Droplet-based protein chip with Ni–Co coated surface

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This Letter describes the implementation of a droplet-based protein chip with an Ni–Co alloy layer for conducting immunoassays. The proposed chip consists of a substrate of a printed circuit board and a microchannel layer. On the substrate surface, an Ni–Co coating was fabricated by electrodeposition to immobilise the histidine-tagged protein. Through droplet fusion and mixing, the fused droplets aligned at the downstream position of the microchannel and were immobilised by the Ni–Co layer for further incubation and fluorescence detection. The experimental results showed that the sample which is encapsulated by the carrier fluid does not have evaporation and contamination problems. Therefore strong fluorescence intensity proportional to the sample concentration can be obtained. The fluorescence intensity is also related to the droplet size. An extremely low concentration of encapsulated content in a smaller droplet might result in arduous fluorescence detection. This proposed droplet-based protein chip allows the operation sequence of the immunoassay to be conducted automatically through the manipulation of droplets. Moreover, the fabrication process is simple and the chip can be implemented to immobilise any functional proteins with His-tag attached.

1. Introduction: Droplet-based microfluidic systems, usually forming droplets of an aqueous liquid in an immiscible carrier liquid, provide an elegant solution for assays to be conducted in miniscale volumes [1–3]. To date, considerable research efforts have focused on the generation of droplets with precisely controlled sizes, shapes and internal compositions [4, 5]. Through droplet transportation, fusion and mixing, droplet-based microfluidic systems can add new reagents to perform multi-step chemical or biological reactions [6]. Therefore droplet-based microfluidic systems are suitable for high throughput screening [7], synthesis of nanoparticles [8] and polymerase chain reaction [9]. Moreover, microfluidic droplet techniques have been implemented for research of protein expression, protein crystallisation and immunoassay [10–12].

In this study, a microfluidic device was designed to generate droplets in water-in-oil emulsions working as microreactors for conducting immunoassay. Immunoassay is a widely used biochemical test for measuring analytes normally presented at very low concentrations. It is based on the high affinity recognition capabilities of antibodies with their antigens. In recent years, immunoassay has been performed on microarray chips because of the maturity of the bio-MEMS technologies. The analyte-specific reagents (ASRs), such as antigens, are spotted on the microarray surface. Each microspot works as a capture molecule, and can be incubated with a specific type of analyte from a complex mixture. A microarray allows high throughput study of protein abundance [13]. However, immunoassay requiring multiple steps is a labour-intensive and time-consuming procedure even though it is conducted by using protein microarray chips. On the contrary, a droplet-based protein chip adopts the microfluidic platform so that the sequence of operations can be controlled through the manipulation of the microfluids. In this study, droplets are formed when the reagents are injected into the carrier flow. Moreover, droplet fusion and transportation improve the mixing of different reagents inside the droplet. Droplets are finally aligned at the downstream position of the microchannel for further incubation and fluorescence detection. Manual operations can be reduced. In addition, the reagents encapsulated by the carrier oil are prevented from contamination.

Furthermore, the surface for the immobilisation of protein samples in this proposed chip is an Ni–Co alloy layer fabricated by electrodeposition. The immobilisation mechanism is based on the technique of immobilised metal affinity chromatography (IMAC). Most protein microarrays based on the theory of IMAC were fabricated by using the chelator or compound of mono-metallic ion, such as Ni^{+2}, to capture the histidine-tagged protein [14–16]. However, the protein immobilisation layer of this proposed droplet-based protein chip possesses bi-metallic elements, that is, by means of adding cobalt to nickle to enhance the specific binding capability to immobilise functional proteins with the His-tag attached [17]. This proposed chip has good performance and, moreover, its fabrication process is simple.

2. Materials and methods: This proposed droplet-based Ni–Co protein chip uses the printed circuit board (PCB) as the substrate on which a microchannel layer is attached. An Ni–Co layer fabricated on the PCB substrate by electrodeposition is used for immobilising the histidine-tagged protein. Then, the droplets are generated and manipulated to flow through the microchannel for rapid mixing and consequent immunoassay.

The microfluidic device is composed of four injection inlets, two T-junctions, a Y-junction and an outlet channel, as illustrated in Fig. 1*a*. Their depths are all 100 μm. The inlet channels indicated as number 2 and 3 in Fig. 1*a* are called carrier channels for introducing the carrier fluid, whereas the other inlet channels are called solution channels for the injection of reagents. Two different channel widths were designed, that is, 300 and 500 μm, for comparison. The outlet channel is arranged in a zigzag pattern for droplet mixing and collection.

In this study, silicon oil was used as a carrier fluid. One set of inlet channels forming a T-junction is used to produce the biotin droplets, whereas the other set of inlet channels is for generating the streptavidin droplets. The monodispersed droplets are generated at the T-junction when the aqueous liquid, that is, biotin or streptavidin, is injected into the silicon oil stream and sheared by the carrier flow. Furthermore, both kinds of droplets contact and fuse at the Y-junction. The design of the Y-junction prevents the reagents from flowing across the opposite channel.

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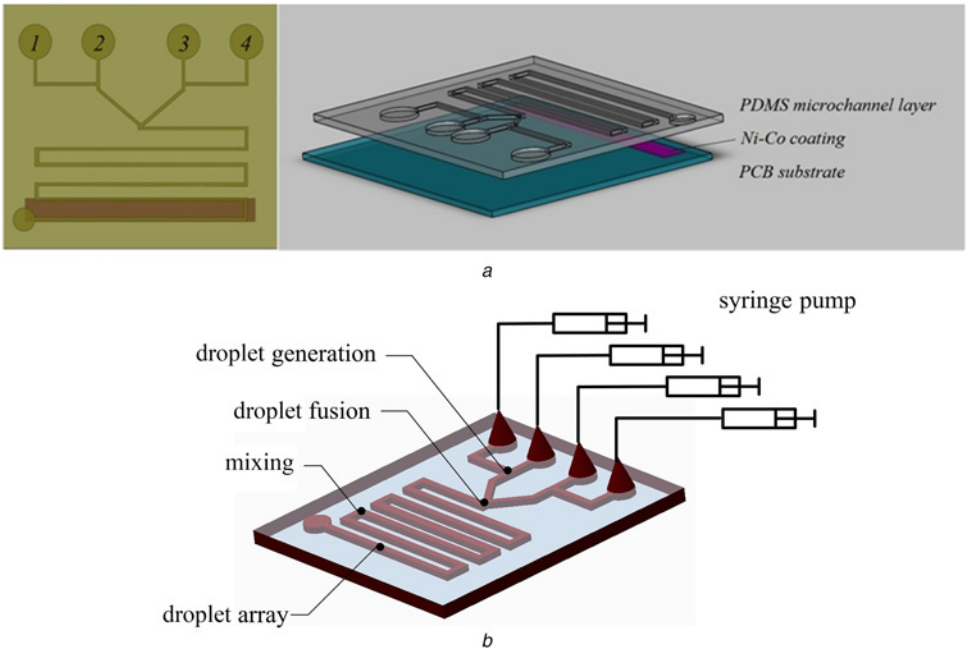

Figure 1 *Design of droplet-based protein chip*
The chip consists of a PCB substrate and a PDMS microchannel layer (Fig. 1*a*)
The experimental setup (Fig. 1*b*)

An SU-8 mould was first prepared by fabricating the opposite pattern of the microchannel on a glass substrate. Then, a microchannel layer was formed by casting polydimethylsiloxane (PDMS) in the SU-8 mould. After curing, the PDMS layer was carefully peeled from the SU-8 mould.

On the PCB substrate, an Ni–Co film covering an area of W 3.6 mm × L 20 mm located at the downstream position of the outlet channel was fabricated by electrodeposition to immobilise the histidine-tagged proteins for immunoassay. PCBs having a conducting layer typically made of thin copper foil can serve as the plating seed layer directly. Since electrodeposition is a well-developed technique, the fabrication of this chip becomes simple.

The PCB was trimmed into the size of 7.5 × 2.5 cm so that it can be placed on the scanner for fluorescence detection. Each substrate contains two droplet-based protein chips. Prior to the electroplating process, photolithography process was used to confine the region of the Ni–Co film. After stripping the photoresist of this region, the electrodeposition process was conducted in a W 18 cm × L 25 cm × H 18 cm electroplating tank. The thickness of the electrodeposited Ni–Co film was 2 μm. Then, the fabrication process of the microstructures on the PCB substrate was accomplished and no further treatment was needed for the other regions. The droplet Ni–Co protein chips were then kept at 45% relative humidity and 23°C for two weeks before usage to avoid the influence of the pH value of the coating buffer on the sensitivities of the Ni–Co film [17].

To bond the microchannel layer with the PCB substrate, oxygen plasma treatment was adopted in this study. Then, the PDMS layer was placed on the substrate and the chip was heated in a 70°C hot circulator oven for 30 min to remove the residual moisture.

The success of this proposed droplet-based Ni–Co protein chip relies on several crucial factors, including the formation, the mixing and the immobilisation of the droplets. To examine the performance of this chip, the possibly mistakable or weak bio-reactions must be excluded so that these factors can be investigated. Since the binding between biotin and streptavidin is the strongest non-covalent biological interaction known [18], we utilised this mechanism to show that this proposed droplet-based protein chip has the ability to accomplish these aforementioned functions by observing the final fluorescence intensity of the streptavidin–Cy5 conjugate. The necessary bio-materials include penta-His biotin purchased from QIAGEN Inc. and streptavidin–Cy5 conjugate obtained from Zymed Laboratories, Inc. All the reagents were of analytical grade. In addition, a diluted buffer which is 1% BSA in a 0.1 M phosphate buffer was used to prepare different concentrations of biotin and streptavidin–Cy5. In this study, the streptavidin–Cy5 conjugate had a constant concentration of 5 μg/ml (200 × dilution). However, the His-tagged biotin solution was diluted to different concentrations: 33 μg/ml (30×), 16.6 μg/ml (60×), 11 μg/ml (90×), 8.3 μg/ml (120×) and 6 μg/ml (150× dilution).

The solutions of His-tagged biotin and streptavidin–Cy5 conjugate are introduced into the solution channels, respectively; whereas silicon oil is introduced into the carrier channels. The syringe pump is used to drive these solutions and oil through their own microchannels. Owing to the symmetric configuration of the microchannels and the same pumping rates, the frequencies of the droplet formations at both T-junctions are identical. That is, both kinds of monodispersed droplets are generated synchronously so that a pair of droplets can precisely contact and, hence, fuse at the Y-junction. Each fused droplet is still isolated by the carrier fluid to prevent further aggregation. Then, the individual fused droplet exits to the outlet channel. Although the fused monodispersed droplet traverses the zigzag outlet channel, its encapsulated contents mix concomitantly and rapidly. After the droplets align at the downstream position of the outlet channel, the PDMS microchannel layer is carefully peeled off. Two-hour incubation in a 37°C hot circulator oven is conducted. Without the limitation of the microchannel, the silicon oil starts to drain away, allowing the His-tagged biotin to be immobilised on the Ni–Co surface through affinity binding between the intermediate metal and histidine. In addition, the interaction between biotin and streptavidin is strengthened. Then, the chip is washed with a washing buffer (phosphate buffer with 5% Tween-20) three times for 1 min per wash in order to remove the unimmobilised or residual biotin and streptavidin. Finally, the fluorescence scan is performed by laser excitation in a scanner Gene TAC LS IV (manufactured by Genomic Solutions, Inc.) with an excitation light wavelength of 635 nm. The fluorescence intensity is analysed using Gene Pix 4.1 software.

3. Results and discussion: This proposed droplet-based Ni–Co protein chip implements the His-tag immobilisation technique to conduct the immunoassay in droplet arrays. To verify the performance of this chip, three different tests were conducted in this study. To obtain the statistical results, three chips were prepared for repetitive experiments in each test. The experimental procedures and results are elaborated as follows.

• *Droplet fusion and mixing*: To perform immunoassay, a pair of droplets containing His-tagged biotin and streptavidin–Cy5 conjugate, respectively, must be fused at the Y-junction and the encapsulated contents must mix thoroughly when the fused droplet traverses the zigzag outlet channel. Hence, in this experiment deionised (DI) water and red dye solution were used as the materials for the mixing test and injected into the solution channels, respectively. In addition, silicon oil was introduced into both carrier channels. A KDS Legato 270 programmable syringe pump (manufactured by KD Scientific Inc.) was utilised to manipulate these fluids simultaneously with the same flow rate. The images of the droplet were captured at different locations, denoted as *a* to *f*, as shown in Fig. 2*c*. Then, the grey scales of the droplet images were analysed by using software to investigate the mixing phenomenon. Chips with a channel size of 500 µm were used for observation.

The grey scale of water is in the range of 50–100, whereas the scale value of the red dye locates in the range of 200–255. In Fig. 2*a*, each bar shows the grey scale and the number above it denotes the spot on the droplet. As shown in Fig. 2*b*, there are nine spots where the grey scales were calculated. Therefore the change of the grey scales within a droplet explains the mixing effect. At location *a* of the microchannel, all the grey scales at the nine spots within a droplet were almost identical in the range of 50–100. This result describes that a DI water droplet passed and its image was captured. Similarly, at location *b* of the microchannel, a red dye droplet passed because the grey scales were in the range of 200–255. At location *c* of the microchannel, the two droplets merged but the contents were not mixed yet. The barchart clearly shows the result because the encapsulated DI water and red dye solution are separated. However, both contents started to mix whenever the fused droplet passed a bending corner. As shown in Fig. 2*a*, the grey scales within a droplet have a significant change after passing through eight or more bending corners and finally become almost identical again. The scale values lie in between the original grey scales of the pure contents. This result verifies that efficient mixing in a fused droplet can be achieved.

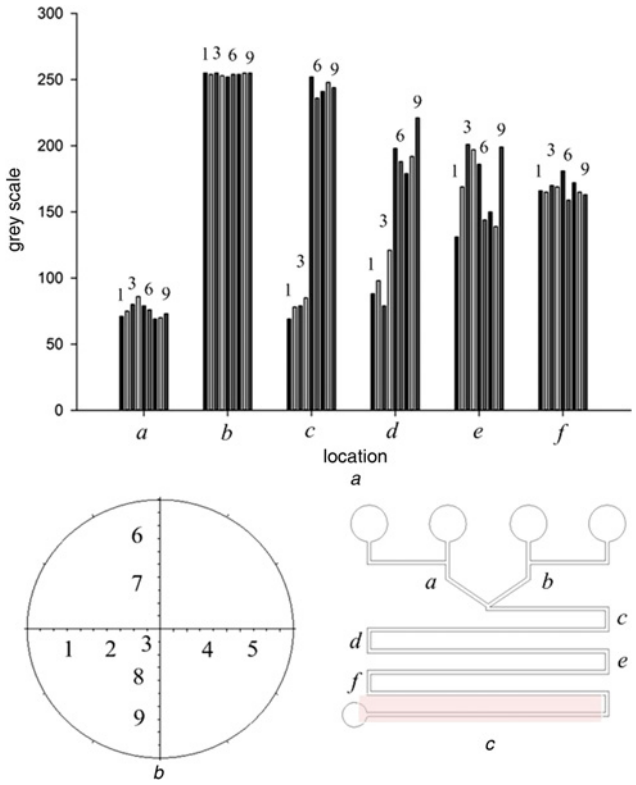

Figure 2 *Droplet fusion and mixing*
At different locations of the microchannel, the spot grey scales of a droplet were detected for analysing the mixing effect inside the droplet (Fig. 2*a*)
The locations of nine spots on a droplet (Fig. 2*b*)
The images of the droplet were captured at locations a–f (Fig. 2*c*)

• *Protein immobilisation*: Since this proposed droplet-based Ni–Co protein chip has been confirmed to have an efficient mixing function using the zigzag outlet channel, its performance for the immunoassays relies on whether the His-tagged biotin can be immobilised on the Ni–Co coating. This issue can be examined by fluorescence detection because of the use of fluorescent dye Cy5. In addition, the fluorescence intensity after the immunoassay represents the binding capability between the His-tagged biotin and the Ni–Co membrane. As shown in Fig. 3, the fluorescence intensity is in linear proportion to the concentration of the His-tagged biotin. That is, the higher concentration (30× diluted biotin) has stronger fluorescence intensity. This result indicates that the Ni–Co alloy surface of the protein chip was able to capture the His-tagged biotin by its functional group to competently immobilise the histidine tag. Moreover, the volume of a droplet generated in a chip with channel size of 500 µm was about 0.5 µl. That is, the sample abundance of His-tagged biotin (or streptavidin–Cy5 conjugate) in a droplet was 0.5 µl at most. It is difficult to handle such a small amount of sample by manual operation and any inaccurate experimental results might lead to incorrect inferences. However, the average detected fluorescence intensity of 30× diluted biotin using chips with a channel size of 500 µm reached 59 411 a.u. (with a coefficient of variation of 1.5%). Compared with our previous experimental result, the detected fluorescence intensity is twice stronger than that using the standard Ni–Co microarray. Obviously, this droplet-based Ni–Co protein chip has excellent performance. A conclusion can be generalised from the result. Under the proper flow rate, all the droplets have almost a uniform size, containing equivalent samples. Furthermore, the sample which is encapsulated by the carrier fluid does not have evaporation and contamination problems. Therefore strong and proportional fluorescence intensities can be obtained.

• *Influence of droplet size upon immunoassay*: For the same biotin concentration, the amount of sample encapsulated in a droplet generated in a channel of 300 µm is smaller than that in a channel of 500 µm. Therefore weaker fluorescence intensities were detected, as shown in Fig. 3. However, the fluorescence intensity is also in linear proportion to the concentration of His-tagged biotin. The average detected fluorescence intensity of 30 × diluted biotin reached 48 095 a.u. (with a coefficient of variation of 3.8%). This result indicates that the chip with smaller channel size, forming smaller droplets and requiring smaller amount of reagents, still can provide good performance for the immunoassay. Nevertheless, the chip performance is worse than that with larger channel size. If we fit straight lines to the experimental data by using the method of least squares, it is worth noting that the slope of the straight line for a channel size of 300 µm is steeper. In a smaller droplet, the encapsulated contents become sparse (e.g. 150× diluted biotin), and the fluorescence detection becomes arduous. By using this proposed chip with a channel size of 300 µm, the detection limit is suggested to be 6 µg/ml (150× dilution). Otherwise, the detected fluorescence intensities

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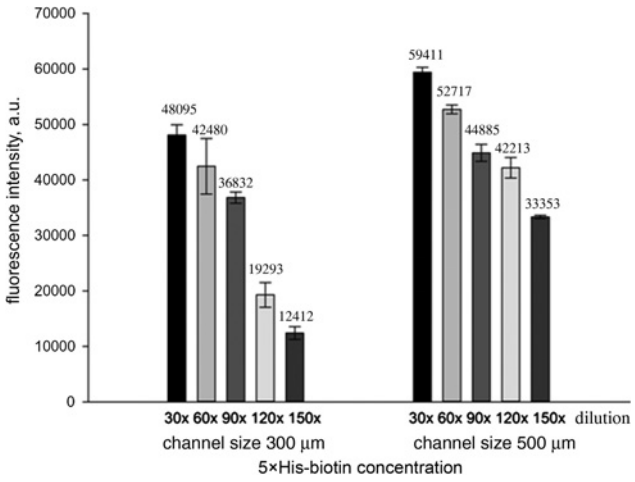

Figure 3 *Fluorescence intensity after the immunoassay is in linear proportion to the concentration of His-tagged biotin*

become too low to be discriminated for the concentration of biotin lower than this limit. However, the chip with a channel size of 500 μm can be used to detect biotin that has even lower concentration.

4. Conclusion: This proposed droplet-based protein chip allows the sequence of operations of the immunoassay to be manipulated automatically through the microfluidic method. Only a smaller consumption volume of reagents, that is, less than 0.5 μl for a channel size of 500 μm, is needed. The Ni–Co alloy coating fabricated by electrodeposition on the substrate surface is used to immobilise the His-tagged proteins. Although biotin was adopted for experiment in this study, the chip can be implemented to immobilise any functional proteins with His-tag attached. The experimental results showed that the sample which is encapsulated by the carrier fluid does not have evaporation and contamination problems. Therefore strong fluorescence intensity which is proportional to the sample concentration can be obtained. In addition, the fluorescence intensity is also related to the droplet size. However, an extremely low concentration of encapsulated content in a smaller droplet might result in arduous fluorescence detection. The development of the chip surface by electrodeposition is an economical approach because electrodeposition is a well-developed technique; in addition, only inexpensive materials, such as PCB and PDMS, are needed for the fabrication of this chip. The advantages of this droplet-based protein chip include good performance, high repeatability and inexpensiveness.

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6 References

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