from substrates. The membranes can be used to pattern surfaces and to fabricate 3D microstructures.

The principle of this method is simple (Figure 4.1): we fill the space between two planar substrates using photosensitive prepolymer; we pattern the photosensitive prepolymer using a mask that selectively blocks light; we remove the two planar substrates from the photopatterned polymer to produce a perforated membrane. The method requires a single step of photolithography only. This method is different from conventional photolithography in several ways: (1) the patterned material is not limited to photoresists but can be fabricated using all photocurable liquids (e.g., optical adhesives); (2) because the liquid is confined in two substrates, it is not necessary to treat the substrates to promote adhesion of each photoresist; (3) the thickness of the membrane is defined using spacers, with which more accurate and more uniform membranes are easier to fabricate compared with methods that use spin-coating; and (4) multi-layer structures can be formed in the perforated membranes using similar procedures. Finally, perforated membranes with moderate quality can be generated with a handheld UV lamp in a common laboratory, eliminating the need for a mask aligner and cleanroom.

4.2 Experimental Section

4.2.1 Fabrication of SU-8 membranes

We thoroughly mixed the two components of PDMS (GE Silicones RTV 615, Wilton, CT, USA) prepolymer (A:B = 10:1) and degassed the mixture under vacuum in a desiccator [31, 147]. This PDMS mixture was spin-coated (4000 rpm, 30 s) on to silicon wafers or other substrates and cured to form a thin PDMS layer on the substrates. We poured ~2 mL SU-8 2050 (MicroChem, Newton, MA, USA) onto a PDMS-coated silicon wafer and heated the
photoresist at 120 °C in an oven for 1.5 h to remove solvent in the photoresist. After being cooled down to room temperature, the pre-baked SU-8 became solid and was stored for use.

Figure 4.1. Fabrication of perforated membranes using SU-8 and NOA. (A) SU-8 prepolymer is sandwiched between two PDMS surfaces, which produces a flat membrane; exposure to light through a photomask produces a perforated membrane that can be simply peeled from the PDMS substrate. (Note: The dark gray colors in the third and forth steps represent polymerized SU-8.) (B) NOA is sandwiched between two substrates ((i) transparency film or (ii) PDMS); one substrate contains a transparency film that contains a pattern, which transmits light in regions and prevents the transmission of light in others; after exposure to light, the membrane can be removed.
We spin-coated PDMS prepolymer on a glass slide (4000 rpm, 30 s) and cured the PDMS in 70 °C oven for 1 h. The excess PDMS at the edges was cut away with a razor to ensure a flat substrate. To make an SU-8 membrane, we took proper amount of pre-baked SU-8 and placed it between two PDMS-coated glass slides, which were separated by two spacers at the ends of the slides. We used three kinds of spacers: Teflon membrane (~15 µm thick), cover slide (~150 µm thick), and silicon wafer (~500 µm thick); other thicknesses were achieved using combinations of these spacers. After being heated in a 120 °C oven for 5 min, the SU-8 melted and re-flowed to fill the gap between the two PDMS layers on the slides. Then a pair of office clips was used to fix the assembly together until it cooled to room temperature. After the top PDMS-coated slide was carefully removed from the SU-8, we gently placed a photomask with the desired pattern onto the SU-8 layer. Subsequently, we exposed the SU-8 layer by illuminating UV light through the photomask (the exposure time depends on the thickness of SU-8). After a post-bake at 95 °C in an oven for 10 min, we disassembled the glass slides and spacers and immersed the SU-8 membrane in its developer until the desired pattern was obtained. The perforated membrane was dried carefully using a nitrogen air gun and stored in a Petri dish for examination under an optical microscope and for future use.

4.2.2 Fabrication of NOA membranes

We spin-coated PDMS prepolymer on a glass slide (4000 rpm, 30 s for ~10 µm) and cured the PDMS in 70 °C oven for 1 h. The excess PDMS at the edges was cut away using a razor; a blank transparency film (PP2900, 3M Visual System Products, Europe) was placed onto the PDMS-covered glass slide. After spacers (e.g., two layers of Scotch™ tape for ~80 µm) were placed at the edges of the transparency film, we added a few drops of NOA 73 (Norland Products Inc., NJ, USA) on the transparency film. The NOA on the film was degassed under vacuum in a desiccator for 10 min. We placed another transparency film that contained printed patterns on one side onto a PDMS-coated glass slide with the blank side of
the film contacting the PDMS. This film was directly placed onto the NOA with the patterned side of the film in contact with NOA. We fixed the device using two clamps, which were positioned at the sides of the glass slides; the NOA was sandwiched into a thin membrane between the substrates.

We used UV light (365 nm, 18 mJ/cm$^2$, 20 s) from a mask aligner to polymerize the NOA membrane. We also used UV light from a thin-layer chromatography chamber (3 min) to polymerize another NOA membrane. We disassembled the device to separate the NOA membrane from both substrates. The NOA membrane was subsequently developed in acetone to remove the unexposed area. After development, the membrane was exposed to UV light to complete polymerization (365 nm, 18 mJ/cm$^2$, 120 s or handheld UV lamp for 15 min). The fabricated NOA membrane was examined under an optical microscope and stored in a Petri dish for future use.

### 4.2.3 Fabrication of NOA membranes that contain a 3D microstructure

We fabricated PDMS slabs that contained features using soft lithography as described previously [31, 147]. We used a glass slide to support one PDMS slab. We aligned the other PDMS slab to a photomask that was attached to a glass slide. We used two 150 μm thick glass slides as spacer. We applied NOA between the two patterned PDMS slabs and we exposed the device to UV light (365 nm, 18 mJ/cm$^2$, 1 min). We removed the exposed NOA membrane, developed it in acetone (0.5 h), and dried it in air. We used this NOA membrane and two flat PDMS slabs (one of which has holes as inlets and outlets) to form a sealed 3D microfluidic system.

### 4.2.4 Micropatterning of protein on PDMS

We exposed a ~100-μm thick SU-8 membrane to oxygen plasma (1 min) and we sealed a layer of PDMS to the SU-8 membrane. We placed FITC-BSA (0.1 mL of a 0.05 mg/mL
solution in PBS, pH 7.4; Sigma) on the membrane. We placed the PDMS in vacuum (5 min), so that the air bubbles that were trapped when adding the solution were removed. We incubated the device (6 h, 4 °C), we removed the FITC-BSA solution, and we rinsed the device in PBS (pH 7.4). We removed the SU-8 membrane and imaged its surface using fluorescence microscopy (Nikon Eclipse TE2000-U, Japan).

4.2.5 Micropatterning of metallic thin films on glass

We fixed SU-8 membranes on a glass slide using Scotch tape or directly attached NOA membranes to a glass slide. We sputtered metallic thin films (5 nm Cr as adhesive layer and then 100 nm Au or Pt) with the desired thickness onto the slides using a DC magnetic sputtering system (ARC-12M, Plasma Science Inc., USA). The perforated membranes were removed and the patterned slides were examined under an optical microscope.

4.2.6 Micropatterning of cells on a Petri dish

HeLa cells were handled in a sterile tissue culture hoods and we maintained the cells in Eagle’s minimum essential medium (MEM)/10% fetal bovine serum (FBS) supplemented with 100 U/mL penicillin and 100 mg/mL streptomycin (all chemicals and reagents were purchased from Gibco, Invitrogen, NY, USA). We incubated the cells in an incubator at 37 °C with 5% CO2. We passed the cells using 0.25% trypsin (Gibco, Invitrogen, NY) and we neutralized the trypsin using MEM medium. We centrifuged the cell suspension (1250 rpm, 3 min) and we resuspended the cells in MEM medium. We directly attached a ~80-μm-thick NOA membrane to a sterile Petri dish and sterilized it using UV light (30 min). We seeded the cells to the NOA covered dish (2x10^5 cells/mL). After two days of incubation, we peeled off the NOA membrane and we examined the patterns of cells using optical microscopy.

4.3 Results and Discussion
We demonstrated our method with two photosensitive polymers: SU-8, a negative photoresist that seals reversibly to PDMS, and Norland Optical Adhesive (NOA), a photocurable adhesive that, when cured, seals reversibly to transparency film and PDMS.

4.3.1 Fabrication of SU-8 membranes

Figure 4.1A schematically illustrates the method that we use to fabricate perforated membranes in SU-8 photoresist. We take an appropriate amount of stock SU-8 photoresist (pre-baked) and place it between two PDMS-coated glass slides that are separated by two spacers at the sides. We heat this pre-baked SU-8 photoresist at 120 °C in an oven for 5 min. After the SU-8 photoresist is melted, we quickly fix the two PDMS surfaces in position with clamps and allow the assembly to cool. After cooling down to room temperature, a dried SU-8 prepolymer membrane is formed between the two flat PDMS surfaces. Because the strength of the clamps are weak and the PDMS layers are relatively thin (~100 µm), the thickness of the formed SU-8 membrane is equal to the thickness of the spacers. The top PDMS is removed and replaced by a photomask for photolithography. After UV exposure and post-bake, SU-8 polymer that has not been exposed to UV light is washed away in SU-8 developer.

We fabricate three SU-8 membranes with feature sizes down to 10 µm and thickness from 15 µm to 500 µm using this method (Figure 4.2A-C). Our method has two technical improvements that facilitate the process: (1) the thickness of formed membranes is accurately controlled. The thickness of the membrane is determined only by the thickness of the spacers. It is also possible to make membranes with thicknesses up to 1 mm using thicker spacers; (2) pretreatment of SU-8 prepolymer in large amounts at one time saves time: a stock supply of soft-baked SU-8 can be stored in lieu of an experiment. For other methods that require the fabrication of SU-8 structures with thicknesses greater than 200 µm, it takes a long time (a few hours) to dry the thick photoresist during the soft-baking step. In our method, the
pre-baked SU-8 prepolymer is stored and divided into several small portions; it is only necessary to reheat an appropriate amount of the prebaked prepolymer at 120 °C for only ~5 min to make it ready for subsequent UV exposure.

4.3.2 Fabrication of NOA membranes

Figure 4.1B illustrates the method for fabricating a perforated membrane using NOA and either 3M transparency film or PDMS as the substrate. We describe the method using 3M transparency film in the following (Figure 4.1B-i). The polymer solution is placed onto the transparency film, which is supported by a PDMS-coated glass slide. We use a layer of PDMS, so that the film that contains printed micropatterns will seal conformally to the glass slide; we cut the higher edge of a spin-coated layer of PDMS to ensure a flat substrate. The degassing step is used to avoid bubbles during sandwiching the liquid NOA prepolymer between two substrates.

Figure 4.2D shows a pattern of square holes that is produced using photolithography with collimated light. In the example described, 3M film – the photomask – is placed in direct contact with the NOA polymer. Because NOA prepolymer is liquid before it is photocured, it can easily stick to the photomask transparency and contaminate it.

To protect the pattern on the film, a thin layer of PDMS is spin-coated and cured on the patterned side of the top transparency film (Figure 4.1B-ii). With the layer of PDMS in the middle, the transparency does not directly contact the liquid NOA prepolymer. This PDMS layer is required to be thin enough (less than 1/10 of the smallest features on the pattern) to maintain pattern fidelity during pattern transfer with UV exposure. Using PDMS also gives this method the potential to fabricate multilayer structures in NOA membranes (see below). Figure 4.2E shows a pattern of circular holes that is produced by exposing the NOA liquid to a non-collimated light source (handheld UV lamp commonly used in thin-layer chromatography). Although the fidelity of pattern transferred is not perfect, the elimination of
mask aligner and cleanroom makes this method convenient and accessible to almost all researchers in related fields.

4.3.3 Smallest feature size of membranes

The smallest feature size of our membranes depends on the thickness of the membranes and the largest aspect ratio that can be achieved. Thin membranes always suffer from low mechanical strength, which makes the membranes delicate and hard to remove from the substrate. As SU-8 and NOA have different mechanical strengths and different bonding strengths to the substrates, membranes fabricated from these materials are easily handled when the thickness of the SU-8 membrane is greater than ~15 μm or the thickness of the NOA membrane is greater than ~50 μm. The aspect ratio is largely determined by the light source; collimated light generates features with higher aspect ratios than non-collimated light (e.g., handheld UV lamp) does. Currently, the smallest features that we can achieve are ~10 μm with a mask aligner and ~200 μm with UV handheld UV lamp.

4.3.4 Criteria for substrate selecting

The choice of substrate is critical in the two strategies presented here. We have identified three criteria for selecting an appropriate substrate. (1) The surface of the substrate does not strongly adhere to the formed membrane so that the membrane can be separated after polymerization. (2) The hardness of the substrate should be appropriate, for example, a hard SU-8 membrane requires a soft and elastic substrate like PDMS, which facilitates easy removal of the membrane from the substrate and prevents cracking of the membrane. (3) The top substrate (other than the photomask) should be transparent to allow light pass for UV exposure.
Figure 4.2. Microphotographs of SU-8/NOA membranes that contain various patterns. (A) An 80-μm thick SU-8 membrane. (B) A 500-μm thick SU-8 membrane. (C) An array of 10-μm diameter circles in a 15-μm thick SU-8 membrane. (D) An array of 100-μm side squares in an 80-μm thick NOA membrane. (E) An array of circles that is fabricated using a membrane that contained an array of 300 μm-diameter circles and and a non-collimated light source. The scale bars in (C), (D) and (E) represent 100 μm, 200 μm and 500 μm, respectively.
**Figure 4.3.** Photographs of surface micropatterning using SU-8 and NOA membranes. (A) FITC-BSA is patterned on the PDMS surface using an SU-8 membrane. (B) A pattern of Au sputtered onto a glass slide using an SU-8 membrane as a template. (C) A pattern of Pt sputtered onto a glass slide using an NOA membrane. (D) A pattern of HeLa cells on a commercial culture dish, which was produced using an NOA membrane. The scale bars in A, C and D represent 200 µm and in B represents 500 µm.

### 4.3.5 Micropatterning

Both SU-8 and NOA microperforated membranes can be used as masks for patterning various materials. We patterned proteins on PDMS using an SU-8 membrane, thin metal films on glass using both SU-8 and NOA membranes, and cells on a commercial Petri dish using an NOA membrane (Figure 4.3). The fidelity of the pattern produced by metal sputtering is acceptable for feature sizes greater than 200 µm with SU-8 membranes; however, for smaller feature sizes, we experience difficulties in producing high-quality patterns with sharp edges. These problems are caused by the stiffness of SU-8 membranes, which are unable to conform...
to substrates. In contrast, NOA membranes adhere to surfaces better than SU-8 membranes, which improves the range of feature sizes that can be produced using the method described (Figure 4.3C). The same strategy can be used to pattern cells; compare to seeding cells followed by selectively surface modification [110, 113, 139, 148, 149], the use of perforated membranes as a mask to pattern cells is more straightforward [133, 140]. The ability of NOA membranes to act as templates for the fabrication of patterns of metal and cells (Figure 4.3D) makes it a viable alternative to PDMS and parylene for these particular applications. One drawback of NOA membranes is that, being soft, the membrane can deform to some extent when they contain certain perforations with large length-to-width ratio, e.g., long lines. For these types of perforations, our experience using several photosensitive polymers indicates that SU-8 (the hardest) is most appropriate, NOA is less appropriate, and PDMS (the least hard) is least appropriate for the fabrication of membranes.

4.3.6 Fabrication of 3D microfluidic devices

A 3D channel structure can be topologically mapped onto a flat surface so that it only contains channels and crossings where channels at different levels overlap on the 2D projection. To reconstruct any 3D channel structure, we can mathematically decompose it into three layers: the top and bottom layers containing fluidic channels that are connected by a middle membrane with perforated holes. The middle perforated membrane is important because it enables two channels at different levels to cross each other without connecting them. Because the surfaces of our perforated membranes are flat, they are ideal components for the construction of 3D microfluidic devices. Figure 4.4A shows an example of fabricating a microfluidic device that contains a double-helix structure with an SU-8 perforated membrane. We have demonstrated the operation of the 3D structures with red and blue inks. We also fabricate other types of 3D channel structures without channel crossings. In Figure 4.4B, a 3D micromixer [150] is generated with a thin perforated SU-8 membrane sandwiched
between two PDMS channel pieces. We use a syringe pump to fill the channels with blue ink and water and this microfluidic device is able to operate well at tested flow rates (1-100 µL/min).

**Figure 4.4.** Fabrication of 3D microfluidic chips using PDMS and SU-8 membranes. (A) A double-helix channel structure. The channel size is 200 µm. Red and blue dyes are used to show the channels. (B) A 3D micro-mixer. Blue dye and water are mixed in the channel. The channel size is 400 µm.

All the formed membranes have two flat surfaces and through holes. However, we can also generate membranes with both through holes and channels on two surfaces. The fabrication process is similar to the membranes shown before; the difference is that the flat PDMS substrates are replaced with patterned PDMS pieces (Figure 4.5A). The patterns on the PDMS substrates have been transferred into the membranes after UV exposure and development. In this manner, the 3D channel crossings are realized in the perforated membrane; to complete the desired 3D microfluidic structure, only two flat PDMS slabs are required to seal the top and bottom of the channels in the membrane. Figure 4.5B-D shows an example of a 3D channel structure that contains a basket-weave pattern using an NOA membrane sandwiched between two PDMS slabs that contain a multilayer structure.
4.5 Fabrication of 3D microfluidic chips using PDMS and NOA membranes. (A) Schematic illustration of the fabrication method. The ‘*’ in the second picture indicates that the dark regions are polymerized NOA. (B, C) Scheme of the design for channels on two PDMS slabs; the combination of these designs is the shape of the channel produced ultimately in the NOA membrane. (D) Photograph of dyes in a 3D microfluidic chip. The channels are 300 μm in width.

4.4 Conclusion

In this chapter, we have demonstrated a convenient method that can fabricate free-standing, perforated membranes in photocurable materials such as SU-8 and NOA polymers. Membranes having 3D microstructures can also be formed with similar procedures. These formed membranes can be used to pattern surfaces and to construct 3D microfluidic structures. Because SU-8 membranes cannot conformally seal to hard materials such as glass and polystyrene, it can be used to pattern certain substrates such as PDMS to which SU-8 can seal conformally. In contrast, NOA membranes can seal conformally to both hard and soft substrates including glass, PDMS, and polystyrene. Noting that conventional
photolithography is limited to the fabrication of SU-8 perforated membranes, our method is advantageous in making perforated membranes in various materials with extended applications. In addition, our method for fabricating membranes presents several other advantages over existing methods: (1) only one step of photolithography is required; (2) the surfaces of the membranes are flat; (3) little treatment of substrate surfaces is needed and (4) the thickness of the membrane can be easily controlled ranging from tens of microns to millimeters. We believe that our method will be of interest to people working in microfluidics and bioengineering.
Chapter 5

A Prototypic Microfluidic Platform Generating Stepwise Concentration Gradients for Real-time Study of Cell Apoptosis

This work describes the development of a prototypic microfluidic platform for the generation of stepwise concentration gradients of drugs. A sensitive apoptotic analysis method is integrated into this microfluidic system for studying apoptosis of HeLa cells under the influence of anticancer drug, etoposide, with various concentrations in parallel; it measures the YFP/CFP FRET signal that responds to the activation of caspase-3, an indicator of cell apoptosis. Sets of microfluidic valves on the chip generate stepwise concentration gradient of etoposide in various cell-culture microchambers. The FRET signals from multiple chambers are simultaneously monitored under a fluorescent microscope for long-time observation and the on-chip results are compared with those from 96-well plate study and the MTT assay. The microfluidic platform shows several advantages including high-throughput capacity, low drug consumption and high sensitivity.

5.1 Introduction

This chapter describes a multiple-dilution microfluidic device for the generation of stepwise drug concentration gradients that is used for studying cell apoptotic processes simultaneously under various concentrations.

It is believed that the key event of programmed cell death process is the activation of intracellular cysteine-aspartic acid proteases (caspases), which induces the start of cell apoptosis [151-153]. Nicholson et al. [154] have identified the tetrapeptide of Asp-Glu-Val-Asp (DEVD) as a consensus cleavage site for caspase-3. Based on this discovery, many fluorescent analysis methods are demonstrated to successfully measure caspase-3 activity during cell apoptosis. For example, a cell-based detection system has been developed
for monitoring the dynamics of caspase-3 activation in live cells. This system uses a fluorescence resonance energy transfer (FRET) probe generated by fusing a cyan fluorescent protein (CFP) and a yellow fluorescent protein (YFP) with a peptide linker containing the caspase-3 cleavage sequence DEVD [155]. Activation of caspase-3 is a hallmark event of apoptosis [151-153]; during cell apoptosis, activated caspase-3 cleaves the peptide linker to separate YFP from CFP and alters the FRET signal. By measuring the relative intensities of the cyan and yellow fluorescence (i.e., the FRET signal) of HeLa cells transfected with the plasmid of this FRET probe at different time, one can determine whether, when and how fast the cells go through apoptosis. Using the stable cell line of HeLa-C3 cells which can constitutively produce this caspase sensor, ~3 fold changes in the emission ratio between the acceptor and donor upon the activation of caspase-3 was detected from previous studies [155, 156]. In this study, we introduce this system into a microfluidic chip that can run multiple apoptotic assays of HeLa-C3 cells at various drug concentrations simultaneously. Different from the conventional MTT assay which measures the activity of dehydrogenase enzymes (UV-Vis absorption is measured) in cell populations, the FRET system directly analyzes the activity of caspase-3 in same cells at different time points. This system is easier to be integrated into a microfluidic chip than conventional methods. Stepwise gradients of drugs in microchambers are generated on the chip with integrated microfluidic valves to mix drug stock solution with culture medium at different ratios. We monitored real-time signal changes of each individual cell during cell apoptosis under the influence of drugs at various concentrations with fluorescence microscopy. The validity of this microfluidic technique is confirmed by comparing the on-chip results with those from standard MTT assay and 96-well plate assay.

**5.2 Experimental Section**

**5.2.1 Cell seeding and culture**
The cell line HeLa-C3 was manipulated under sterile tissue culture hoods and maintained in Eagle minimum essential medium (MEM)/10% fetal bovine serum (FBS) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin (all purchased from Gibco, Invitrogen, NY), 50 μg/mL G-418 solution (Roche, Switzerland). The cells were cultured in humid incubator at 37 °C with 5% CO₂. The device was sterilized under the UV treatment for 30 min before use. The surface of channels was treated with a 0.01% poly-L-lysine (Sigma) solution for 30 min to enhance cell adhesion. The cell passage was achieved with standard 0.25% trypsin (Gibco, Invitrogen, NY) and neutralized with MEM medium. The cell suspension solution was centrifuged at 1250 rpm for 3 min and the cells were resuspended in the MEM medium. The cells was counted with a hemocytometer and diluted to the density of 5×10⁷ cells/mL. A programmed syringe pump (Harvard Apparatus, MA) injected the cell suspension into the microfluidic device.

5.2.2 Drug exposure and analysis

The stock solution of etoposide (Sigma) was 100 μM. After cells were seeded on the chip for 12 h, the drug was perfused into the cell chambers. Multiple concentrations of etoposide were generated in different microchambers on the microfluidic chip for apoptosis study in parallel. Fluorescent images of the HeLa-C3 cells were captured by an inverse fluorescence microscope (Nikon Eclipse TE2000-U, Japan) at different times (0 h, 12 h, 24 h, 36 h, 48 h) with excitation filter at 436 ± 20 nm, a dichroic mirror at 455 nm, and emission at 535 ± 15 nm (YFP) and 480 ± 20 nm (CFP). Images were recorded with a computerized cooled CCD camera (Diagnostic Instruments, MI). The cell culture chip was kept in a cell incubator at 37 °C with 5% CO₂ supplement at all time expect for imaging.

The fluorescence signals from apoptosis markers of FRET probes (CFP and YFP fusion protein) were analyzed using image processing and analysis software (AlphaEaseFC, CA). The fluorescence of each cell was corrected by subtracting the averaged local background.
To verify cell apoptosis, immediately after the on-chip study, 4 μg/mL Hoechst 33342 (Molecular Probes, Eugene, OR) was perfused into the channel and incubated at 37 °C for 20 min. The cells were rinsed with PBS and imaged immediately.

5.2.3 Measurement of FRET signals on a 96-well plate

Cells were grown in a 96-well plate overnight with a density of 5×10^3-8×10^3 per well and treated with etoposide at different concentrations. At different time points, emission signals of YFP (535 ± 8 nm) and CFP (486 ± 8 nm) were measured by a fluorescent plate reader (Perkin–Elmer Victor, MA) with excitation at 440 ± 10 nm. The background fluorescence was measured from the wells containing only the culture medium.

5.2.4 MTT assay

Cells were grown in a 96-well plate and treated with the drug at different concentrations. At the indicated time points, 10 μL of the MTT labeling reagent (Sigma) was added into each well and the plate was incubated for 4 h in a cell incubator before a 100 μL solubilization solution was added into each well. The plate was incubated overnight in the incubator before its absorbance at 595 nm was measured with a plate reader (Perkin–Elmer Victor, MA).

5.3 Results and Discussion

5.3.1 Microchip fabrication

The microfluidic device was fabricated with standard multilayer soft-lithographic technology with slight changes (Figure 5.1) [63, 157]. In normal PDMS peristaltic valves [63, 158], fluid channels are normally low (maximum height of ~10-20 μm) with a rounded cross section; the maximum height of the channel is limited by the thickness of positive photoresist that can be obtained with spin-coating during photolithography. Cells (~10-20 μm in size) that flow in such low channels are very easy to stick to channel walls and to break. To avoid this
problem, we made high microchannels (~80 μm high) with a rounded cross-sectional profile by replicating high features of positive photoresist from negative SU-8 photoresist, which is well-known for tall features to 1 mm thick. Silicon masters with SU-8 photoresist are generated with photolithography. The channel design is patterned into SU-8 photoresist with ~130 μm thick, which had a rectangular cross section. A PDMS channel is then molded from this SU-8-silicon master at a 70 °C oven for 1 h. After conformally sealed with a glass slide, the PDMS channel is filled with positive photoresist and placed in a hood overnight to remove the solvent in the photoresist. After the photoresist is dry, the PDMS channel is peeled off to leave a photoresist mold (~80 μm thick with solvent removed) on the glass. This glass slide is subsequently placed on a 100 °C hotplate for 10 min to melt and round the photoresist pattern. A thin layer of PDMS prepolymer is spin-coated onto this rounded photoresist pattern; heating the PDMS prepolymer in a 60 °C oven for ~8 min partly cures the PDMS layer.

Another SU-8 master with different design is used to generate open PDMS channels for valve control. The valve control channels are aligned and placed onto the partly cured PDMS layer on the rounded photoresist pattern; heating in a 70 °C oven fully cures the PDMS and bonds the two PDMS layers together. After plasma treated for 1 min, this multilayer PDMS piece is permanently bonded with a flat PDMS substrate to form the entire PDMS device. The control channels are filled with water and controlled by screw-driven structures [158] (See Chapter 2), which make the setup convenient to place the whole setup in cell incubator.

5.3.2 Generation of stepwise gradients of drug concentration on microfluidic chip

One major advantage gained by using microfluidic chips is that multiple samples can be studied simultaneously, thus increasing the throughput and reducing the error by running the samples in parallel.
Figure 5.1. Schematic illustration of the fabrication of the microfluidic device.

Figure 5.2 shows the design of the microfluidic device that is used to form stepwise concentration gradients of compounds in parallel. It can be conveniently fabricated by soft lithography and has two layers. The bottom layer is the fluidic channel network in a “ladder” shape with four fluid inlets/outlets. The top layer is a system of three groups (Group 1, 2 & 3) of microvalves for controlling the fluid flow underneath; each group consists of many microfluidic valves that are connected to each other and synchronized in operation. Valve Group 2 blocks all the middle channels at the rung position of the ladder in the fluidic layer and forms one microchamber (middle chamber) in each “rung” channel. When closed, valve
Group 2 and Group 3 form two side microchambers (side chambers) in each “rung” channel. The position of each valve of Group 3 is shifted from its neighbors so that the volume ratio of solutions at the two side chambers is changed along the ladder. If the solution at one side is buffer for dilution, stepwise gradient of solution concentration can be created in the microchambers along the ladder. The exact concentration in each microchamber is determined by the original concentration of the solution and the volume ratio of the side chambers. We achieved two orders of magnitude of dilution in our current device. In principle, more dilutions can be obtained. Valve Group 1 is used to close the side channels to ensure that an injected solution, particularly cell culture, will flow through the “rung” channels without any bubble trapped; which is important to seed cells in the middle chamber (see section below).

We demonstrate the capability of this device for the generation of stepwise concentration gradients by mixing an acid solution and DI water at increasing ratios to create solutions with increased pH values in the chambers. Figure 5.2A is the illustration of the process with corresponding images of the microchip in Figure 5.2B. After Valve Group 1 is closed, a 5% crystal violet solution (a pH indicator) is filled into the entire channel. Valve Group 2 is closed to trap a small amount of the solution in the middle microchambers and segregate the flows in the channels at the two sides of the ladder, which are filled with an acid solution (0.5 M sulfuric acid) and DI water, respectively. Valve Group 3 is closed to accurately meter an intended amount of acid solution and water in the side chambers. Valve Group 2 is opened to allow the acid and water to mix and generate stepwise pH gradients in the microchambers along the ladder. The pH change in different microchambers can be clearly seen from the color variation of the pH indicator.
**Figure 5.2.** Operation of the microchip to generate stepwise gradient. (A) Schematic illustration of the operation. Valve channels (Groups 1, 2 & 3) are shown in red (valve is closed) and pink (valve is open). The fluidic channels are in the shape of a “ladder” (blue, grey, and black colors stand for different solutions). (B-D) Generation of stepwise gradient of pH in the microchambers. Crystal violet (a pH indicator) is added into the solution to visualize the formed pH values. The lower three figures in A correspond to B, C and left half of D, respectively.

### 5.3.3 Quantification of solution concentrations in the microchambers

We quantify the concentrations of solutions formed in the microchambers with sodium
fluorescein. Fluorescein solutions with different concentrations (0-10 mM) are injected into the microchannels and fluorescent intensity in the microchambers are measured. All the fluorescent intensities are offset with the fluorescent intensity of pure water (0 mM fluorescein). Within the measured concentration region, fluorescent intensity increases linearly with concentration (Figure 5.3A).

With the operation in Figure 5.2, stepwise gradient of fluorescein solution is generated in the microchambers. The concentration of solution in each chamber can be calculated by multiplying the original solution with the dilution factor, which is the ratio of the channel volumes between valve 2 and valve 3. This calculated value of each chamber is compared with the corresponding measured value (Figure 5.3B). The two values match very well with each other. For convenience, we directly use the calculated concentrations in the microchambers in subsequent experiments.

**Figure 5.3.** Quantification of fluorescein concentrations in the microfluidic chambers. (A) Fluorescent intensity of fluorescein solution in the microchamber changes linearly with its concentration. (B) Comparison of measured fluorescein concentrations in the microchambers and calculated values of concentrations. The calculated values are obtained by multiplying the original fluorescein solution concentration with dilute factors, which is determined by the chip design.
5.3.4 Cell seeding and culture on chip

The same chip design is used for drug screening of cytotoxicity (only apoptosis is monitored). Cells are loaded into the microfluidic chambers by following an adapted procedure. (1) Surface treatment. To enhance cell attachment and spreading on PDMS surface, PDMS pieces are oxidized in oxygen plasma before they are sealed to form microfluidic channels. The surface of the sealed microchannels is immediately treated in a 0.01% poly-L-lysine (PLL) solution for 30 min. The positively-charged PLL polymers adsorb to the negatively-charged PDMS surface by electrostatic force. PLL treatment has been demonstrated previously to be able to enhance cell attachment onto surfaces. Our results show that all HeLa-C3 cells can attach to the PLL-treated hydrophilic PDMS substrate within 2 h after seeding and spread well within 4 h. We found that, for cell attachment and spreading, the PLL-coated PDMS substrate is comparable to the commercial polystyrene culture dishes with tissue culture treatment. (2) Load cells. Valve Group 1 is closed to force the cells flow through the “rung” channels. After cells are distributed in all the “rung” channels, the flow is stopped and cells are allowed to settle and attach to the bottom surface of the PDMS channels. (3) Trap cells. Valve Group 2 is closed to form isolated microfluidic chambers. During the cell loading step, cells are seeded in both the “rung” channels and the side channels. Because the size of each cell is about 1/20 of the channel size, the cells that attach on the side channels need to be removed to meter solutions accurately in the next step. With valve Group 2 closed, sterilized DI water is flowed into the side channels of the system until all the cells outside are swelled and broken because of osmotic pressure. The cells in the middle chambers are not influenced with valve Group 2 closed. (4) Meter solutions and generate stepwise gradients. Culture medium and drug-containing culture medium are injected into the two side channels, respectively. Valve Group 3 is closed to form side chambers that trap the two solutions with accurate volume. Finally, the valve Group 2 is opened to allow the solutions from the two side chambers to mix; because the volume ratios of solutions at the two side microchambers are
different in each “rung” channel, stepwise gradient of drug concentration is created along the system. (5) Cell culture. The whole chip is placed into a cell incubator at 37 °C for cell culture. The use of a screw-driven microvalve design that we developed previously [158] (See Chapter 2) avoids complicated connections of bulky external pressure source; the entire microfluidic device can be placed into a conventional cell incubator for the optimum control over both temperature and CO₂ concentration (PDMS is gas permeable).

The side channels are ~1-1.5 mm long and the microchamber size is 300 μm by 500 μm. The diffusion constant of small molecules in water at 37 °C is in the order of 10⁻⁵ cm²/s. Estimated by the diffusion equation \(x^2 = 2Dt\), where \(x\) is the diffusion distance within time \(t\) and \(D\) is the diffusion coefficient, it takes about 20 min for the drug molecules to diffuse over this distance and form homogeneous solutions in each microchamber. This time can be reduced with short side channels. Compared with the time required to induce cell apoptosis with etoposide, which is normally over 10 h [156, 159], this time for mixing is short and negligible.

The medium used on the microchip is supplied with 15% fetal bovine serum (FBS). For screening assays, it is important that the nutrient in the enclosed environment (both the middle microchamber and the side channels) is sufficient to support the cell growth. On average, each microchamber is loaded with around 20 HeLa-C3 cells. The total volume of the enclosed space is tens of nL. We found that this volume can sustain cells to live healthy for at least two days. If needed, fresh culture medium can be changed by repeating the procedure above.

5.3.5 Single-cell apoptosis assay under drugs with stepwise concentration gradients

To demonstrate the capability of our system for real-time study of cell apoptosis at the single cell level, we chose etoposide, a commercial anticancer drug commonly used in chemotherapy, as cell apoptosis inducer on HeLa-C3 cells. The stepwise gradients between 0-100 μM are generated with the device shown above. The moment when the drug solution
starts to be diluted is set as the zero time point. Phase contrast image, YFP and CFP fluorescence images of each cell in all microchambers are recorded every 12 h for two days. The ratio of YFP and CFP fluorescence intensities of each cell is used as the indicator of cell apoptosis. YFP and CFP ratios of each cell at different times are normalized by setting the ratio of the cell at the zero time point as 1.

In order to quantitatively define the inducement of the apoptosis in gradients, we could detect the activation of caspase-3 inside each living cell by calculating the relative emission ratio of YFP/CFP in the fluorescence images and determine the temporal relationship between caspase-3 and the apoptotic event. Before caspase-3 is activated, the cells are in normal cell shape and the fluorescent light is emitted primarily from the YFP. When caspase-3 is activated during drug-induced apoptosis, the sensor C3 is cleaved and the fluorescent light is emitted predominantly from the CFP (Figure 5.4).

Figure 5.5 shows typical FRET results of five individual cells at different drug concentrations. In general, the YFP fluorescence decreases while CFP fluorescence increases with time. The relative YFP/CFP ratio begins to decrease gradually, and a fast drop of this ratio indicates the irreversibility of cell apoptosis. Individual cells show significantly different profiles with different apoptosis-initiation times even under the same conditions. The result suggests that individual cells can have different sensitivity to the same drug. We found that at low drug concentrations a few cells (less than 10%) show distinct profiles where the relative YFP/CFP ratio increases slightly at the beginning of the experiment. The exact reason needs further investigations.
Figure 5.4. Representative phase contrast and fluorescent images of HeLa-C3 cells in a typical microchamber treated with etoposide (37 μM) at different time points. (A-E), (a-e) and (α-ε) show the phase contrast, YFP fluorescent and CFP fluorescent images, respectively. The line-shaped shadow in the phase contrast images is from the diffraction of light at two sides of the microchamber, which are curved under the influence of the valve control channels.
Figure 5.5. Single-cell apoptosis analysis. (A-C) show the monitoring of apoptotic process of five individual cells in three (low, medium and high) etoposide concentrations at 9 μM, 55 μM and 83 μM, respectively.
Cell apoptosis is confirmed by two other commonly used methods. The first one is to monitor the change of cell morphology, which can indicate cell apoptosis. From the phase contrast images, cell morphology changes little within the first 12 h at all concentrations of etoposide. With longer treatment time, the cell shape becomes abnormal with cells starting to shrink. The other method is to observe chromatin condensation and fragmentation, which are the symbols of apoptosis. Hoechst, a cell permeable fluorescent nucleic acid dye, is applied to the cells to reveal these phenomena by staining the condensed nuclei of apoptotic cells. In Figure 5.6, the cells with shrunk cell morphology displayed chromatin condensation and fragmentation; while others with normal cell shape appeared with much weaker Hoechst staining. The results of both methods are consistent with the FRET analysis of cell apoptosis.

To check the quality of the culture medium in the chamber, we set a control group that has no anticancer drug in the MEM 15% FBS medium for 48 h. By analyzing the relative YFP/CFP ratio, it is evident that the nutrient is sufficient for the cells (~20 cells/chamber) and the caspase-3 is not activated under that condition (Figure 5.7).
Figure 5.7. Averaged results of on-chip apoptosis assay. The relative Y/C ratio from YFP and CFP fluorescence images of each cell in all microchambers was recorded every 12 h for two days and averaged to give the relative Y/C ratio for each concentration at each time point. The number of individual cells recorded at each concentration is ~100.

5.3.6 Comparing on-chip apoptosis assay with conventional assays

The single-cell results are averaged from over ~100 cells and compared with two other assay methods (Figure 5.5 and Figure 5.6).

In the first, cell apoptosis is studied in a 96-well plate with each well containing 5-7 thousands of HeLa-C3 cells (Figure 5.8A). The results in the relative emission ratio of YFP/CFP show the same trend as those obtained from the microchip. However, the FRET signal obtained from a large number of cells grown in the 96-well plate with the data obtained from a plate reader is not as strong as the data obtained from a small number of cells during the on-chip study. The YFP/CFP emission ratio of the plate system changes approximately 2-3
folds in two days after the drug addition, while from our chip-based single call analysis, the ratio is around 5 folds. The major reason comes from the difference of optical filter systems of the fluorescent microscope and the plate reader we have. The emission peaks of YFP and CFP are 526 nm and 480 nm, respectively. The emission filters in the microscope for YFP and CFP are 535±15 nm and 480±20 nm, respectively; both cover well the emission peaks of YFP and CFP. However, the corresponding plate reader’s filters are 535±8 nm and 486±8 nm, which barely cover the emission peaks of both fluorescent proteins. Another contributing reason is the capability of collecting light: objective of a fluorescent microscope can certainly collect more light than a plate reader for a unit area.

We also compared the cell apoptosis assay results with the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay; in living cells, the yellow colored MTT molecules are reduced to purple colored formazan products by the mitochondrial reductase enzymes. This color change is quantified by measuring the light absorbance at 595 nm by a spectrophotometer, which is commonly used as a standard colorimetric assay to determine the cytotoxicity of certain agents that may cause cell toxicity, metabolic dysfunction and decreased performance. Figure 5.8B gives the results of MTT assay of etoposide on HeLa-C3 cells. Compared with the FRET assay shown above, the MTT assay gives similar trends. However, it shows slower response (decrease of cell viability) than the FRET assay. In the early stage of apoptosis, although caspase-3 is activated, the activity of the mitochondrial reductase enzymes, which the MTT measures, will mostly not be affected. Therefore, this cell viability test shows time delay in response to cytotoxic drug. The HeLa-C3 FRET system gives more accurate temporal sensitivity for real-time quantifying the cell apoptotic process.
Figure 5.8. Other assay methods of detection of apoptosis. (A) caspase-3 activity assay with a 96-well plate. (B) MTT assay (the viability is relative to the control). All results are obtained from plate readers.

5.4 Conclusions

We have developed a prototypic microfluidic platform for the generation of stepwise concentration gradients of drugs. We also demonstrated the application of this microsystem to study cell apoptosis by integrating into the chip a sensitive FRET-based cell apoptotic analysis.
method, which was previously demonstrated to directly quantify the apoptotic process by measuring the activity of caspase-3 under the influence of anticancer drug, etoposide. Sets of microfluidic valves are used to accurately meter different solutions and mix them to generate stepwise gradients of drugs to test their cytotoxicity with cell apoptotic assay. This microfluidic platform can provide several advantages: (1) cytotoxicity of drugs at multiple concentrations can be simultaneously studied in parallel on one chip, thus saving time and labor and reducing systematic errors; (2) the sample size can be largely reduced, (3) microfluidic chambers facilitate long-time tracking of individual cells; and (4) microscopic imaging can give higher optical sensitivity than fluorescent scanner or UV-Vis absorption. This microfluidic platform can also avoid possible shear force that exists on other cell-study microfluidic chips. Also, it resembles the traditional well plate format for drug screening, but with miniaturized sizes and higher throughput. This chapter is focused on the establishment of the microfluidic prototype for the study of cell apoptosis and we have tested five “ladders” channels on single chips; higher throughputs should be possible. This prototypic system should be useful in the areas of biology and bioengineering, especially for discovering apoptosis-inducing agents as potential anti-cancer drug candidates.
Chapter 6

Printing Concentration Gradients of Solutions in Microwell Arrays

This chapter describes a convenient method that can establish concentration gradients in multiple microwell arrays by transferring the gradients from a gel slab. We first make a hydrogel slab containing two parallel reservoirs. After adding two solutions with different concentrations into the two reservoirs respectively, a concentration gradient is generated in the middle area of the hydrogel by diffusion. The existing gradient in this gel slab is transferred to a gel-filled well array. The gel-filled well array that has concentration gradient can be used for establishing gradients in a solution-filled well array by assembling the two well arrays together and allowing the solutions in the corresponding wells to mix. We demonstrate that one gel slab with gradient can be used to print multiple gel-filled microwell arrays. Furthermore, the obtained concentration gradients in the gel-filled array can be transferred to another gel-filled array so that two gradient-printed arrays can be combined to form complex, orthogonal gradients of different chemicals. We successfully use the gradients generated with our technique to study the effects of Zn\(^{2+}\) concentrations on the proliferation of *E. coli* bacteria.

6.1 Introduction

This chapter describes a new method that can conveniently establish concentration gradients in microwell arrays by transferring the gradient pre-formed in a hydrogel slab to a microwell array. The hydrogel slab with two parallel reservoirs is made of agarose. Two solutions with different concentrations are added in the reservoirs, a concentration gradient is formed between the reservoirs by diffusion. This gel slab with the concentration gradient is brought in contact with a gel-filled microwell array to print the gradient. After the gel slab is removed, we can modify the profile of concentration gradient in the gel-filled array by
aligning it to other microwell arrays. At last, we align a solution-filled microwell array that requires gradient to this gel-filled one, and the concentration gradient is transferred to the solution-filled microwell array (Figure 6.1).

**Figure 6.1.** Schematic illustration of printing concentration gradient into microwell array. This schematics starts with an agarose gel slab containing two parallel reservoirs. A concentration gradient is formed in the gel slab when two solutions with different concentrations are added into the reservoirs respectively. The concentration gradient in the gel slab is transferred to a gel-filled well array. At last, the gel-filled well array is assembled to a microwell array filled with solution, transferring the concentration gradient to the microwell array.
6.2 Experimental Section

6.2.1 Formation of hydrogel slab

An aluminum master with wanted structures (two reservoirs) was made by the machine shop. If not specifically pointed out, we molded the gel slabs (40 mm X 19 mm, 4 mm thick) which have two parallel reservoirs (3 mm X 3 mm X 30 mm, 3 mm apart) from this aluminum master by pouring a hot agarose solution (2%) on the master and leaving it cooling down to be gel. The thickness of the gel slab is determined by the structure on the master.

6.2.2 Establishment of concentration gradients in a hydrogel slab

We used two methods to establish concentration gradients in the hydrogel. The first method is non-continuous flowing method. The gradient was generated by adding certain amount of solutions (200 μL each) into reservoirs and stored it in humid container to avoid drying. The second method is continuous flowing method. The gradient was generated by continuously flowing solutions through the reservoirs (4.5 μL/min); we clamped the gel slab between two PMMA plates and flowed the wanted solutions through the holes on the PMMA plates. After wanted periods of time, we removed the solutions in the reservoirs and dried the gel surface in the air before printing.

6.2.3 Generation and filling of PDMS microwell array

We made PDMS microwell arrays by soft lithography [22, 23, 63]. The period of the wells (200 μm in diameter, 50 μm in depth) was 300 μm. In the case where we needed to reduce molecular absorption in PDMS, we modified the PDMS micowell array with paraffin wax [160]. We placed the PDMS micowell array in a vacuum desiccator for 5 min. We melted the paraffin wax (Aldrich) at 100 °C followed by adding it onto the vacuumed PDMS micowell array. The melted wax was kept for 1 min at 100 °C on a hotplate before we removed the PDMS from the hotplate and cooled it down to room temperature. Before use,
we peeled off the PDMS microwell array from the solidified paraffin and used Scotch tape to clean the PDMS surface.

We used discontinuous dewetting to fill solutions into wells [161]. We pretreated the PDMS microwell array in a vacuum chamber, and added the solution on the microwell array. The pretreatment under vacuum helped the removal of any trapped air bubble in the microwells. We tilted the PDMS slab and imbibed extra solution with Kimwipe paper. To fill agarose gel into wells, we covered a microporous polycarbonate membrane (PC membrane, 5.0 μm, GE Water & Process Technologies) onto microwell array followed by vacuum pretreatment. We poured the hot agarose solution on the PC membrane and cooled down to gel the solution at room temperature. We removed the extra hydrogel on PC membrane and peeled off the PC membrane. This filled array was ready for use without any other treatment. If it needs to store for a long time before subsequent use, it is possible to preserve the gel-filled microarray in silicon oil to prevent drying.

6.2.4 Establishment of equilibrium between the gel slab and gel-filled microwell array

We immersed the 2% agarose gel slab in a 0.1 mM sodium fluorescein solution overnight. After we dried the surface of the gel slab in the air, we placed the gel slab onto the microwell array that was filled with 2.0% agarose for a short time before separating them. Fluorescence images were taken under a fluorescent microscope (AZ100, Nikon). We tested the contact times of 1s, 5s, 10s, 20s, 30s, 40s, 50s, 60s, 120s, 180s, 240s, and 300s in our experiments.

6.2.5 Transferring concentration gradient from gel slab to microwell array

We aligned the gel slab to the gel-filled microwell array using the reservoirs in the gel slab and the wells on the PDMS slab as registration marker. After contact for 2 min, we peeled off the gel slab and the gradient was transferred into the microwell array. We could repeat this transferring step many times to print the same gradient into different microwell
arrays using the same gel slab that contained the concentration gradient.

6.2.6 Generation of standard calibration curve of concentration versus fluorescent intensity

We immersed 2% agarose gel slabs into sodium fluorescein solutions (overnight) with known concentrations (100 µM, 80 µM, 60 µM, 40 µM, 20 µM, 10 µM, 5 µM, 0 µM). After drying the surface of gel slab in air, we printed these gel slabs onto gel-filled microwell arrays (2 min). Fluorescence images of the microwell arrays were taken with a fluorescent microscope after peeling off the gel slabs. The fluorescence intensities were obtained from the fluorescence images using the NIS-Elements BR software for the plot of the intensity-concentration calibration curve.

6.2.7 Establishment of combined gradients in microwell arrays

We used 0.1 mM sodium fluorescein aqueous solution and 0.1 mM Rhodamine B aqueous solution to establish two individual concentration gradients in two gel slabs by discontinuous flowing method, respectively. We printed the sodium fluorescein gradient into a PDMS microwell array and Rhodamine B gradient into another PDMS microwell array which had been modified by paraffin. We aligned the two microwell arrays together (2 min) with their gradients orientated orthogonally. After disassembling the microwell arrays, images were taken under fluorescent microscope.

6.2.8 Effects of ZnCl$_2$ on the proliferation of *E. coli* bacteria

We autoclaved LB medium (tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L, pH~7.4) and 2X LB medium (tryptone 20 g/L, yeast extract 10 g/L, NaCl 20 g/L, pH~7.4). We prepared the gel slab with 2% agarose in the 2X LB medium. We introduced the 2X LB medium with and without 2 mg/mL ZnCl$_2$ through two channels (4.5 µL/min, 12 h) through
the reservoirs respectively to establish ZnCl$_2$ gradient in the gel slab. We filled 2% agarose in the 2X LB media into one PDMS microwell array, and printed this gel-filled array with the previously prepared gel slab (which contained ZnCl$_2$ gradient). We incubated *E. coli* bacteria (PS2164, with green fluorescent protein expressing and ampicillin resistance in plasmid) in the LB culture medium (37 °C, 250 rpm, 14 h) to be in the exponential growth phase. We centrifuged 1 mL of this *E. coli* suspension solution (7000 rpm, 2 min) and removed the supernatant. We used 10 mM HEPES to re-suspend the tablet and repeated the process twice. We filled this *E. coli* suspension solution (in HEPES) into one microwell array and aligned this array with the gel-filled one which contained the ZnCl$_2$ concentration gradient. We incubated the two assembled well arrays in saturated humidity (37 °C, 24 h) before images were taken under fluorescent microscope.

6.3 Results and Discussion

6.3.1 Establishment of concentration gradients in a gel slab

We use two diffusion-based methods to establish gradients in gel slabs: (1) continuously flowing solutions through two parallel channels that are inside the hydrogel slab (continuous flowing method); and (2) filling solutions into two parallel reservoirs at two sides the hydrogel slab (discontinuous flowing method). With the former method, we can establish a stable linear gradient of sodium fluorescein in the hydrogel between the two reservoirs after 8 h (Table 6.1). However, this method requires relatively large amount of samples. With the latter method, the gradient can slowly shift with the diffusion time (Figure 6.2). But its low consumption of sample and easy operation makes it an alternative choice, especially when sample solutions are limited. In the experiment showed in Figure 6.2, we used 0.1 mM sodium fluorescein and water to establish the gradient. For the gradient that is formed by the discontinuous flowing method, the highest concentration is about 50 μM because the blank gel slab itself is a sink which can constantly remove the solute from the gel. In the continuous
flowing method, because the solutions in the reservoirs can be renewed continuously for several hours, the highest concentration is almost the same as the original solution (0.1 mM). In the gradient generate with the continuous flowing method, the lowest concentration is about 50 μM, much higher than the concentration in water (0 μM). This difference in concentration is resulted because the low flow rate (4.5 μL/min) of water is not enough to completely remove all the fluorescein molecules in time. We have two solutions to address the issue: (1) increasing the flow rate; and (2) reducing the molecular diffusion by decreasing the depth of the reservoirs.

<table>
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</tr>
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</table>

Table 6.1. Linear fitting results of concentration gradient data from the continuous flowing method. The original data are from the central 10 lines (line 11-20) of the arrays (Figure 6.2A).

6.3.2 Filling well arrays with hydrogel

We have tried to directly print the concentration gradient into a solution-filled microwell array. However, no matter whether we make the well surface to be hydrophilic or hydrophobic, the solution escapes from the wells when the gel slab is peeled off. If the microwell array is filled with hydrogel, the solution can stay in the wells. If needed, we can transfer the gradient in a gel-filled microarray to microwells that are filled with free solution by bringing them into contact. During the step of gel filling, we cover a microporous polycarbonate (PC) membrane onto the PDMS microwell array. We pretreat the PDMS
microwell array under vacuum so that the hot agarose solution can be imbibed into the microwells through the holes on the PC membrane. In addition, the PC membranes forms a conform contact with the PDMS surface very well so that only the wells are filled with gel after extra gel on the PC membrane is removed.

Figure 6.2. Concentration gradients of sodium fluorescein in microwell arrays that are printed from gel slabs. (A) The concentration gradient of sodium fluorescein in a microwell array; the dashed lines areas represent the positions of reservoirs in the gel slab. The diameter of each microwell is 200 μm. (B) Concentration gradients that are established with and without continuous flow of the solutions in reservoirs, respectively. We use 0.1 mM sodium fluorescein and pure water to form the gradients in both cases. Each data dot is averaged from the intensities of the 30 wells along the line.

6.3.3 Establishment of equilibrium between gel slab and microwell array

During the printing step, we ensure that equilibrium has been reached between the solutions in the gel slab and in the microwells before removing the gel slab. We determine the time that is needed for reaching the equilibrium of sodium fluorescein between a gel slab and 50 μm deep wells is ~1 min (Figure 6.3). In the experiments, 2 min is applied to ensure that
the equilibrium is reached. We also print four microwell arrays with the same gel slab. We found that such short time does not affect the gradient in the gel slab obviously (Figure 6.4); therefore we can print the gradient to multiple microwell arrays with one gel slab.

![Time-dependent fluorescence change in the gel-filled microwell array during printing, showing the equilibrium established between gel slab and gel-filled microwell array.](image)

**Figure 6.3.** Time-dependent fluorescence change in the gel-filled microwell array during printing, showing the equilibrium established between gel slab and gel-filled microwell array. The gel slab (2% agarose) is immersed into a 0.1 mM sodium fluorescein solution overnight and assembled to a gel-filled microwell array. The microwells are 200 μm in diameter and 50 μm in depth.

### 6.3.4 Printing gradient from gel slab to microwell array

By using the edge of reservoirs and microwells as registration marker, we print the gradient from the gel slab that contains the concentration gradient into the gel-filled microwell array. As Figure 6.2A shows, the microwell array that we use has 900 wells in 30 rows. The ten rows in the middle fit right in the area between the two reservoirs in the hydrogel. The gradients in these ten rows are linear with both the continuous and discontinuous flowing methods (Figure 6.2B).
6.3.5 Modification of the gradient in microwell arrays

In addition to the gradient printing, in which we transferred the gradient from the gel slab to a gel-filled microwell array, we can transfer the gradient between two gel-filled arrays by bringing the corresponding wells into contact. In the process, the concentration of solution in each well becomes half. Figure 6.5A shows that the gradient slope is reduced to be half after the transferring.

Figure 6.4. Concentration gradients transferred from the same gel slab to four different gel-filled microwell arrays: (1) the first microarray, (2) the second microarray, (3) the third microarray and (4) the fourth microarray. The four concentration gradients have the same concentration gradient (within the range of error) of sodium fluorescein.

More interestingly, we can also construct combinational orthogonal gradients of two ingredients in a similar process with two different chemicals. Figure 6.5B and 5C shows the example of complex gradients of sodium fluorescein and Rhodamine B in the same microwell array. Because Rhodamine B can absorb into PDMS significantly, we treat the PDMS
microwells with paraffin wax to greatly reduce the absorption of Rhodamine B into PDMS bulk [160].

**Figure 6.5.** Modification of the gradient in gel-filled microwell array. (A) The profiles of the original gradient and the transferred gradient which is formed by assembling the microwell array containing original gradient and a blank one. (B and C) Fluorescence images of a gel-filled microwell array with orthogonal gradients of sodium fluorescein and Rhodamine B. The diameter of each microwell is 200 μm. Fluorescence image (B) is taken under a green filter, showing the concentration gradient of sodium fluorescein (top to bottom), and fluorescence image (C) is taken under a red filter, showing the concentration gradient of Rhodamine B (left to right).
6.3.6 Effects of ZnCl\(_2\) concentrations on the proliferation of *E. coli* bacteria

With gradients generated in microwell arrays, we investigate the effects of metal ion concentrations on the proliferation of *E. coli* bacteria in the wells. We choose Zn\(^{2+}\) ion for its wide use as a model in related studies. We use the fluorescently-modified *E. coli* stain that expresses green fluorescent protein (GFP) to facilitate the quantification of the bacteria number under a fluorescent microscope. Because the proteins in the LB media can render native PDMS surface into hydrophilic by adsorption, LB media is not suitable for discontinuous dewetting. To address this problem, we replace the culture media from LB media to a HEPES solution before loading the bacteria into the microwells. Figure 6.6 shows the images of the device just after the assembling of the two microwell arrays (Figure 6.6A) and after 24 h of incubation (Figure 6.6B), respectively. Because ZnCl\(_2\) and sodium fluorescein are small molecules with similar diffusion rates, the ZnCl\(_2\) gradient in the well array is predicted by the result of sodium fluorescein shown previously (Figure 6.2B). The profile of the gradient is linear where the concentration of ZnCl\(_2\) in the top row of the wells is \(~1\) mg/mL and that in the bottom \(~0.5\) mg/mL. The difference of fluorescent intensity of GFP between the rows of wells is clearly observed from the array. From the result, we can conclude that, in the range of 0.5 mg/mL to 1 mg/mL, higher ZnCl\(_2\) concentrations can suppress the proliferation of *E. coli* more strongly.

![Figure 6.6](image_url)

**Figure 6.6.** Fluorescence images of *E. coli* proliferated under different concentrations ZnCl\(_2\)
solution. (A) At the beginning, the fluorescent intensities in the wells are nearly the same; (B) after 24 h, the bacteria in the wells with low ZnCl₂ concentrations (bottom wells) proliferate faster than those in high concentrations (top wells). The diameter of each microwell is 200 μm. Solution gradient is formed (with LB culture media with and without 2 mg/mL ZnCl₂) in a microwell array that is filled with *E. coli* suspension in 10 mM HEPES. Proliferation of these GFP-express *E. coli* is monitored under a fluorescent microscope.

6.4 Conclusion

In this work, we have demonstrated that hydrogel slabs can be used as the media to print concentration gradients into microwell arrays. We use two methods to establish the gradient in the gel slab—the discontinuous flowing method requires less samples than the continuous flowing method but the latter forms more stable gradients. The concentration gradient can also be transferred between different microwell arrays; therefore the gradient profile in a microwell array can be tuned (e.g., orthogonal gradients of two chemicals in one microwell array). The gradient in a hydrogel slab can be transferred into multiple microwell arrays by repeating the printing step. With the established gradient, the effects of ZnCl₂ concentration on the proliferation of *E coli* bacteria are studied in the microwell array.

Compared with the other methods [87, 102], our method provides the following advantages: (1) the gradient is originally from diffusion in hydrogel (thus it is stable and easy to predict); (2) the final gradient is established in separated wells so that the solution concentration in each well is fixed without interference from neighbor wells (this is important for experiments such as long-term culture of bacteria shown above); (3) the gradients in different microwell arrays can be combined to form complex gradients of multiple chemicals, which make it easier to study the combined effects of different chemicals.
Chapter 7

Establishing Concentration Gradient of Solutions in Microspot Arrays with a Droplet-based Microfluidic System

This chapter describes a microfluidic method for the establishment of concentration gradients in separate droplets on a microspot array. We align a PDMS microchannel with a hydrophilic microspot array on a hydrophobic surface. When a buffer solution is flowed through the channel and removed, a small volume of buffer is left on each hydrophilic spot. When a plug of the wanted aqueous solution (other phase is oil) flows through the channel, this solution plug consecutively merge with and separate from each buffer spot. This solution is sequentially diluted at each buffer spot; after the solution plug has been flowed through, the solution concentrations at the spots form a gradient that is determined by the volume ratio of the solution plug and the buffer at each spot. The major advantages of this method are (1) concentration gradients can be generated in thousands of arrayed spots conveniently, (2) the gradient profiles can be theoretically determined and thus be well controlled, and (3) wide ranges of concentrations can be formed in one experiment.

7.1 Introduction

In microchannels, two immiscible phases can easily form droplets or plugs of one phase in the continuous phase. In recent years, the creation of femtoliter to microliter droplets (mostly water in oil and oil in water) in microfluidic channels offers a new and potentially powerful tool for microanalysis of chemical and biological samples [162]. Because of its advantages of rapid mixing, small sample consumption and the potential for parallelization of multiple microreactions [163, 164], this microfluidic droplet system has been widely explored in various applications ranging from screening of protein crystallization conditions [165, 166], synthesis of microparticles [167, 168] to single-cell manipulation [169-171].
Figure 7.1. Formation of concentration gradients in microspot arrays using microfluidics. (A-E) schematics showing the formation of concentration gradient in a microspot array by aqueous solution plug in oil. (F-G) Photographs showing the formation of the gradient of sodium fluorescein solution in a microspot array.

Here, we introduce a convenient method to use this microdroplets system to create concentration gradient. We align a PDMS microchannel with a hydrophilic microspot array on a hydrophobic surface. When a buffer solution is flowed through the channel and removed, a small volume of buffer is left on each hydrophilic spot. When a plug of the wanted aqueous solution (other phase is oil) flows through the channel, this solution plug consecutively merge with and separate from each buffer spot. This solution is sequentially diluted at each buffer spot. After the solution plug has been flowed through, a small volume of diluted solution with varying concentrations is left on each hydrophilic spot. The solution concentrations at the spots form a gradient that is determined by the volume ratio of the solution plug and the buffer at each spot. (Figure 7.1)

7.2 Experimental Section

7.2.1 Fabrication of PDMS microperforated membrane and PDMS channel slabs
We made both PDMS microperforated membranes [113] and PDMS channels by [22] the soft lithographic method. The width and height of the PDMS channel were 500 μm and 200 μm, respectively. The master was made of SU-8 on silicon wafer by conventional photolithography. After pouring PDMS prepolymer (A:B = 10:1) onto the master, we degassed the PDMS by vacuum and solidified the PDMS in 70 °C oven for more than 30 min. After peeling off the PDMS channel slab from the master, we punched two holes at inlet (~0.5 mm in diameter) and outlet (~1.7 mm in diameter). For PDMS microperforated membrane, we prepared SU-8 microposts on silicon wafer by photolithography (the diameter and height of the post were both 200 μm and the space between two posts along the channel direction was 200 μm or 1 mm) and spin coated PDMS prepolymer (A:B = 10:1) onto the microposts array by 1500 rpm for 30s. After solidifying in a 70 °C oven for 20 min, a small amount of PDMS prepolymer was carefully painted at the peripheral areas on the silicon water (NOT on the microposts) that formed a rim for easy manipulation of the membrane. The final PDMS microperforated membrane was obtained after further 30 min of incubation in a 70 °C oven. The membrane was peeled off the master and stored for use.

7.2.2 Hydrophobic modification of glass surface

We cleaned the glass slides in Piranha solution (3:1 mixture of sulfuric acid and 30% H₂O₂) by sonication for 15 min. We then washed the glass slides in de-ionized water for 15 min and in ethanol for 15 min by sonication successively. The clean glass slides were ready for modification after drying in 120 °C oven for 0.5 h. For hydrocarbon modification, we immersed the clean glass slides into a 0.1% solution (v/v) of octadecyltrichlorosilane (OTS, Sigma) in toluene (VWR International) for 1 h before we washed the slides in water for 15 min and ethanol for 15 min by sonication successively. The hydrocarbon modified slides were ready for use after drying by compressed air. For fluorocarbon modification, we placed the clean glass slides in a vacuum desiccator containing 20 μL exposed
trichloro(1H, 1H, 2H, 2H-perfluoroctyl) silane (Sigma) overnight. We washed the modified slides by sonication in ethanol for 20 min. The fluorocarbon modified slides were ready for use after drying by compressed air.

7.2.3 Device setup

We covered the hydrophobic modified glass slide with the PDMS microperforated membrane. Then, a PDMS slab with registration marks is placed at the other side of the slide by aligning it with the membrane. After alignment, the assembly was treated by oxygen plasma for 2 min (Plasma cleaner/sterilizer, Harrick). The PDMS membrane was peeled off from the glass slide while the PDMS slab remained unmoved on the other side. Finally, by aligning to the registration marks on the PDMS slab, the PDMS microchannel was placed onto the side where the original PDMS membrane was. After the PDMS slab with registration marks was peeled off, liquids were filled into the channel.

7.2.4 Construction of concentration gradients of sodium fluorescein and green fluorescent protein (GFP) in droplet arrays.

We connected the outlet of the microchannel to a syringe pump through a plastic tube. After adding a drop of mineral oil (white, light, Sigma-Aldrich) at the inlet, we used the pump to pull the syringe and vacuumed the oil into the microchannel. Then we applied one or two relatively large water droplets (~ 2 μL) into the channel to form microarray of water droplets. Finally, we used a micropipette to add a drop of sodium fluorescein solution (500μM, 300 nL) or green fluorescent protein solution (2 mg/mL) to the inlet.

7.2.5 Construction of the gradients of human chorionic gonadotropin (HCG) for enzyme-linked immunosorbent assay

We constructed the gradients of human chorionic gonadotropin (HCG) in microdroplet
array and used it an enzyme-linked immunosorbent assay (ELISA) to analyze HCG gradient in solutions. We made the solutions of coating antibody (goat anti-HCG IgG, 15 µg/mL), HCG (10 IU) and first antibody (mouse anti-HCG IgG, 10 µg/mL) in a phosphate buffer (0.02 M, pH 7.4), respectively. We used Tris buffer saline (50 mM Tris, 100 mM NaCl, pH 8.0) to dissolve second antibody (goat anti-mouse IgG, alkaline phosphatase conjugate, Invitrogen, 1 µg/mL). We used a glycine buffer (10 mM Glycine, 10 mM NaCl, 1 mM ZnCl₂ and 1 mM MgCl₂) to dissolve the phosphatase substrate (ELF 97 phosphate substrate, Invitrogen, 50 µM/mL).

We treated a PDMS surface with the coating antibody at 4 °C overnight. After washing the PDMS surface with PBST (0.1% Tween-20 in phosphate buffer silane) for three times, we used 5% albumin from bovine serum (BSA, Sigma) to block the surface at 37 °C for 1 h. After washing the treated surface with PBST for three times, we dried the PDMS with compressed air. We followed the same procedure of sodium fluorescein and GFP to establish a gradient of HCG from a 10 IU HCG (300 nL plug) solution in a microspot array. After peeling off the PDMS channel slab from the microspot array, we assembled the modified PDMS surface onto the microspot array and incubated the device at 37 °C for 1 h. After disassembling the device and washing the PDMS surface with PBST for three times, we treated the PDMS surface with the first antibody at 37 °C for 2 h and second antibody at 37 °C for another 1 h. Then we washed the PDMS surface with Glycine buffer for five times and incubated the surface with the phosphatase substrate at 37 °C for 0.5 h. The final product of the reaction was fluorescent precipitates which can be examined under a fluorescent microscope.

7.2.6 Formation the gradient of NaCl crystals in varying sizes

We followed the same procedure of sodium fluorescein and GFP to establish a gradient of NaCl solution in the microspot array from an original saturated NaCl solution. Then we peeled off the PDMS channel slab followed by immersing the microspot array under mineral
oil. We placed the array in mineral oil (glass slide with microspots array and mineral oil) on a 37°C hotplate overnight to dry the solution. The NaCl crystals were examined under an optical microscope.

7.2.7 Calculation of the volumes of microspots and solution plugs

To calculate the volume of solution plug in microchannel, we took images of the plug in the microchannel and the cross section of the microchannel. The plug volume was calculated as the product of the area of the plug in the channel and the height of the channel. To estimate the volume of solution at each microspot, we calculated the volume change of a water plug before it contacted the first microspot and after it left the last spot in the array. This volume difference was the total volume ($V_{\text{total}}$) of all the solutions on the entire microspot array. It is reasonable to assume that the volume of the solution at each microspot is the same and to be $V_{\text{total}}/N$ (N is the number of microspots in the array).

7.3 Results and discussion

7.3.1 Device setup

We fabricate microperforated membrane in PDMS because PDMS membranes form a conform contact to the hydrophobic treated glass surface. The PDMS rim around the micropost array facilitates the manipulation of the microperforated PDMS membrane.

The PDMS channel slab is reversibly and conformed to the modified glass slide by van der Waals interactions. Because this sealing is relatively weak, we inject liquids into the channels by withdrawing instead of pressuring the liquid into the channel to avoid possible leakage of liquids. Withdrawing solution into the channel also makes it easier to add solutions into the inlet and to change solutions with a micropipette. In comparison to normal injection method of filling syringe and connecting tubes, this method lowers the required amount of sample solutions to the minimum.
7.3.2 The profile of the gradients

After the dilution buffer is generated on the hydrophilic microspots, we introduce a plug of a known-concentration solution with certain volume into the channel. The solution plug will merge and mix with the buffer on the hydrophilic spots; during this mixing, the solution is diluted by the buffer on the microspot. After the plug flows beyond the microspot, a new solution is left on the microspot with the same concentration as the solution plug when it separates from that particular microspot. This concentration is dependent on the volume ratio of the solution plug and the buffer solution at that microspot. Because the plug of solution is diluted every time when it meets a microspot, this concentration is decreasing in the microspots along the microchannel; therefore a gradient of concentration has been generated among the solutions in the microspot array. Figure 7.2 shows that if the volume ratio of the solution plug and microdroplets at the spot is larger, the dilution of the solution plug is less by the solution at each microspot and thus milder gradient profile is resulted.

Figure 7.2. The relationship between the plug volume and the gradient profile. The concentrated solution is 150 μM of sodium fluorescein in water. The black profile, dark gray profile and light gray profile refers to the three different concentrated solution plugs of 1000 nL, 400 nL and 150 nL, respectively.
The gradient profile created in this method can be predicted for some simple cases. Here we assume that (1) the solution plug contacts and mixed with the solution at no more than one spot at any time, and (2) the mixing between the solution at each spot and the plug of solution is complete. Under these conditions, the concentrations at the \((n+1)\)th and the \(n\)th spot have the following relationship:

\[
C_{\text{spot}(n+1)} = \left(\frac{C_{\text{spot}(n)} \cdot V_{\text{plug}}}{V_{\text{plug}} + V_{\text{spot}}}ight)
\]

where \(C_{\text{spot}(n+1)}\) and \(C_{\text{spot}(n)}\) are the concentrations of the solution at the \((n+1)\)th and the \(n\)th spot, respectively. \(V_{\text{plug}}\) and \(V_{\text{spot}}\) are the solution volumes of the plug and at each spot, respectively. 

\[
k = \frac{V_{\text{plug}}}{V_{\text{plug}} + V_{\text{spot}}}
\]

and is a constant. Therefore,

\[
C_{\text{spot}(n)} = k^n C_0
\]

where \(C_0\) is the concentration of the original plug.

We use sodium fluorescein as the model to test the prediction because its fluorescent intensity versus concentration has a wide linear dynamic range when its concentration is below 150 \(\mu\text{M}\) (Figure 7.3A).

\[
I_{\text{spot}(n)} = a C_{\text{spot}(n)}
\]

\(I_{\text{spot}(n)}\) is the fluorescent intensity of the solution on \(n\)th spot; \(a\) is a constant. By combining equation (2) and (3) together,

\[
I_{\text{spot}(n)} = a k^n C_0
\]

So,

\[
\log(I_{\text{spot}(n)}) = \log(a C_0) + n \log(k)
\]

As a result, \(\log(I_{\text{spot}(n)})\) has a linear relation with the spot number \(n\) as shown in Figure 7.3B.

The deviation of the prediction from the experimental results comes from several factors. A solution plug (especially when it is large) contacts and merges with several spots simultaneously. When the flow rate is fast or the solution plug is large, the mixing of the solutions may not be complete. There are some other factors that can affect the true volume of solutions at the microspots; we will discuss these factors in detail in section below.
Figure 7.3. Comparison of the experimental and predicted gradients. (A) shows the relationship between fluorescent intensity and the concentration of sodium fluorescein solution is linear when the concentration is less than 150 μM. (B) Comparison of the experimental and predicted profiles of generated gradient. We use a 500 nL plug of 150 μM sodium fluorescein in the experiment; the average spot volume is 0.8 nL.

Equation 5 suggests that (1) the steepness of the gradient can be easily tuned by changing the value of $k$, and (2) the concentration range in the gradient can be enlarged by increasing the number of the microspots. Because it is not easy for us to accurately quantify the fluorescent intensity of fluorescein at very low concentration, we form the concentration gradient of NaCl and examine the sizes of the NaCl salt crystals at each microspot after the
solution is dry. Figure 7.4 shows the NaCl solution gradient with the concentration variation of ~1000 fold using a microspot array of 3150 spots. After the solutions at the spots are dried, the sizes of formed NaCl microcrystals give a good indication of the dilution at each spot. Wider ranges of concentration can be achieved by simply increasing the number of the microspots.

![Figure 7.4. Dilution result of saturated NaCl solution in a microspot array containing 3150 spots (35 rows X 90 spots/row). The entire array has a size of 2.5 cm X 3.5 cm. (A) Microscopic images of NaCl crystals in different rows of the array. The scale bar represents 500 µm. (B-C) Microscopic images of two NaCl crystals at 1st row and 35th row, respectively. The ratio of their short edge of the single crystal from the pictures is ~10. Therefore the size ratio of the two crystals is ~1000, so is the concentration ratio of the corresponding solutions.](image)

**7.3.3 Surface modification of glass surface**

In the experiment, we modify the entire glass surface hydrophobic before selectively treating the glass surface into a hydrophilic array with plasma through a microperforated membrane. We have tried this surface modification with two different hydrophobic molecules: hydrocarbon and fluorocarbon. If the hydrophobic surface is modified with the hydrocarbon,
the volume water retained at each spot is ~1 nL while for fluorocarbon modification, this volume is decreased to ~0.3 nL (the continuous phase in both cases is mineral oil). Because of higher similarity of molecular structure of hydrocarbon to mineral oil than that of fluorocarbon, it is expected that the surface tension $\gamma$ between mineral oil and fluorocarbon surface is bigger than that between mineral oil and hydrocarbon surface. While the other two interfaces (water/hydrophilic and water/mineral oil) of the droplet at the microspot are the same in both cases, the contact angle of the water droplet is larger on the hydrocarbon modified surface by balancing the three interfacial surface tensions (Figure 7.5). In my experiment, we choose the hydrocarbon modification to obtain larger dilution at each spot.

![Figure 7.5](image)

**Figure 7.5.** Schematic illustration of water spots on selectively modified fluorocarbon surface (A) and hydrocarbon surface (B). The area under the water droplets is plasma treated into hydrophilic.

### 7.3.4 Influence of the velocity of the solution plug and its size on spot volume

At very high flow rate in the channel, long plugs of solution are easy to break into
smaller plugs, which will complicate the prediction of the formed concentration gradient. At low flow rate, it takes quite long time to establish concentration gradients among a microarray with an area larger than 5 cm$^2$ (large area with large number of microspots can give a wide range of concentrations). In the NaCl dilution experiment (Figure 7.4), the microarray covers ~9 cm$^2$ with a density of 360 microspots/cm$^2$. With this condition, 5 μL/min is found to be the optimal flow speed. In addition, careful examination of the solutions at the microspots shows that the solution volume at the microspots is slightly dependent on the plug flow rate; a faster flow gives smaller volume of solution left on the hydrophilic spots. To avoid this effect, we keep the flow rate constant at 5 μL/min in all our experiments.

![Graph showing the relationship between plug volume and the microspot volume.](image)

**Figure 7.6.** The relationship between plug volume and the microspot volume.

We also found that, other than the property of hydrophobic modification and plug flow rate, the volume of the solution plug can also slightly affect the volume of the solutions left at each spot. Figure 7.6 shows that, when the plug volume is less than 400 nL (the flow rate of the syringe pump is set at 5 μL/min for all data points), the effect of this volume on the
solution volume left at the microspots is not obvious. However, when the plug volume is over 400 nL, the results clear indicate that a larger plug gives less solution left at each microspot.

**Figure 7.7.** The relationship between plug volume and the plug flow rate. (A) Two plugs with different sizes are introduced into the channel successively. There are 16 rows of hydrophilic spots along the channel. The white arrows show the flow directions of the plugs. The solid line and dashed line show the areas in (B) and (C), respectively. (B-C) show the distance of two plugs at two time points: $t_0$ and $t_1$ ($t_1 > t_0$), respectively.

To understand this relation, we carefully examined the microscope videos of our experiments (frames show in Figure 7.7A) during the droplet formation at the microspots. In Figures 7.7B and 7C, two separate plugs of solution with different sizes are running at the same channel with the smaller plug at the front. The results show that with increasing time, the distance between these two plugs increases, indicating that the larger plugs flows faster than smaller plugs of solution in the microchannels when the flow rate is set constant. Note that the plugs of aqueous solution are dispersed in a continuous oil phase in the microchannel. There is a gap between the plugs of solution and the channel wall that is filled with the oil phase. A larger solution plug causes this gap to be narrower and longer along the channel.
direction. When the total flow rate of all liquids in the microchannel is the same, the oil phase around a larger solution plug (narrower and longer gap) flows more slowly than that around a smaller plug. Therefore, this larger solution plug runs faster than the smaller one to make the total flow rate equal. This difference in flow rates of plugs causes different volumes of solutions left at the microspots (see above in this section). In order to compensate this effect during the analyses in the following section, we flow five dilution buffer plugs which have the same volume as the solution plug during the step of forming buffer droplets array.

### 7.3.5 Generation of gradients of protein solutions

Because the hydrophobicity/hydrophilicity property of the involved surfaces is critical in this method, surfactants and certain macromolecules in solution can change the hydrophobicity/hydrophilicity of the surface and also the final solution gradients. For example, surfactant molecules can lower surface tension and reduce the contact angle of solutions at the microspot (Figure 7.4); therefore the volume of solution left at each hydrophilic spot will be reduced. Our experiments show that 0.1% sodium dodecyl sulfate (SDS) in the solution renders that there is no solution on the spots after the solution plug flows by. An additional possible reason for the disappearing of solution at the microspots is that SDS molecules absorb on the channels wall and change it into hydrophilic.

We also tested different protein solutions by comparing with sodium fluorescein. The gradient profile varies with the property of the protein. With the same condition, GFP gives almost the same gradient profile as sodium fluorescein (Figure 7.8) while the gradient profile of HCG shows a steeper trend. That is, the HCG solution is diluted faster than sodium fluorescein and GFP (Figure 7.9). We think that HCG is easier to absorb on the channel wall while little GFP and fluorescein can absorb to the channel wall.
**Figure 7.8.** Comparison of the gradient of GFP and sodium fluorescein.

**Figure 7.9.** Comparison of the gradient of HCG and sodium fluorescein. (A) HCG gradient which is shown by ELISA. (B) Gradient of sodium fluorescein solution in the microspot array.

### 7.4 Conclusion

In this chapter, we have introduced a convenient method to establish various gradients of solution concentrations in microspot arrays. A plug of wanted aqueous solution is flowed through an array of droplets that are pre-formed on hydrophilic patches across a hydrophobic surface. The solution in the plug consecutively contacts, merges, mixes with and separates from each microdroplets in the array. The solution is diluted by the microdroplet one by one.
and leaves a new microdroplet with the equilibrated concentration. After the plug of solution flows out from the microchannel, a concentration gradient has been generated among the microdroplets in the array; the droplets close to the inlet have the highest concentration while those close to the outlet have the lowest. The formed gradient profile is determined by the volume ratio of the solution plug and the microdroplet of the array and can be well predicted with a simple equation. The concentration range of the gradient can be widened by tuning both the volume ratio above and the number of total microspots in the array. This gradient can also be affected slightly by other parameters including surface hydrophobicity, the flow rate and the size of the solution plug. We use this system to establish the gradient of several different solutes including inorganic salts, small organic molecules and biomolecules. Molecules that can strongly absorb onto channel walls will dramatically change the hydrophobicity of the walls and thus damage the gradient formation. They are typically highly amphiphilic molecules such as SDS and certain macromolecules (HCG). This method works best for most small molecules and other macromolecules such as GFP that do not strongly absorb to the channel walls.

The major advantages of this method are (1) concentration gradients can be generated in thousands of arrayed spots conveniently, (2) the gradient profiles can be theoretically determined and thus be well controlled, and (3) wide ranges of concentrations can be formed in one experiment. Also, only minimum amount of samples is required.
Conclusion Remarks

In summary, this thesis has presented three new fabrication techniques of microfluidic chips and a few applications of fabricated microfluidic chips.

Currently there is no universal material and fabrication technique in microfluidics. There are still great interests in the exploration of new materials and new fabrication techniques for various applications in this fast growing area. In this study, I have made substantial improvements on microfluidic fabrication techniques. I have also demonstrated the applications of microfluidics in chemical and biological researches by establishing stepwise concentration gradients on chip. The results and findings can be summarized as follows:

Three practical problems in microfluidic fabrication are solved.

I have greatly improved the portability of the control setup of the PDMS pneumatic valve for miniaturized and portable devices. A screw-actuated system has been used for controlling the pneumatic valves where a polymethylmethacrylate (PMMA) scaffold is used for holding the screws. Compared with conventional gas cylinder and syringe pump for pressure control, the screw-actuated system has greatly reduced the size and cost of the device. The convenience of this screw actuated system has been demonstrated for cell culturing on the microfluidic chip.

A general strategy to fabricate microchannel systems in hydrogels has been developed. The mixture of two different hydrogel ingredients which have different gelation conditions is used for microfluidic fabrication. The gelation of the first ingredient transfers the microstructure into mixed hydrogel slabs by replica molding and fixed the structure in the molding shape, while the gelation of the second ingredient bonds two hydrogel slabs in contact together by covalent bonding or reversible bonding forces. Microchannels in an agarose-alginate mixed gel system and an agarose-polyacrylamide mixed gel system have
been successfully demonstrated.

I have developed a new technique to fabricate free-standing microperforated membrane from photosensitive prepolymer such as SU-8 photoresist and Norland optical adhesive (NOA). The membrane is formed by sandwiching the liquid prepolymer between two flat surfaces which can be separated from the polymerized membrane. The microhole pattern through the membrane is defined by photolithography. The formed microperforated membrane has been used for surface micropatterning and 3D microfluidic channel system fabrication.

In addition, three techniques for establishing concentration gradients in microarrays have been developed for chemical and biological analyses: (1) in the first, the gradients are generated with microvalves to control the dilution factor of a concentrated solution in different microchambers; (2) the second method forms concentration gradients in microwell arrays by printing a pre-established gradient from a gel slab into the solutions in the microwells; (3) in the third, a concentrated solution plug is flowed through in microchannel across a pre-formed microdroplet array of buffer on a hydrophobic substrate; the gradient is established when the solution plug merges and splits from each microdroplet in the array along the microchannel (the plug of solution is diluted once when it merges with a microdroplet). The screening of etoposide (an anti-cancer drug) and the effect of Zn$^{2+}$ on *E. coli* proliferation have studied with the formed gradients.
References


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Publications


