On-chip immuno-agglutination assay based on a dynamic magnetic bead clump and a sheath-less flow cytometry

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ABSTRACT

Immunoagglutination assay is a promising approach for the detection of waterborne analytes like virus, cells, proteins with its advantages such as a smaller amount of reagents and easier operation. This paper presents a microfluidic agglutination assay on which all the assay processes including analyte capture, agglutination, and detection are performed. The chip integrates an on-chip pump for sample loading, a dynamic magnetic bead (MB) clump for analyte capture and agglutination, and a sheath-less flow cytometry for particle detection, sizing, and counting. The chip is tested with streptavidin-coated MBs and biotinylated bovine serum albumin as a model assay, which realizes a limit of detection (LOD) of 1 pM. Then, an antigen/antibody assay using rabbit IgG and goat anti-rabbit IgG coated MBs is tested and a LOD of 5.5 pM is achieved. At last, human ferritin in 10% fetal bovine serum is tested with Ab-functionalized MBs and the detection achieves a LOD of 8.5 pM. The whole procedure takes only 10 min in total.

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I. INTRODUCTION

Immunoagglutination assay is a homogeneous assay based on agglutination of antibody-coated particles in the presence of specific antigens.^{1–3} Compared with heterogeneous immunoassays, the immunoagglutination assay has advantages like less complex protocols and shorter assay time.⁴ It has been, therefore, widely used in veterinary, food safety,^{6,7} rapid clinical and field diagnosis,⁸ and so on.

Microfluidic systems take advantage of the integration of multiple processes on a single platform, small reaction volume, and short diffusion lengths, thus reducing sample consumption and the assay time, and potentially improving the sensitivity.⁹ Performing immunoagglutination assay on chip has become an attractive choice, especially those based on magnetic bead (MB). Surface-functionalized MB can be magnetically manipulated independently of normal microfluidic or biological processes^{10–14} and has been widely used as capture support in sample enrichment and purification,^{15,16} cell and molecule manipulation,^{17–19} and biomagnetic sensing.^{20–24}

Typically, trapping MBs within a small section of a microfluidic channel to form a dense clump is an effective way to realize analyte capture.^{25,26} A MB clump can be realized by applying a magnetic field generated by external permanent magnets,^{27–31} electromagnets,^{32–35} or on-chip magnetizable microstructures.^{36–38} MB clumps based on static magnetic field suffer from a relatively low capture efficiency because the clumps cannot fill the cross section²⁶ or require a large amount of MBs.³¹ However, with a dynamic magnetic field applied, MBs reciprocate in the cross section and fill the channel, resulting in a higher capture rate.³⁹ By introducing a MB clump into a microfluidic chip for immunoagglutination assay, the whole assay process including analyte capture, agglutination, and detection can be performed on a single chip.^{25,26}

The level of agglutination can be estimated by holistic indicators from clump images, like the swelling area of the clump after withdrawing the magnetic field,²⁵ or the gray level of the suspension,^{40,41} but this approach is not accurate enough because the changes of these indicators are very small. Another method is to

count monomers and aggregates from images and calculate the percentage of monomers or aggregates,^{26,42} in which the sensitivity is significantly improved because the percentage of monomers or aggregates is only up to the amount of analyte except for non-specific agglutination. However, data processing of images is time-consuming. Light scattering detection is inherently quick and high throughput. Measuring the turbidity of the bead suspension is a simple method to estimate the level of agglutination,^{43,44} but its sensitivity is relatively low. Particle sizing and counting based on scattering detection, which can accurately count monomers and aggregates in the bead suspension, have the advantages of high sensitivity and high throughput⁴⁵ but often require complex and expensive equipment.

Previously, we proposed an approach for agglutination measurement based on on-chip sheath-less flow cytometry using light scattering detection.⁴⁶ The approach realized a high throughput, without using complex flow actuation and optical system. However, analyte capture and agglutination were conducted off chip by manual operation. In this paper, an on-chip agglutination assay, on which all the assay processes including sample loading, analyte capture, immunoagglutination, and detection are automatically conducted, is demonstrated. A dynamic MB clump is generated at the reaction chamber on the chip by the rotation of two permanent magnets to enhance the analyte capture and agglutination. The relationship between the level of agglutination and analyte concentration is firstly verified using streptavidin-coated MBs and biotinylated bovine serum albumin (bBSA) as a model assay and then tested by an antigen/antibody agglutination assay using rabbit IgG and goat anti-rabbit IgG coated MBs. Finally, human ferritin in 10% FBS (Fetal Bovine Serum) is tested with Ab-functionalized MBs. The assay realizes a fast, easily manipulated assay method with high sensitivity.

II. MATERIALS AND METHODS

A. Materials and reagents

In the first set of experiments, biotinylated bovine serum albumin (bBSA) (Biosynthesis biotechnology Co., China) is used as the analyte and diluted by PBS (Phosphate Buffered Saline) to a concentration range of 0.15 pM, 1.5 pM, 15 pM, 150 pM, 1.5 nM, and 15 nM. Pure BSA (bs-0292P, Bioss Antibodies Co., China) is used as negative control. Streptavidin-coated MBs of 1 μ m in diameter (Dynabeads Myone Streptavidin C1, ThermoFisher Scientific Co., USA) with a nominal concentration of 1010 beads/ml is diluted by a factor of 10 000 in PBS with 0.1% Tween-20. In the second set of experiments, goat anti-rabbit IgG coated MBs are prepared by adding protein A-coated MBs suspension of 5 μ l (Dynabeads Protein A for Immunoprecipitation, ThermoFisher Scientific Co., USA) to 10 μ g goat anti-rabbit IgG (bs-0295G, Bioss Antibodies Co., China) diluted in 40 μ l PBS with 0.1% Tween-20, followed by surface-sealing with goat serum (C-0005, Bioss Antibodies Co., China) of 50 μ l. The processed rabbit IgG (bs-0295P, Bioss Antibodies Co., China) is then diluted by factors of 103–108 with PBS and used as analyte. Mouse IgG (bs-0296P, Bioss Antibodies Co., China) is prepared in accordance with the product protocols and used for negative control. In the last set of experiments, human ferritin (bs-41188P, Bioss Antibodies Co., China) is used as the analyte and diluted by 10%

FBS (1921005PJ, ThermoFisher Scientific Co., USA) to a concentration range of 0.1 pM, 1 pM, 10 pM, 100 pM, and 1000 pM. Ab-functionalized MBs are prepared by adding 10 μ l biotin conjugated rabbit antiferritin antibody (bs-5907R-Bio, Bioss Antibodies Co., China) to 10 μ l streptavidin-coated MBs diluted in 40 μ l PBS with 0.1% Tween-20. The antibodies would be attached on the MBs through the streptavidin-biotin reaction. Then, Ab-functionalized MBs suspension is diluted to 1 ml for later use.

B. Chip design and working principle

The chip is composed of three layers: a PMMA base layer (BL) as a solid support, a PDMS main channel layer (ML), which performs most assay steps, and a glass detection channel layer (DL) [Fig. 1(a)]. The ML's and DL's channels are connected through two holes to form a continuous flow. The chip integrates an annular peristaltic pump (on ML), three valves (on ML), a reaction region (on ML), and a detection region (on DL) [Fig. 1(b)].

The micropump is composed of an annular channel covered by a flexible PDMS membrane, described in our earlier work.⁴⁷ Briefly, three steel balls are pressed on the annular channel by a stepper motor and rotate driven by a DC motor, resulting the flexible PDMS membrane to deform peristaltically and driving the fluid in the channel to flow in the rotating direction. Push-pull electromagnets are used as check valves to shut down the flow by pressing the PDMS membrane on the channel. A photograph of the chip is shown in Fig. S1 of the [supplementary material](#). The volume of the total channel on the chip is 200 μ l.

In the reaction region, a dynamic MB clump is generated and controlled by two cylindrical permanent magnets located above and below the reaction region, which are driven by servomotors, as shown in Fig. 1(c). Initially, the magnet above is vertical to the channel facing the chip and the other is parallel to the channel. When magnets start swinging with the same frequency, MBs in the reaction region move up and down. When the sample flows through the reaction chamber, analytes are captured by the surface-functionalized MBs. After that, servo motors stop and the magnetic pole of the magnet above faces the chip, drawing MBs to the channel wall to accelerate the agglutination. The length, width, and depth of the reaction chamber are 4 mm, 3 mm, and 0.6 mm, respectively.

The detection region, as shown in Figs. 1(d) and 1(e), is integrated with the sheath-less flow cytometry. If a particle flows through the laser spot located in the center of the detection region, the scattered light will be detected. Details can be seen in our previous work.⁴⁶ The main channels of the chip are designed to be 2 mm wide and 200 μ m deep, and the detection channel has the same width with a depth of 60 μ m, in order to reduce overlapping in the vertical direction. A rectangular hole is cut on the BL and ML at the location to eliminate scattering from PDMS and PMMA.

C. Chip fabrication

The PDMS structure layer is fabricated by soft lithograph and bonded with a 120 μ m thick PDMS membrane by plasma treatment to form ML. The inlet, outlet, and two holes connecting to the detection region are drilled on the membrane after that step. Two glass slats are adhered together with a patterned dual adhesive tape (ARclear

FIG. 1. Schematic of the microfluidic chip. (a) Explosive view of multilayer structure of the chip, from bottom to top: PMMA base layer (BL), PDMS main channel layer (ML), glass detection channel layer (DL), (b) functional structures of the chip, (c) schematic of the MB clump, (d) cross section of the detection region, and (e) the detection region in top view.

8932, Adhesives Research) to form DL, one of which has two holes to connect with the two connecting holes on the ML. The DL is aligned and bonded onto ML to connect the channels. The rectangle holes on ML and BL at the detection region are opened by laser cutting. Finally, the assembled layers of DL and ML are stuck on BL.

D. Experimental setup

The experimental setup for the microfluidic chip is composed of a control module and an optical detection module as shown in Fig. 2. A photograph for the setup is shown in Fig. S2 of the [supplementary material](#). The control module integrates valve and pump drivers and a MB clump controller [Fig. 2(a)], which mainly includes a rotary motor (GA12-N2, JCS, Shenzhen, China) for pumping, a stepper motor for pump vertical positioning, three electromagnets (JF-0530B, JF, Leqing, China) for valve control, and two cylindrical permanent magnets (Magpanda, Ningbo, China) driven by two servo motors (16G214E, ESCAP, Switzerland) for the MB clump control.

The optical detection module is essentially a sheath-less flow cytometry [Fig. 2(b)], which was reported in our previous work⁴⁶ and adopted here to fulfill the agglutination detection. Basically, a laser beam of 140 μm in diameter is focused onto the center of the detection region. The scattered light within a certain angle range from particles is collected by a photodiode (BPW 21, Siemens, Germany) through a 10 $\times$ microscope objective and transformed to a voltage signal. Finally, the signal is processed by a LabVIEW program, which was designed to distinguish monomers and aggregates.

E. Operating procedure

The whole procedure includes the following six steps as shown in Fig. 3:

- MBs suspension loading. Both inlet valve and outlet valve are open and the circulating valve is closed. 200 μl MBs suspension is pumped into the main channel from the inlet. In this step, the magnet above faces the chip and MBs flowing through the reaction region will be captured on the channel wall [Fig. 3(a)].
- MBs collection. Phosphate Buffered Saline (PBS) is loaded and pushes all the MB suspension through the reaction region so that all MBs are collected [Fig. 3(b)]. The states of valves and magnets remain the same as step (a).
- Sample loading and analyte capture. The sample is loaded intermittently. When the sample is being loaded, the magnets swing continuously and MBs fill the cross section of the reaction region [Fig. 3(c)] to capture analyte in the sample. Then, the sample flow stops, and the magnet above faces the chip and draws MBs to the channel top wall for a short time to promote agglutination. This loading and capture step is repeated for several times until all sample is loaded.
- Agglutination. MBs are drawn to the top of the channel to agglutinate for a relatively long time [Fig. 3(d)].
- MBs spreading. The inlet and outlet valves are closed, and the circulating valve is open. The pump runs at high speed. MBs are released by turning both magnets parallel to the chip, and the suspension is circulated in the channel at a high flow rate

FIG. 2. Sketch of the experimental setup, including (a) the control module and (b) the optical detection module.

FIG. 3. Operating procedure of the assay shown by the top view of the chip and side view of the reaction region. (a) MBs loading, (b) MBs collection, (c) sample loading and analyte capturing, (d) agglutination reaction, (e) beads spreading, and (f) detection. The black arrows represent the flow direction in each step.

[Fig. 3(e)], leading MBs to distribute uniformly in the channel and breaking up the nonspecific aggregates.

- (f) Detection. By circulating the suspension slowly, monomers and aggregates are counted by the sheath-less flow cytometry [Fig. 3(f)].

III. RESULTS AND DISCUSSION

A. Determination of the actuation frequency

In order to obtain a high efficiency of analyte capture, the MB clump should fill the cross section of the channel, which is governed by the actuation frequency of the magnets and the flow rate. To determine the actuation frequency, the flow is kept stagnant and the distribution of MB clump is imaged. Figure 4(a) shows the images of the MB clump with actuation frequencies of 1 Hz, 2.5 Hz, 5 Hz, 7.5 Hz, 10 Hz, and 12.5 Hz. When the actuation frequency is lower than 5 Hz, MBs fail to fill the cross section of the channel. If the frequency increases to 10 Hz or higher, a tapered tail of high density MBs is formed in the middle of the cross section [Fig. 4(b)]. The higher the frequency is, the larger the percentage of beads the tail contains. The MBs in the tail vibrate with a much smaller amplitude compared with the channel dimension.

Those MBs can be regarded static in the middle of the channel, which causes gaps near the channel wall. This phenomenon is easy to explain. At a high frequency, most beads do not have enough time to travel across the channel before the direction of magnetic field changes. Accordingly, the actuation frequency should be between 5 Hz and 10 Hz.

The shape of the MB clump is also influenced by the flow rate. When there is a sample flow, the clump location shifts slightly to the downstream of the magnets' position, and the MBs will be broken into a series of satellite MBs if the flow rate is too high [Fig. 4(b)]. For a low actuation frequency, the clump is easily broken. The allowable maximum flow rate is related to the actuation frequency of the MB clump, as shown in Fig. 4(c).

We further studied the relationship between the actuation frequency and the capture efficiency of the clump. Capture and agglutination are conducted in the chip with MB clump actuated under different frequencies (5 Hz, 7.5 Hz, and 10 Hz), and the agglutination product is analyzed by a commercially available flow cytometry (BD LSRFortessa, BD, America). 200 μ l streptavidin-coated MBs suspension of 10^6 beads/ml and 600 μ l fluorescein isothiocyanate (FITC) marked bBSA (Biosynthesis Biotechnology Co., China) of 1 nM is used as a sample. Forward scattering light (FSC) and fluorescent light (FITC) of the product is measured and shown in

FIG. 4. The distribution of MB clump and the allowable maximum flow rate under different actuation frequencies. (a) MB clump images under different actuation frequencies without flow, (b) MB clump without flow (up) and with a flow rate of 2400 μ L/min (down) at actuation frequency of 12.5 Hz, and (c) the relationship between the max flow rate and actuation frequency. The error bars represent the standard deviation of five measurements.

FIG. 5. Capture efficiency presented by mean FITC intensity under different actuation frequency measured by a commercially available flow cytometry. (a) FSC and FITC plots with the product with 5 Hz and (b) the relationship between the average FITC fluorescent intensity of all population and actuation frequency. At each frequency, three measurements are conducted.

Fig. 5(a). Three subpopulations can be distinguished which represent species of monomers, dimers, and trimers. The average FITC fluorescent light intensity reflects the amount of captured bBSA molecules, which indicates the capture efficiency. Figure 5(b) shows the average FITC intensity vs actuation frequency, indicating that the highest capture efficiency occurs at 5 Hz. This is attributed to the increasing motion speed of MBs as the actuation frequency rises, which hampers the combination of MBs and analytes. So, the actuation frequency of 5 Hz is chosen for subsequent experiments.

B. Determination of time parameters

Biotinylated BSA and streptavidin are widely used as a model assay of immunoagglutination because of their easy access. More importantly, the reaction between bBSA and streptavidin is analogous to the specific combination of antigen/antibody systems. So as the first step, we used the model assay of bBSA and streptavidin-coated MBs to determine several time parameters in the operating procedure. The flow rate is determined to be 200 $\mu\text{L}/\text{min}$ for MBs suspension and sample loading. 200 μL MBs suspension containing 2×10^5 MBs, and 200 μL PBS buffer is loaded for MB collection.

In sample loading and analyte capture step, the parameters are determined based on experimental trials. Firstly, the sample is loaded for 2 s and then the pump rests for 4 s, during which magnets keep swinging, to ensure analytes are fully captured. This process repeats for 1 min as a cycle. Between two cycles, there is 5 s for agglutination, during which the pump and the magnets stop working, and MBs are attracted to the channel wall to promote agglutination. Five cycles are included for whole sample loading and analytes capture, and totally 330 μL sample

is loaded. Finally, agglutination is conducted for an additional time of 30 s.

In order to fully disperse MBs after agglutination, we tested the influence of different spreading times. PBS is used as the sample and other experimental conditions are the same as described above. Figure 6(a) shows the relationship between the event counts per second and the spreading time, at a flow rate of 800 $\mu\text{L}/\text{min}$. After 60 s, the event count becomes stable, indicating that MBs distribute uniformly in the channel. Therefore, the spreading time is chosen as 90 s for subsequent experiments.

The threshold to distinguish monomers and aggregates is set to be 0.7 V. The details can be seen in Figs. S3 and S4 of the [supplementary material](#). In the detection step, a proper detection time needs to be determined to obtain stable results. To do so, the percentages of monomers for different event counts are recorded in Fig. 6(b) for bBSA concentration of 1 pM and 1.5 nM, respectively, showing that after 3000 events, the percentage of monomers barely changes. Considering about 300 events are collected every second, 10 s of detection is enough to get a relatively stable result and 30 s is chosen for safe margin. The whole process takes a total time of about 10 min.

C. Model assay of bBSA

To evaluate the assay performance, bBSA is tested with streptavidin-coated MBs as a model assay, using BSA as a negative control. Both bBSA and BSA of different concentrations are tested. The original event counts of monomers and aggregates are shown in Fig. S5 of the [supplementary material](#), which are processed to obtain the relationship between the percentage of monomers and

FIG. 6. Determination of spreading time and detection time. (a) The relationship between the event number detected per second and the spreading time, and (b) the relationship between detected events and the percentage of monomers for sample concentration of 15 pM and 1.5 nM in the detection step. For each concentration, three experiments were repeated in the same condition.

sample concentration as shown in Fig. 7(a). For the bBSA sample, the percentage of monomers decreases as concentration rises, while the percentage of monomers of the BSA sample barely changes, which confirms the assay specificity. Figure 7(b) shows the normalized signal (defined by $1 - P/P_0$, where P is the percentage of monomers at a certain concentration and P_0 is the percentage at zero concentration) in relation to the sample concentration for

bBSA. The SD (standard deviation) of zero bBSA concentration is 0.017. According to the linear fitting result, a concentration of 1 pM corresponding to 0.051 (3 times of the SD) is determined to be the LOD of the model assay.

In Fig. 7, we can see that the percentage of monomers decreases as sample concentration rises, showing good linearity over a wide range of concentrations. In our previous work using off-chip assay,⁴⁶

FIG. 7. Dose-response curve of the model assay for bBSA. (a) The percentage of monomers vs sample concentration of both bBSA and BSA and (b) the normalized signal vs bBSA concentration. Each dot is the average result of three measurements, and the error bars represent SD.

the measurement has the same trend only when the concentration is lower than 150 pM, and the percentage of monomers increases when the sample concentration is above 1.5 nM. And compared with the previous work, there is a small improvement in LOD (1 pM vs 1.5 pM). These differences are probably caused by different reaction environments. In off-chip situation, the distances among MBs are large so that it is difficult for MBs to agglutinate. If the analyte concentration is above a certain value, very few free binding points are left for agglutination and the percentage of monomer increases. In on-chip experiments here, the concentration of MBs in the reaction region is much higher, resulting in sufficient agglutination. So, the detectable range of the on-chip assay extends to higher concentration and the sensitivity is improved.

It seems that MB spreading is not well-done, leading to the high level of nonspecific agglutination. In Fig. 7(a), up to 50% of MBs in both BSA and bBSA samples form nonspecific aggregates without analytes, considerably higher than the value of 14% which is the case in off-chip agglutination.⁴⁶ The nonspecific agglutination could be reduced by MB spreading with a higher flow rate, a longer spreading time, or a lower MB concentration.

D. Assay for IgG

In this part of the experiment, rabbit IgG samples of different concentrations are tested with goat anti-rabbit IgG decorated MBs. Mouse IgG is used as a negative control. The original event counts of monomer and aggregate are shown in Fig. S6 of the [supplementary material](#), and the dose-response curves of rabbit IgG sample and negative control are shown in Fig. 8(a). The percentage of monomer decreases as the sample concentration rises for rabbit IgG, while stays nearly stable for negative control. Figure 8(b)

shows the relationship between the normalized signal of monomer's percentage and concentration for rabbit IgG. According to the linear fitting result, a concentration of 5.5 pM is determined to be the LOD of the assay.

Compared with the model assay of streptavidin/biotin, the IgG assay is less sensitive and has a higher LOD. This can be explained by a lower affinity constant of the IgG assay compared with the model assay of streptavidin/biotin. For a lower affinity constant, it will be harder for analyte and MBs to bind and the level of agglutination is less obvious, especially in low sample concentration situations.

E. Assay for ferritin

To evaluate the feasibility of the assay in real use, human ferritin in 10% FBS of different concentrations is tested with Ab-functionalized MBs. The original event counts of monomer and aggregate are shown in Fig. S7 of the [supplementary material](#), and the dose-response curve and the relationship between the normalized signal of monomer's percentage and concentration of human ferritin are shown in Figs. 9(a) and 9(b), respectively. According to the linear fitting result, a concentration of 8.5 pM is determined to be the LOD of the assay.

Experiments of ferritin detection in undiluted FBS were carried out and the result can be seen in Fig. S8 of the [supplementary material](#). Although the dose-response curve shows a decreasing trend of the monomer percentage as sample concentration rises, the difference in monomer percentages under different concentrations is very small, indicating a low sensitivity. This might be due to that serum interferes the reaction between Ab-functionalized MBs and analyte and then influences the

FIG. 8. Dose-response curve of the antigen/antibody assay for IgG. (a) Percentage of monomers vs sample concentration of both rabbit IgG and negative control and (b) normalized signal vs concentration of rabbit IgG. Each dot is the average result of five measurements, and the error bars represent SD.

FIG. 9. Dose-response curve of human ferritin in 10% PBS. (a) Percentage of monomers vs sample concentration and (b) normalized signal vs concentration of human ferritin. Each dot is the average result of three measurements, and the error bars represent SD.

agglutination of MBs. With diluted FBS, the influence is reduced, thus increasing the slope of the dose-response curve. However, the dilution decreases the concentration of the pristine sample, which means that the LOD of 8.5 pM for ferritin in 10% FBS corresponds to the LOD of 85 pM for pristine serum samples. The dilution rate should be further optimized for obtaining a lower LOD in the future.

IV. CONCLUSION

In this paper, a novel microfluidic agglutination assay chip based on a dynamic MB clump and a sheath-less cytometry is demonstrated. The MB clump captures analytes and leads MBs to agglutinate efficiently. An on-chip peristaltic pump provides the driving force of flow. The sheath-less flow cytometry is realized by collecting scattered light from particles flowing through the laser spot focused at the middle of the channel. All the processes including sample loading, analyte capture, agglutination, and particle detection are automatically conducted in the platform. The model assay of bBSA and streptavidin-coated MPs achieves a LOD of 1 pM. For the assay of rabbit IgG and goat anti-rabbit IgG, the LOD is 5.5 pM. Finally, the detection of human ferritin in 10% FBS achieves a LOD of 8.5 pM. The whole procedure takes only 10 min. This assay has potential for wider applications. In the future, the operating procedure should be optimized for different realistic clinical assays, and the sensitivity should be further improved.

SUPPLEMENTARY MATERIAL

See the [supplementary material](#) for photographs of the chip and experimental setup and more details about experimental data.

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