A multiplexed immunoaggregation biomarker assay using a two-stage micro resistive pulse sensor

Y. Han,^{1,a)} H. Wu,^{2,a)} F. Liu,¹ G. Cheng,^{2,b)} and J. Zhe^{1,b)}

¹Department of Mechanical Engineering, University of Akron, Akron, Ohio 44325, USA

²Department of Chemical and Biomolecular Engineering, University of Akron, Akron, Ohio 44325, USA

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We present an immunoaggregation assay chip for multiplexed biomarkers detection. This chip is based on immunoaggregation of antibody functionalized microparticles (Ab-MPs) to quantify concentrations of multiple biomarkers simultaneously. A mixture of multiple types of Ab-MPs probes with different sizes and magnetic properties, which were functionalized by different antibodies, was used for the multiplexed assay. The interactions between biomarkers and their specific Ab-MPs probes caused the immunoaggregation of Ab-MPs. A two-stage micro resistive pulse sensor was used to differentiate and count the Ab-MP aggregates triggered by different biomarkers via size and magnetic property for multiplexed detection. The volume fraction of each type of Ab-MP aggregates indicates the concentration of the corresponding target biomarker. In our study, we demonstrated multiplexed detection of two model biomarkers (human ferritin and mouse anti-rabbit IgG) in 10% fetal bovine serum, using anti-ferritin Ab and anti-mouse IgG Ab functionalized MPs. We found that the volume fraction of Ab-MP aggregates increased with the increased biomarker concentrations. The detection ranges from 5.2 ng/ml to 208 ng/ml and 3.1 ng/ml to 5.12×10^4 ng/ml were achieved for human ferritin and mouse anti-rabbit IgG. This bioassay chip is able to quantitatively detect multiple biomarkers in a single test without fluorescence or enzymatic labeling process and hence is promising to serve as a useful tool for rapid detection of multiple biomarkers in biomedical research and clinical applications. © 2016 AIP Publishing LLC. [<http://dx.doi.org/10.1063/1.4944456>]

I. INTRODUCTION

Quantitative detection of macromolecular biomarkers, indicators of biological states, is an important task in disease diagnosis,^{1–3} biodefense,⁴ environmental monitoring,^{5,6} and biological research.⁷ Many conventional immunosensors including surface plasmon resonance (SPR),^{8–10} quartz crystal microbalance (QCM),^{9,11–13} and electrochemical sensors^{14–16} have been demonstrated for the detection of single biomarker.¹⁷ However, single biomarker detection is unable to provide sufficient information for disease diagnosis due to the complexity of human biology and heterogeneity of diseases.^{18–20} Detection of multiple biomarkers associated with different stages or classification of diseases is important in increasing the accuracy in disease diagnosis.^{21–24}

Immunoassay is a prevalent method for biomarker detection due to its high specificity. However, conventional immunoassays, such as enzyme-linked immunosorbent assay (ELISA), require multistep labeling of antibodies, long assay time, and complicated detection instruments.^{25,26} Recently, development of microfluidic immunosensors enables multiple biomarkers detection with various methods, including optical (fluorescent,^{19,27–29} luminescent,^{30,31} or

^{a)}Y. Han and H. Wu contributed equally to this work.

^{b)}Authors to whom correspondence should be addressed. Electronic addresses: gc@uakron.edu and jzhe@uakron.edu.

colorimetric^{32,33}), electrochemical,^{23,34–36} SPR,^{37–40} surface-enhanced Raman scattering (SERS),^{41,42} and capillary electrophoretic immunoassays (CEIA).^{43,44} However, these methods require complex setups, expensive detectors, and/or complicated encoding and decoding process,⁴⁵ making them impediment for point-of-care biomarker detection for applications including disease diagnosis and therapy monitoring. Additionally, while surface modification with antibodies is crucial for achieving specificity and high sensitivity of an immunosensor,⁴⁶ it remains a large challenge in a microchannel; maintaining surface functionality and regenerating the surface modification are also difficult due to the instability of antibodies.^{47,48} Recently, microparticle-enhanced immunoassays have attracted many attentions because surface functionalization of microparticles (MPs) is flexible and fast.^{49,50} Among these microparticle-based immunoassays, immunoaggregation assays based on the aggregation of antibody functionalized MPs (Ab-MPs) triggered by target biomarkers enable direct measurement of a target biomarker concentration with only one step⁵¹ without fluorescent, enzymatic, or radioactive labeling. However, traditional immunoaggregation detection method such as turbidimetry, nephelometry, and optical detection can only detect biomarkers with a relatively high concentration.^{50,52}

To address the above limitations, namely, the low sensitivity and low throughput, we report a multiplexed immunoaggregation biomarker assay based on a two-stage resistive pulse sensor (RPS) for simultaneous detection of multiple biomarkers. Resistive pulse sensing based immunoaggregation assay has advantages to quantitatively detect macromolecules.^{53,54} Because of the large surface area/volume ratio of microparticles, aggregates can be formed conveniently and quickly even at a low biomarker concentration. Resistive pulse sensors are highly sensitive to the size difference and can accurately measure every single aggregate even if the number of aggregates is small at a very low biomarker concentration.⁵³ Hence, the combination of immunoaggregation assay and resistive pulse sensing enables rapid biomarker detection with high sensitivity. In this work, we used a two-stage resistive pulse sensor to measure different aggregates formed by microparticles with different sizes and magnetic properties. We demonstrated that this multiplexed assay was able to quantitatively measure multiple biomarkers in a complex medium with one single test, without the need for fluorescence and enzyme labeling of antibodies.

II. SENSING PRINCIPLE

Fig. 1(a) shows the detection mechanism of the multiplexed immunoaggregation assay. To detect two biomarkers, BM1 and BM2, two antibody functionalized microparticles (Ab1-MP1 and Ab2-MP2) with different sizes are used as probes; microparticle 1 (MP1) is selected to be smaller than microparticle 2 (MP2). Sample solution containing two biomarkers is mixed with the two types of Ab-MPs to form immunoaggregates. Biomarker 1 (BM1), specific to antibody 1 (Ab1), triggers the aggregation of Ab1-MP1s. Note that due to the use of relatively large micro-sized particles for the immunoaggregation, the number of formed doublets is much higher than that of the formed triplets.⁵³ The volume fraction of Ab1-MP1 doublets to all single Ab1-MP1 probes and their doublets is indicative of the BM1 concentration. Similarly, BM2 in the sample induces the formation of Ab2-MP2s doublets. The formed doublets are detected by the 1st stage RPS in Fig. 1(b), which can accurately measure the sizes and count the number of Ab-MPs and their aggregates. By selecting appropriate microparticle sizes, in the 1st stage RPS, Ab1-MP1 doublets and Ab2-MP2 doublets can be differentiated and measured by size. The concentration of two biomarkers can be measured by the 1st stage RPS from the volume fraction of the doublet induced by this biomarker.

Fig. 1(b) also shows the differentiation of the aggregates with different magnetic properties by a two-stage resistive pulse sensor. If MP2 is a magnetic particle while MP1 is a non-magnetic particle, Ab2-MP2s and their aggregates are captured in the capture chamber where an external magnetic field is applied. Hence, only non-magnetic particles (Ab1-MP1s) and their aggregates are detected by the 2nd stage RPS, while the 1st stage RPS detects all particles and aggregates. The difference of aggregates measured by the 1st stage RPS and the 2nd stage RPS

FIG. 1. Schematic of the multiplexed immunoaggregation assay and design concept of the two-stage resistive pulse sensor. (a) A mixture of antibody functionalized microparticles (Ab-MPs) containing two probes, Ab1-MP1 and Ab2-MP2, is used to detect and measure the concentrations of two biomarkers, BM1 and BM2, via immunoaggregation; (b) the immunoaggregation is analyzed by a pair of resistive pulse sensors (RPSs) separated by a capture chamber; and (c) the equivalent circuit of the two-stage RPS; 1st and 2nd stage RPS are modeled as R_1 and R_2 in the circuit, respectively.

is the magnetic particle aggregates, which are indicative of concentration of the BM2. If four types of Ab-MPs are used, two are magnetic particles with different sizes, and other two are non-magnetic particles with different sizes, up to four biomarkers can be detected; the formed non-magnetic doublets are detected and differentiated by the 1st stage RPS, while the magnetic doublets are detected and differentiated by the 2nd stage RPS. Hence, the two-stage resistive pulse sensing device can detect up to four biomarkers in terms of size and magnetic property of the formed aggregates. Fig. 1(c) shows the equivalent circuit of the two resistive pulse sensors which are modelled as two variable resistors, R_1 and R_2 , connected in series. Measurement, V_{out} , will be taken at the central electrode. The measurement details are discussed in Section IV and supplementary material.⁵⁵

III. DEVICE FABRICATION AND TESTING PROCEDURE

The resistive pulse sensor was fabricated using the standard soft lithography method. It consists of (1) two sensing channels with a width of $10\ \mu\text{m}$ and a length of $30\ \mu\text{m}$ to detect aggregates, (2) a capture chamber with a width of $1\ \text{mm}$ and a length of $15\ \text{mm}$ to capture magnetic aggregates and particles, (3) a pair of inlet and outlet reservoirs, (4) three Ag/AgCl electrodes to measure the resistive pulses, and (5) a filter structure with a pore size of $10\ \mu\text{m}$. The on-chip filter is used to block any objects (i.e., cells and debris) larger than $10\ \mu\text{m}$. For a real sample with a high concentration of large cells (i.e., a blood sample), the cells may clog the filter during the test. A simple separation step (e.g., centrifugation) is needed to separate the blood cells from the sample. A two-layer SU8 mold, consisting of patterns for the sensing channels and the on-chip filter (with a thickness of $10\ \mu\text{m}$), the capture chamber and reservoirs (with a thickness of $40\ \mu\text{m}$), was created by a two-step photolithography.⁵³ The microchannels, capture chamber, and reservoirs were formed by pouring polydimethylsiloxane (PDMS) onto the

two-layer SU8 mold followed by degassing and curing. The two layer structure enables a high sensitivity of the sensing channels and low flow resistance of the capture chamber at the same time. Next, the PDMS layer was bonded onto a glass substrate after an oxygen plasma treatment (200 mTorr, 50 W, 50 s). Three Ag/AgCl electrodes (1 mm in diameter) were inserted in each side of the sensing channel via three punched 1 mm holes (see Fig. 2(d)) to finish the fabrication of the resistive pulse sensor. For each test, 50 μl sample was loaded to the inlet reservoir, then driven through the two-stage RPS at a flow rate of 20 $\mu\text{l}/\text{h}$ by a syringe pump (KDS Legato 270, KD Scientific). 10 μl sample was measured in 30 min for each test. The total testing time was 1 h for each sample including another 30 min for mixing a biomarker sample with Ab-MPs probes. An external magnet (Grade N42, 3.2 mm $\times$ 3.2 mm $\times$ 3.2 mm, K&J Magnetics, Inc.) was used to capture magnetic MPs in the capture chamber. After each test, the remaining sample was removed from the inlet reservoir; the external magnet was removed to release the magnetic particles captured in the capture chamber. Then, the device was washed by phosphate-buffered saline (PBS) buffer for three times at a flow rate of 100 $\mu\text{l}/\text{h}$ for 15 min per wash. Resistive pulse responses were recorded by a data acquisition card (NI USB-6251, National Instruments) at a sampling rate of 500 kHz (see the measurement circuit in the supplementary material).⁵⁵ Note that the external magnet was placed 10 mm away from the 1st stage RPS, as shown in Fig. 2. Using such a long distance reduces the possibility of magnetizing the magnetic microparticles before they enter the 1st stage RPS; magnetized microparticles tend to form nonspecific aggregates, which will be counted as immunoaggregates and lead to errors in biomarker concentration measurement.

To prepare a mixture of MPs with different antibody functionalization, sizes, and magnetic properties, two types of Ab-MPs were prepared separately. First, streptavidin functionalized polystyrene microparticles (MP1) with an average diameter of 2.0 μm (MP1, Polybead Microspheres, Polysciences, Inc., USA) were diluted in 1/20 in PBS (pH 7.4, Sigma-Aldrich,

FIG. 2. Microscopic images of the two-stage micro RPS. (a) On-chip filter; (b) microscopy image of 1st stage RPS; (c) magnetic microparticles were captured in the capture chamber; (d) 2nd stage RPS; and (e) image of the two-stage RPS including 1st stage and 2nd stage RPS, a pair of inlet and outlet reservoir, an on-chip filter, three Ag/AgCl electrodes, and an external magnet.

USA) containing 0.1% bovine serum albumin (BSA, Sigma-Aldrich, USA). The biotinylated rabbit anti-mouse IgG (Ab1) (1 mg/ml, Life Technologies, USA) was diluted 1/180 in PBS with 0.1% BSA. Next, 166.7 μ l of diluted MP1 solution was mixed with 166.7 μ l diluted anti-mouse IgG Ab solution for 30 min in a thermal mixer at a speed of 650 rpm at room temperature. MP1s conjugated with biotinylated anti-mouse Ab1 through the streptavidin-biotin binding. The Ab1-MP1 solution was then centrifuged at 10000 rpm to separate Ab1-MP1s from the solution and the supernatant containing unconjugated Ab1 was removed. And this washing process was repeated for three times. Ab1-MP1s were then resuspended in PBS with 0.1% BSA to a concentration of 142.3 μ g/ml. Another type of Ab-MP was goat anti-human ferritin Ab (Ab2) (6.5 mg/ml, US Biological, USA) functionalized magnetic microparticle (MP2) with an average diameter of 2.8 μ m (Dynabeads M280, Life Technologies, USA) and the preparation procedure was reported previously.⁵³ Two types of Ab-MPs were mixed together with equivalent volumes (166.7 μ l each); the mixed solution was used as a mixture Ab-MPs probe.

Two biomarker solutions, mouse anti-rabbit IgG (BM1) (Life technologies, USA) with concentrations ranging from 3.1 ng/ml to 5.12×10^4 ng/ml, and human ferritin (BM2) (US Biological, USA) with concentrations ranging from 5.2 ng/ml to 416.0 ng/ml were prepared by serial dilutions with 10% fetal bovine serum (FBS) (Sigma-Aldrich, USA). Two types of mixed biomarker solutions were prepared as follows: (1) BM1 concentration was ranged from 3.1 ng/ml to 5.12×10^4 ng/ml, while BM2 concentration was kept a constant at 41.6 ng/ml. (2) BM2 concentration was ranged from 2.6 ng/ml to 416.0 ng/ml, while BM1 concentration was kept constant at 24.0 ng/ml. An amount of 333.4 μ l Ab1-MP1 and Ab2-MP2 mixture solution was mixed with 166.7 μ l the two biomarker solutions mentioned above separately for 30 min in a thermal mixer at the speed of 650 rpm at room temperature. The biomarker causes specific aggregations of Ab1-MP1 and Ab2-MP2 independently, which were detected by the two-stage resistive pulse sensor.

IV. RESULTS AND DISCUSSION

To prove the multiplexed biomarker concentration measurement, the mixture of Ab1-MP1s and Ab2-MP2s probes was mixed with two model biomarkers, BM1 with a concentration of 24.0 ng/ml and BM2 with a concentration of 208 ng/ml. The mixture of Ab-MPs probes consisted of 5.8×10^3 counts/ μ l of Ab1-MP1s (2.0 μ m in diameter) and 1.4×10^4 counts/ μ l of Ab2-MP2s (2.8 μ m in diameter) in all experiments presented in this paper.

Fig. 3(a) shows typical output voltage pulse, V_{out} , generated by the two RPSs when Ab1-MP1, Ab2-MP2, and their aggregates transited through the two RPSs. As shown in the equivalent circuit (Fig. 1(c)), the two-stage RPS is modelled as two variable resistors connected in series. The output voltage, V_{out} , was measured at the central electrode. A particle passing through the sensing channel of 1st stage RPS increases the resistance of the channel, causing a positive voltage pulse in Fig. 3(a) (see details of the RPS response measurement in the supplementary material).⁵⁵ Similarly, a particle passing through 2nd stage RPS also causes an increase in resistance of the second sensing channel but generates a negative voltage pulse in Fig. 3(a). Hence, each positive pulse represents a particle passing through the 1st stage RPS, and the negative pulse represents a particle passing through 2nd stage RPS. The voltage pulse (ΔV_{out}) can be converted to resistive pulses ($\Delta R/R$) of each RPS from the equivalent circuit^{47,56} (shown in Fig. 1(c) and Fig. S1 in the supplementary material).⁵⁵ The magnitude of the resistive pulse ($\Delta R/R$) is proportional to the particle volume^{57,58}

$$\Delta R/R = (d^3/LD^2) \cdot [(D^2/2L^2) + 1/\sqrt{1 + (D/L)^2}] \cdot F(d^3/D^3), \quad (1)$$

where R is the resistance of the sensing channel, d is the equivalent diameter of the particle, D and L are the characteristic diameter and the length of the rectangular sensing channel, and F is the correction factor. In our design, D was calculated to be 11.29 μ m by $D = (4 \cdot A/\pi)^{1/2}$, where A is the cross-sectional area of the sensing channel. F was taken to be 1.0.⁵⁹ After a sample

FIG. 3. (a) Typical output voltage pulse, ΔV_{out} , caused by microparticle transits. Positive and negative pulses represent passages of microparticles through the 1st stage RPS and the 2nd stage RPS, respectively. The red circle, blue square, orange triangle, and green diamond markers represent the voltage pulses caused by Ab1-MP1s, Ab1-MP1 doublets, Ab2-MP2s, and Ab2-MP2 doublets, respectively. (b) Typical counts and size distributions of two types of Ab-MPs and their aggregates. The inset is a microscopy image of Ab1-MP1s ($2.0 \mu\text{m}$), Ab2-MP2s ($2.8 \mu\text{m}$), and their doublets. The BM1 concentration was 24.0 ng/ml , and the BM2 concentration was 208 ng/ml . The mixture of Ab-MPs probes consisted of $5.8 \times 10^3 \text{ counts}/\mu\text{l}$ of Ab1-MP1s and $1.4 \times 10^4 \text{ counts}/\mu\text{l}$ of Ab2-MP2s.

was tested in the device, equivalent volume spherical diameter of each particle/aggregate was back calculated from its resistive pulse ($\Delta R/R$), using Equation (1). Fig. 3(b) shows the counts and size distributions of the two types of Ab-MPs and their aggregates by the 1st stage RPS. From left to right, the first two peaks centered at $1.99 \pm 0.06 \mu\text{m}$ and $2.50 \pm 0.09 \mu\text{m}$ represent the distributions of singlets and doublets of Ab1-MP1. The measured diameters matched well with calculated equivalent volume spherical diameter of Ab1-MP1 doublets, which are $2.52 \mu\text{m}$. The third and fourth peaks centered at $2.87 \pm 0.07 \mu\text{m}$ and $3.60 \pm 0.11 \mu\text{m}$ represent the single and doublet of Ab2-MP2s. The calculated equivalent spherical diameter of Ab2-MP2 doublet is $3.53 \mu\text{m}$, which also matched with the measured result ($3.60 \mu\text{m}$). The volume fraction of Ab1-MP1 doublets (f_1) is defined as the total volume of Ab1-MP1 doublets to total volume of detected Ab1-MP1 singlets and their doublets. Similarly, the volume fraction of Ab2-MP2 doublets is defined as the volume ratio of Ab2-MP2 doublets to all detected Ab2-MP2 singlets and their doublets. The result shown in Fig. 3(b) demonstrated that the 1st stage RPS is able to differentiate Ab1-MP1 and Ab2-MP2 probes and their doublets according to their size distribution.

Next, to prove the volume fraction f_1 can be correlated to the concentration of BM1, similar tests were conducted at various BM1 concentrations ranging from 3.1 ng/ml to $5.12 \times 10^4 \text{ ng/ml}$, while BM2 concentration was kept a constant (41.6 ng/ml). Note that the detection limit of the immunoaggregation method is mainly controlled by the non-specific aggregation of Ab-MPs.^{53,60} The nonspecific aggregation of Ab1-MP1s in 10% FBS without BM1 was tested and the volume fraction of Ab1-MP1 doublets was $17.9 \pm 0.4\%$. After subtracting the volume fraction of non-specific doublets, volume fractions of Ab1-MP1s doublets as a function of the BM1 concentrations were plotted in Fig. 4. To ensure repeatability, at each BM1 concentration, five immunoaggregation samples were prepared and measured. The error bar represents the standard deviation of the five measurements.

Fig. 4 shows the correlation between the volume fraction of Ab1-MP1 doublets and BM1 ranging from 3.1 ng/ml to $5.12 \times 10^4 \text{ ng/ml}$ (red dot), which can be fitted with a 4-parameter logistic function (red solid curve)

$$f_1(x) = 0.03 + 0.37 / (1 + (x/106.25)^{-0.69}). \quad (2)$$

The coefficient of determination (R^2) of the fitted curve is 0.9883. The volume fraction of Ab1-MP1 doublet, f_1 , increased with the increase of the BM1 concentrations in the range of 3.1 ng/ml to $5.12 \times 10^4 \text{ ng/ml}$. The maximum volume fraction ($f_1 = 40.3\%$) occurred at $5.12 \times 10^4 \text{ ng/ml}$. The volume fractions of Ab2-MP2 doublets in all samples were also measured (blue curve), which remained nearly a constant; the average value was $11.1 \pm 2.0\%$. Note that

FIG. 4. Measured relationship between the concentration of mouse anti-rabbit IgG (BM1) ranging from 3.1 to 5.12×10^4 ng/ml and volume fraction of Ab1-MP1s doublets (f_1).

the calculated average equivalent spherical diameter of Ab1-MP1 triplets was $2.88 \mu\text{m}$, which overlapped with the diameter range of Ab2-MP2s and may cause a false count of Ab2-MP2s. To evaluate the volume fraction of formed Ab1-MP1 triplets, control experiments were conducted using only BM1 with a concentration from 3.1 ng/ml to 5.12×10^4 ng/ml, and only Ab1-MP1s with a concentration of 5.8×10^3 counts/ μl , the same as used in Ab-MPs probe mixture. From the RPS measurement, the triplet concentration was from 154 to 500 counts/ μl . Hence, when Ab2-MP2 had a concentration of 1.4×10^4 counts/ μl , the volume fraction of Ab1-MP1 triplets to total volume of Ab2-MP2s and aggregates were estimated to range from 1.5% to 3.8% at all tested BM1 concentrations. The value was 1.5% at a BM1 concentration of 24 ng/ml. Hence, the error caused by the size overlapping of Ab1-MP1 triplets and Ab2-MP2 singlets can be neglected.

Next, we proved that the volume fraction of Ab2-MP2 doublets, f_2 , is related to the concentration of BM2. In this experiment, the concentration of BM2 was varied from 5.2 ng/ml to 208 ng/ml, while BM1 concentration was kept a constant at 24.0 ng/ml, as shown in Fig. 5. To ensure repeatability, at each BM2 concentration, five immunoaggregation samples were prepared and measured. The error bar represents the standard deviation of the five measurements.

The correlation between the volume fraction of Ab2-MP2 doublets and BM2 ranging from 5.2 to 208 ng/ml was also fitted with a 4-parameter logistic curve (blue solid curve)

$$f_2(x) = 0.02 + 0.30 / (1 + (x/55.00)^{-3.02}). \quad (3)$$

The coefficient of determination (R^2) of the fitted curve is 0.9870. The volume fraction of Ab2-MP2 aggregates is increased from 2.7% to 33.1% with the increase of BM2 concentration in the range of 5.2 ng/ml to 208 ng/ml. With a fixed BM1 concentration of 24 ng/ml, the volume fraction caused by the Ab1-MP1 triplets was estimated to be approximately 1.5%. Hence, using the multiplexed immunoaggregation assay, we can confidently measure the BM2 concentration as low as 5.2 ng/ml. The maximum volume fraction (33.1%) occurred at the BM2 concentration of 208 ng/ml. Above 208 ng/ml, the volume fraction f_2 reduced with the increase of BM2 concentration. This is because the high concentration of BM2 would saturate Ab2 on the surfaces of Ab2-MP2s; hence, the number of unreacted Ab2 on Ab2-MP2s is too low to cause the aggregation of Ab2-MP2s. The measured average volume fraction of Ab1-MP1 doublets was $11.6 \pm 1.4\%$ for the constant BM1 concentration of 24 ng/ml. Using Equation (2) and BM1 concentration of 24 ng/ml, the calculated volume fraction of Ab1-MP1 doublets is 12.8%, which matched with the measurement value ($11.6 \pm 1.4\%$) well. Using Equation (3) and the BM2 concentration of 41.6 ng/ml, the calculated volume fraction of Ab2-MP2 doublets is 11.3%, which also matched well with measurement result in Fig. 4 ($11.1 \pm 2.0\%$). The results shown in Figs.

FIG. 5. Measured relationship between the concentration of human ferritin (BM2) ranging from 5.2 ng/ml to 208 ng/ml and volume fraction of Ab2-MP2 doublet (f_2). The volume fraction of non-specific Ab2-MP2 doublets was $5.3 \pm 0.5\%$.

4 and 5 clearly demonstrate: (1) the volume fractions of Ab1-MP1 doublets and Ab2-MP2 doublets are correlated to the concentrations of BM1 and BM2 in a mixture and (2) the 1st stage RPS is capable of differentiating Ab1-MP1 and Ab2-MP2 singlets and their doublets according to their size difference.

Next, experiments were conducted to prove that the two biomarkers (BM1 and BM2) can also be detected via the magnetic property of the aggregates. The magnetic Ab2-MP2 aggregates are correlated to the BM2 concentrations, and the non-magnetic Ab1-MP1 aggregates are correlated to the BM1 concentrations. An external magnet was used to capture magnetic particles in the capture chamber, while the flow-through non-magnetic particles were counted by the 2nd stage RPS.

To prove that the external magnet is able to efficiently capture magnetic Ab2-MP2s and their aggregates, we compared the count and size distribution measured by the 1st and 2nd stage RPSs in the following experiment condition: BM2 concentration = 208 ng/ml, without the presence of BM1. The mixture of Ab1-MP1s and Ab2-MP2s was used at the same concentration as the previous experiments. The counts of different-sized particles are shown in Fig. 6(a). The capture efficiency was defined as a ratio of the counts difference of magnetic particle (magnetic Ab2-MP2s and their aggregates) between the 1st and the 2nd stage RPS over all magnetic particle counts measured by the 1st stage RPS. From the measurements shown in Fig. 6(a), the capture efficiency was calculated to be larger than 98.0%, which is sufficiently high to ensure accurate counting of magnetic particles. Note that if a larger flow rate is used, a large number of magnetic microparticles/aggregates may be washed away from the capture chamber,

FIG. 6. (a) Counts of two types Ab1-MP1s and Ab2-MP2s and their aggregates measured by the two-stage RPS. (b) Comparison of the volume fraction of the non-magnetic Ab1-MP1 doublets measured by 1st stage and 2nd stage RPS, respectively.

causing large errors in biomarker detection. A stronger external magnetic field can be used to achieve a high throughput detection. Alternatively, throughput can be improved by integrating multiple RPSs into one chip via parallel detection.

Next, to prove that the 2nd stage RPS can accurately count and size the non-magnetic Ab1-MP1s, we compared the measured volume fraction of non-magnetic Ab1-MP1 doublets measured by the 1st stage and 2nd stage of RPS when BM1 concentration was varied from 3.1 ng/ml to 5.12×10^4 ng/ml. The result is shown in Fig. 6(b). As shown in Fig. 6(b), the volume fraction ratio measured by the 1st stage RPS and 2nd stage RPS matched reasonably well when the BM1 concentration ranges from 24.0 ng/ml to 5.12×10^4 ng/ml; the difference between the volume fraction ratios measured by these two RPSs ranges from 0.2% to 4.1%, which could be caused by nonspecific attachment of nonmagnetic Ab1-MP1 singlets and their aggregates in the chamber. The nonspecific attachment also caused larger difference (5.5% to 7.3%) at lower BM1 concentrations (3.1 ng/ml and 12 ng/ml). At low biomarker concentrations, the counts of formed doublets were small; hence, even a small amount of doublets attached to the capture chamber may lead to a large error in the volume fraction of doublets. The use of antifouling materials on the surface of the capture chamber is a possible solution to reduce the non-specific attachments.

The immunoaggregation method requires that the biomarker has more than one binding site for affinity probe and this method can detect any biomarker as long as a corresponding antibody can be found that triggers biomarker-microparticle aggregation. The lower detection limit of the multiplexed immunoaggregation assay is 3.1 ng/ml and 5.2 ng/ml for BM1 and BM2, which are comparable to that of commercial ELISA kits (~ 6 ng/ml for BM1, and ~ 5 ng/ml for BM2). Three possible solutions to further expand the lower detection limit are (1) using antibodies with higher binding affinity, which will increase the volume fraction of aggregates at lower biomarker concentration, (2) using antifouling materials to avoid nonspecific aggregation,⁶¹ and (3) using various MP concentrations to adjust the detection range, as demonstrated in our previous paper.⁵³ With the demonstration of multiplexed immunoaggregation biomarker assay in terms of size and magnetic property of Ab-MPs, if four types of Ab-MPs are used (two different-sized magnetic Ab-MPs and two different-sized non-magnetic Ab-MPs), up to four biomarkers can be detected using the two-stage RPS sensor: The 2nd stage RPS measures the concentration of two biomarkers that trigger the aggregation of nonmagnetic particles, while 1st stage RPS measures all four aggregates. By deducting the nonmagnetic particle counts from all particle counts of the 1st stage RPS, two additional biomarker concentrations can be measured in terms of the magnetic aggregate counts. If multiple two-stage RPSs are integrated into a device, a large number of biomarkers can be detected via parallel detection.⁵⁶

The aggregation of Ab-MPs can form conveniently and rapidly; the total testing time (needed for both immunoaggregation and the detection) is approximately 1 h, which is faster than the conventional ELISA (requiring several hours to 2 days).⁴⁹ The sensor chip, made of PDMS and glass, is cost effective and portable, making it suitable for rapid, onsite, quantitative detection of multiple biomarkers. Recently, Hauer *et al.* reported a two-stage device consisting of a tunable RPS to measure the size and concentration of microparticles, and fluorescence spectroscopy to detect the fluorescent labels simultaneously.²⁸ However, the fluorescence spectroscopy detection required a much longer sampling time (~ 4.3 ms) than the RPS. To accurately detect all microparticles using the fluorescence spectroscopy, the flow velocity had to be set very low. Because of the large sensing zone (~ 200 μ m) of the fluorescence spectroscopy, particle in close succession cannot be distinguished as different events. Hence, the device has a low throughput and accuracy. In addition, although fluorescence spectroscopy may be potentially used to detect multiple types of microparticles with different fluorescence labels, it remains a challenge to integrate all optical components onto a chip. In comparison, the present two-stage RPS device can be operated with a shorter sampling time; the device can detect micro aggregates quickly and accurately; up to four biomarkers can be detected based on size and magnetic properties of microparticles with a simple structure and setup. Nevertheless, the tunable pores²⁸ can be potentially applied to our two-stage RPSs to achieve a larger dynamic range and avoid channel clogging.

V. CONCLUSIONS

We demonstrated multiplexed biomarker detection method based on a two-stage resistive pulse sensor and multiplexed immunoaggregation assay, without a need for fluorescence or enzymatic detection. With the method, the biomarker concentration is correlated to the volume fraction of Ab-MP doublets formed by the target biomarker and its corresponding Ab-MPs. The Ab-MPs and their doublets were differentiated by the two-stage RPS, according to their size and magnetic property. Detection ranges from 5.2 ng/ml to 208 ng/ml and 3.1 ng/ml to 5.12×10^4 ng/ml were achieved for human ferritin and mouse anti-rabbit IgG detection. The testing results demonstrated that this device is able to quantitatively measure the concentrations of multiple biomarkers in a single test, with a detection limit comparable to commercial ELISA kits. With the advantages, this two-stage resistive pulse sensor is promising to be used for rapid detection of multiple biomarkers with simple setup especially for disease diagnosis and therapy monitoring at patient's bedside.

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