Droplet-based microfluidic washing module for magnetic particle-based assays

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In this paper, we propose a continuous flow droplet-based microfluidic platform for magnetic particle-based assays by employing in-droplet washing. The droplet-based washing was implemented by traversing functionalized magnetic particles across a laterally merged droplet from one side (containing sample and reagent) to the other (containing buffer) by an external magnetic field. Consequently, the magnetic particles were extracted to a parallel-synchronized train of washing buffer droplets, and unbound reagents were left in an original train of sample droplets. To realize the droplet-based washing function, the following four procedures were sequentially carried in a droplet-based microfluidic device: parallel synchronization of two trains of droplets by using a ladder-like channel network; lateral electrocoalescence by an electric field; magnetic particle manipulation by a magnetic field; and asymmetrical splitting of merged droplets. For the stable droplet synchronization and electrocoalescence, we optimized droplet generation conditions by varying the flow rate ratio (or droplet size). Image analysis was carried out to determine the fluorescent intensity of reagents before and after the washing step. As a result, the unbound reagents in sample droplets were significantly removed by more than a factor of 25 in the single washing step, while the magnetic particles were successfully extracted into washing buffer droplets. As a proof-of-principle, we demonstrate a magnetic particle-based immunoassay with streptavidin-coated magnetic particles and fluorescently labelled biotin in the proposed continuous flow droplet-based microfluidic platform. © 2014 AIP Publishing LLC.

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I. INTRODUCTION

Droplet-based microfluidic platforms have been shown with the use of immiscible carrier oil that enables all molecular reactions to occur in the confined small volume of a single droplet for the isolation of reagents.^{1–4} The manipulation of individual droplets allows the reagents to be transported, merged, mixed, and analyzed as a micro-reactor effectively and quickly.^{5–13} The technology has been considered as a promising platform due to the need for automated high-throughput systems for various chemical and biological analyses.¹⁴ Moreover, when compared to conventional methods, this approach is more attractive since it does not require costly and time-consuming experimental procedures.^{15,16}

In the past decade, there has been a proliferation of research into microfluidic systems with the use of magnetic particles as a solid support.^{17–20} This is because the magnetic particles can easily be manipulated in a fluidic environment by using external magnetic fields without complex or expensive equipment to handle them. Moreover, they offer high surface-to-volume ratio resulting in relatively large binding sites for efficient biochemical reactions.^{21–23} In this field, several groups have revealed work relying on magnetic particles for DNA separation,²⁴ protein digestion,²⁵ immunoassays,²⁶ and cell capture.²⁷ In particular, Peyman *et al.* demonstrated an

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advanced on-chip multi-step procedure in a continuous flow, wherein functionalized magnetic particles were deflected to perform consecutive binding and washing steps on their surface by traversing multi-laminar reagents streams.²⁸ Despite the recent progress in this field, the continuous flow magnetic handling has limitations in re-configurability and compartmentalization for isolated magnetic bead-based bioassays to prevent contamination and dilution effects between multiple reagents. In addition, any increase in the incubation time of reagents for post processing is limited, due to a short traversing time across the multi-laminar flow streams.

Our approach is to demonstrate a feasibility of magnetic particle based immunoassay on a droplet-based microfluidics platform. However, in conventional droplet-based microfluidics technology, additive functions (e.g., particle/cell encapsulation, sequential fusion, rapid mixing and dilution) are well demonstrated, whereas subtractive functions (e.g., in-droplet particle separation) have been barely attempted, unlike the continuous flow and electrowetting-based digital microfluidic systems. For example, Lombardi and Dittrich showed a concept of magnetic particle separation in a droplet-based microfluidic platform for investigating drug-protein interactions.²⁹ In their system, the magnetic particles were separated into one side of a droplet by a magnetic field and the droplet was then split into two at a T-shape junction, one containing the majority of magnetic particles and the other almost devoid of them. Similarly, in our previous work, we demonstrated a magnetic particle manipulation for separation in a magnetic particle-based immunoassay.³⁰ Despite their huge potential, both systems have so far suffered from the lack of washing of unbound reagents within a droplet. Sista *et al.* and Vergauwe *et al.* have reported digital microfluidic platforms where a droplet is electrostatically controlled on an array of insulated electrodes to perform magnetic particle-based immunoassays.^{31–33} In these systems, a serial dilution method was used to wash unbound reagents from magnetic particles. The serial dilution process was repeated until a sufficient background noise reduction was achieved. Although the digital microfluidics format on flat surfaces is useful for constructing magnetic bead-based assays with discrete droplets, the throughput is limited to several hundred or fewer assays and the droplet volumes are somewhat large (e.g., $\sim 1 \mu\text{l}$). Therefore, a robust and simple washing step in a continuous flow droplet-based microfluidics platform is highly demanded for magnetic particle-based assays. Ali-Cherif *et al.* demonstrated a multi-step assay by using magnetic particles in a droplet microfluidic device.³⁴ Their approach is to use a capillary tube to generate droplets and a magnetic tweezers to extract magnetic particles for a full automatic droplet-based system.

The motivation in our study was to overcome the technical challenges in developing a droplet-based washing module that allows for significant reduction of unbound reagents in droplet-based microfluidic devices. A unique approach to realize such a droplet-based washing function is to traverse the mobile solid supports (e.g., functionalized magnetic particles) from one side (containing sample) of a parallel-merged droplet to the other side (containing buffer), perpendicular to the direction of the flow, by the use of an external magnetic field. In this paper, we present the demonstration of magnetic particle-based in-droplet separation, operating a droplet-based washing step in a continuous flow droplet-based microfluidic platform without a sophisticated system to manipulate the droplets. Droplet generation, mixing, synchronization, electrocoalescence,^{35,36} in-droplet magnetic particle separation, and splitting are sequentially performed on a microfluidic device to achieve such a droplet-based washing function for magnetic particle-based immunoassays. The key approach is that as soon as the electrocoalescence occurs between two paired droplets in a parallel manner (one containing streptavidin conjugated with magnetic particles and biotin with a fluorescent marker that are already mixed prior to the electrocoalescence and the other containing a washing buffer), the magnetic particles bound with the biotin are extracted towards the washing buffer droplet by an externally applied magnetic field, leaving the unbound excess biotin behind in the sample droplet. Thus, this washing step allows for a significant decrease in the level of background noise to be detected within the washing buffer droplets. In this work, the optimized condition of flow rate ratios was investigated to increase the parallel synchronization and lateral electrocoalescence efficiency. Then, the fluorescent intensity emitted from droplets was analyzed before and after the washing step. This platform has a great potential for practical use due to the unique magnetic particle-based in-droplet separation.

II. EXPERIMENTAL

A. Working principle

A total workflow of the proposed droplet-based microfluidic device for the in-droplet magnetic particle separation including a washing function is illustrated in Fig. 1. The droplet-based washing function is performed by a series of the following procedures. (i) Two trains of droplets (D1 and D2) are generated at droplet generation cross-junctions 1 and 2 at the given flow rate condition (see Table I).³⁷ D1 represents a droplet with washing buffer (①). D2 denotes a sample droplet containing the mixture of fluorescently labelled biotin (②) and streptavidin conjugated with magnetic particles (③) that are co-infused at the cross-junction 2. (ii) The sample droplets are fully mixed to form the streptavidin-biotin complex (④) in a serpentine channel region.³⁸ (iii) The sample droplets are introduced into a ladder-like channel network to make them synchronized with the washing buffer droplets.^{39,40} The droplet synchronization unit enables the two trains of the droplets to be merged laterally. (iv) The two parallel-synchronized droplets are merged at an entrance of the X-shape separation junction by an electric field, which can lead to destabilization of the surface of the droplets to be easily merged.⁴¹ (v) At this X-shape junction, the magnetic particles are traversed to the washing buffer side (top of the merged droplet) by an externally applied magnetic field, leaving the unbound biotin behind in the sample droplet side (bottom of the merged droplet). (vi) Subsequently, the merged droplet is split into two daughter droplets at the junction:⁴² one (D3) containing the majority of magnetic particles (⑤) and the other (D4) containing the excess unbound biotin (⑥). In order to increase the washing efficiency and minimize the dilution in the merged droplet, the asymmetric splitting is performed by a different fluidic channel resistance at the two outlets: the fluidic resistance of the channel for D3 = $1.5\times$ and the fluidic resistance of the channel for D4.

B. Materials and operation

For magnetic fields, a rectangular neodymium-iron-boron magnet ($12.7 \times 3.2 \times 3.2 \text{ mm}^3$, Emovendo Magnets & Elements, Petersburg, WV, USA) was used. Mineral oil (M8410, Sigma-Aldrich) was used as an immiscible phase and 2% nonionic surfactant (Span-80, S6760, Sigma-Aldrich) was mixed to prevent accidental coalescence. The channels were treated with a commercial surface coating agent (Aquapel, 47100, Pittsburgh Glass Works, PA, USA) to enhance the hydrophobicity of microchannels.⁴³ The concentration of fluorescently labelled biotin (biotin-4-fluorescein, Molecular Probes, Invitrogen) was $2.5 \mu\text{g/ml}$ for washing performance and varied

FIG. 1. (a) Schematic workflow of the proposed droplet-based microfluidic device for magnetic particle-based assays including a washing function by a series of procedures: (i) droplet generation; (ii) mixing; (iii) parallel synchronization; (iv) lateral electrocoalescence; (v) in-droplet magnetic particle separation; and (vi) asymmetrical lateral splitting. (b) Simple streptavidin-biotin immunoassay requiring a single binding and washing step.

TABLE I. Flow rate conditions to generate the droplets at the cross-junctions 1 and 2.

Flow rate conditions	Cross-junction 1 ($\mu\text{l/h}$)		Cross-junction 2 ($\mu\text{l/h}$)		
	Q_{Oil}	$Q_{\text{Water}} \text{ (①)}$	Q_{Oil}	$Q_{\text{Water}} \text{ (②)}$	$Q_{\text{Water}} \text{ (③)}$
A	35	10	35	5	5
B	35	20	35	10	10
C	35	30	35	15	15
D	35	40	35	20	20
E	35	60	35	30	30
F	35	80	35	40	40

from 62.5 to 500 ng/ml for quantitative analysis. It should be noted that the concentration of biotin is relatively high since our detection system was limited for lower biotin concentrations. But we expect that there is potential for this system to detect lower biotin concentrations with a more sophisticated detection system. The magnetic beads (2.8 μm diameter, Dynabeads M-270 streptavidin, Invitrogen) were diluted in deionized water, giving $\sim 6 \times 10^8/\text{ml}$ concentration. The DC voltage (50 V_{DC}) was supplied by a high-voltage source (HVS448–1500, Lab-Smith, Livermore, CA, USA) connected to a computer controller to generate an electric field through indium tin oxide (ITO) electrodes. The fabricated microfluidic device was connected through a silicone tube to a syringe pump (KDS100W, Fisher Scientific, IL, USA). All experimental results were captured by a CCD camera mounted on a Nikon stereo microscope.

C. Microfluidic device fabrication

The microfluidic device was fabricated by using conventional soft-lithography technique with poly-(dimethylsiloxane) (PDMS).⁴⁴ First, prior to ITO deposition as the electrodes, a positive photoresist (S1818) was patterned to define the position of electrodes on the surface of the glass slide substrate. The ITO electrodes (300 nm) were deposited on the substrate by DC sputter. The remaining positive photoresist was removed by a lift-off process. Second, in order to create a master mould, a negative photoresist (SU-8 2050) was spin-coated on 3-in. silicon wafer with a thickness of 50 μm and then patterned for the designed microchannels by the soft-lithography technique. Third, a prepolymer of PDMS and curing agent were thoroughly mixed at a ratio of 10:1 (wt/wt). The mixed PDMS was degassed in a vacuum chamber. A permanent magnet for the magnetic particle manipulation was directly placed on the SU-8 master mould, which was preformed about 1 mm away from the channel for defining the location of the magnet. The PDMS was carefully poured onto the SU-8 master mould and cured in an oven. The PDMS replica was peeled off, defining the position of the magnet. Finally, the replicated PDMS channel was aligned to the electrodes under the microscope and bonded irreversibly by O_2 plasma treatment.

D. Fluorescence detection and analysis

The fluorescence images were captured by the CCD camera mounted with the microscope. Then, the images were analyzed by image software (HCImage) to quantify the fluorescence intensity emitted by biotin. In order to characterize the performance of the washing step, the fluorescence intensity before and after the washing step without the magnetic particles was measured at the region of magnetic particle separation: the droplets containing biotin at the bottom channel (D2) before the electrocoalescence and the droplets containing washing buffer at the top channel (D3) after the splitting.

III. RESULTS AND DISCUSSION

In our device, the droplet size needs to be optimized to stabilize both the synchronization and electrocoalescence. We investigated the droplet size by adjusting the flow rate ratio of

water and oil ($Q_{\text{Ratio}} = Q_{\text{Water}}/Q_{\text{Oil}}$) that was varied from 0.29 to 2.29 at the droplet generation cross-junctions 1 and 2 as listed in Table I. The washing buffer was injected at the cross-junction 1. Both the streptavidin coated with the magnetic particles and the fluorescently labelled biotin were co-infused at the same ratio at the cross-junction 2. The Q_{Oil} was fixed at $35 \mu\text{l/h}$, while Q_{Water} was varied from 10 to $80 \mu\text{l/h}$, resulting in the different diameter of droplets as shown in Fig. 2(a). In the optimized condition (case C), the volume of droplet was about 11 nl. In order to perform the parallel synchronization of droplets, the ladder-like channel network was used in which the top and bottom channels were interconnected with narrow fluidic channels.^{39,40} The mechanism of synchronization relies on the automatic tuning of velocity of droplets due to the pressure difference between the two channels. As the droplets pass through the ladder-like channel network, the existing pressure difference caused by the droplets squeezed between the channels (top and bottom channel width: $200 \mu\text{m}$) allows the oil phase to flow through the narrow fluidic channels until the pressure is matched. As a result, the lateral synchronization is passively achieved. However, it should be noted that, for the complete synchronization, the size of droplets needs to be larger than the channel width, resulting in a plug-shape droplet to enable the oil phase to flow across the narrow channels rather than across the droplets. Another requirement for successful synchronization is that the droplet generation frequency must be matched between the cross-junctions 1 and 2. In our experiment, the optimized flow rate ratio was $Q_{\text{Ratio}} = 0.85$, the corresponding droplet size was $265 \mu\text{m}$, and the droplet generation frequency was 3 droplets/s (case C in Fig. 2).

FIG. 2. (a) Droplet size as a function of flow rate ratio. (b) Electrocoalescence efficiency as a function of flow rate ratio. Our optimal working point is indicated by C.

The lateral electrocoalescence of the synchronized droplets occurred at the beginning of the X-shape junction in which ITO electrodes were placed. To ensure the successful electrocoalescence, we applied a DC voltage of 50 V_{DC}. The electrocoalescence efficiency, defined by the number of successfully merged droplets over the total number of generated droplets, was plotted as a function of flow rate ratio (or droplet size) as shown in Fig. 2(b). In most cases, the efficiency was above 80%, except for the case A. The maximum efficiency of 94% was achieved at the optimized flow rate ratio of $Q_{\text{Ratio}} = 0.85$ (case C). As detailed in our previous studies, if the droplet length and the generation frequency are perfectly matched, 100% synchronization efficiency can be attainable,^{39,40} after the seamless electrocoalescence at the X-shape junction.

In the electrocoalescence, two failure mechanisms were observed as shown in Fig. 3. In case A, no electrocoalescence happened at all at the X-shape junction (Fig. 3(a)). This failure occurred due to the small size of the droplets. When the droplet size is smaller than the channel width, there are leak flows of the carrier oil across the un-squeezed droplets rather than across the interconnected narrow fluidic channels. Furthermore, in this condition (case A), the droplets could not touch each other at the X-shape junction due to the small size of the droplets. On the other hand, in case F, accidental droplet merging happened as large droplets got close to each other (Fig. 3(b)). This failure occurred with short-spaced large droplets.

In order to demonstrate washing performance, we investigated the fluorescence intensity emitted from the fluorescently labelled biotin in D2 and D3, without injection of magnetic particles. For successful washing, we need to minimize the internal mixing inside the merged droplet, mainly caused by the counter-rotating recirculation, prior to splitting into two lateral droplets (D3 and D4) at the end of the X-shape junction. To have better performance, an asymmetrical lateral droplet splitting was conducted by making the size of D3 smaller than that of D4. By the asymmetrical lateral droplet splitting, the unexpected dilution of the fluorescently

FIG. 3. (a) Photograph of no electrocoalescence due to the small size of droplets (case A, flow rate: $Q_{\text{Oil}} = 35 \mu\text{l/h}$ and $Q_{\text{Water}} = 10 \mu\text{l/h}$). (b) Photograph of accidental electrocoalescence with short-spaced large droplets (case F, flow rate: $Q_{\text{Oil}} = 35 \mu\text{l/h}$ and $Q_{\text{Water}} = 80 \mu\text{l/h}$). The scale bar is 200 μm .

FIG. 4. Comparison of fluorescence intensity in droplet D2 before the lateral electrocoalescence and in droplet D3 after the asymmetrical lateral splitting. The insetted fluorescent image shows the minimized dilution effect by the asymmetrical splitting. The scale bar is $200\ \mu\text{m}$.

labelled biotin in D2 with the buffer in D3 was minimized, which could significantly decrease the background noise in D3. The average fluorescence intensity in droplet D2 before the lateral electrocoalescence and in droplet D3 after the asymmetrical lateral splitting was measured as shown in Fig. 4. The intensity was calculated by measuring 20 droplets. The distribution of fluorescence intensity in D3 was $\sim 4\%$ compared to that in D2. The excess biotin in D3 was significantly removed by more than a factor of 25 through such a single washing step. This washing step through the lateral synchronization-electrocoalescence-splitting will increase the relative fluorescence signal from magnetic particles by removing the background noise signal in D3.

After the demonstration of the washing step, we added magnetic particles in the sample droplets to study in-droplet magnetic particle separation. The calculated mass of magnetic particle included in a single droplet was about $0.11\ \mu\text{g}$. Fig. 5 shows the sequential photographs of the in-droplet magnetic particle manipulation. The red arrows indicate the clusters of the magnetic particles. In this experiment, the optimized condition (case C) was used for the stable in-droplet separation. A permanent magnet was positioned about 1 mm away from the X-shape junction where the in-droplet magnetic particle separation occurred perpendicular to the direction of flow. The magnetic field force was calculated to be about 320 mT at the junction. As the magnetic particles approached near the magnetic fields, they were magnetized, locally in the form of clusters in each droplet. Under the magnetic fields, the clusters were concentrated towards the magnet, so that the clustered magnetic particles easily traversed to the washing buffer side without leaving the clusters in the unbound biotin sample side. Instead of using the permanent magnet, an electromagnet can be used since it is advantageous in terms of controllable electrical signals and strength of magnetic field. As a result, the droplet-based washing step was successfully achieved by extracting the magnetic particle from the sample droplets to the buffer droplets. As shown in Fig. 5, the lateral synchronization-electrocoalescence-splitting process with the in-droplet magnetic particle separation was successfully demonstrated.

As a proof-of-principle of the proposed in-droplet magnetic particle separation, a well-established streptavidin-biotin binding immunoassay involving only a single washing step was performed (see supplementary material⁴⁵). The assay was performed by conducting a series of procedures with the optimized experimental conditions (case C) in the proposed droplet-based microfluidic device.

FIG. 5. Sequential photograph images to show the manipulation of the magnetic particles within the droplets. The magnetic particles are already concentrated to one side of the droplet as they approach the permanent magnet so that they are extracted into the washing buffer side. Red arrows indicate the clusters of the magnetic particles. The scale bar is 200 μm .

FIG. 6. (a) The separated magnetic particles with the streptavidin-biotin complex with the complex emitted a fluorescence signal and (b) the excess unbound biotin to the waste droplets. The scale bar is 200 μm .

FIG. 7. Fluorescent intensity emitted from the magnetic particles as a function of the biotin concentration. The detection limit was 62.5 ng/ml on the proposed microfluidic device including the washing step.

At the outlet 1, the separated magnetic particles with the streptavidin-biotin complex (⑤) were collected in each droplet (D3, top in Fig. 6(a)). The clusters of the magnetic particles conjugated with the complex emitted a strong fluorescence signal with considerably reduced background noise signal (bottom in Fig. 6(a)). The proposed droplet-based washing function could wash away the excess unbound biotin to the waste droplets (D4). The droplets without the magnetic particles were discarded to the outlet 2 (left in Fig. 6(b)). The background signal from D4 was uniform because of the uniformly distributed biotin (⑥) that were fluorescently labelled (right in Fig. 6(b)). For quantitative analysis as a function of biotin concentration ranging from 62.5 to 500 ng/ml, the fluorescent intensity was investigated as shown in Fig. 7. In order to measure the intensity of magnetic particles, the droplets (D3) were collected at outlet 1 and analyzed using Olympus Wasabi imaging software package. Since our detection system is limited to measure the intensity of moving droplets, the collected droplets (D3) were investigated in the stationary state. With increasing the concentration, the fluorescent intensity was increased and the detection limit of the device was 62.5 ng/ml including the washing step.

The above concept-of-principle has validated the potential applicability of the droplet-based microfluidic platform technology to magnetic particle-based immunoassays, reducing the background noise caused by the excess biotin. By integrating multiple droplet-based washing modules in series, one can apply generic magnetic separation procedures requiring more than one washing step (e.g., sandwich immunoassay involving two binding-washing steps). Therefore, the proposed platform has strong potential applications for droplet-based sample preparation of cells, antibodies, antigens, proteins, viruses, DNA, RNA, etc., by binding them to the mobile magnetic beads. The transformative potential use of the proposed droplet-based magnetic particle separation technology is significant.

IV. CONCLUSIONS

We have successfully demonstrated an efficient way to significantly remove unbound reagents by more than a factor of 25 in the droplet-based microfluidic platform. This was accomplished by traversing functionalized magnetic beads from one side (containing sample) of a parallel-merged droplet to the other side (containing buffer) and washing away the excess unbound reagents to the waste droplet. The robust parallel synchronization of two trains of droplets was achieved by the ladder-like channel structure, followed by seamless lateral electro-coalescence at the entrance of the X-shape junction. The proposed in-droplet separation of the magnetic particles enabled a straightforward magnetic particle-based immunoassay involving a single washing step. With various surface functionalities of the magnetic particles, the proposed

method has great potential for many magnetic particle-based immunoassays in the continuous flow droplet-based microfluidic platform.

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