Homogeneous agglutination assay based on micro-chip sheathless flow cytometry

Zengshuai Ma, Pan Zhang, Yinuo Cheng, Shuai Xie, Shuai Zhang, and Xiongying Ye^{a)}

*State Key Laboratory of Precision Measurement Technology and Instruments,
Department of Precision Instruments, Tsinghua University, Beijing, China*

(Received 2 September 2015; accepted 18 November 2015; published online 1 December 2015)

Homogeneous assays possess important advantages that no washing or physical separation is required, contributing to robust protocols and easy implementation which ensures potential point-of-care applications. Optimizing the detection strategy to reduce the number of reagents used and simplify the detection device is desirable. A method of homogeneous bead-agglutination assay based on micro-chip sheathless flow cytometry has been developed. The detection processes include mixing the capture-probe conjugated beads with an analyte containing sample, followed by flowing the reaction mixtures through the micro-chip sheathless flow cytometric device. The analyte concentrations were detected by counting the proportion of monomers in the reaction mixtures. Streptavidin-coated magnetic beads and biotinylated bovine serum albumin (bBSA) were used as a model system to verify the method, and detection limits of 0.15 pM and 1.5 pM for bBSA were achieved, using commercial Calibur and the developed micro-chip sheathless flow cytometric device, respectively. The setup of the micro-chip sheathless flow cytometric device is significantly simple; meanwhile, the system maintains relatively high sensitivity, which mainly benefits from the application of forward scattering to distinguish aggregates from monomers. The micro-chip sheathless flow cytometric device for bead agglutination detection provides us with a promising method for versatile immunoassays on microfluidic platforms.
© 2015 AIP Publishing LLC. [<http://dx.doi.org/10.1063/1.4936926>]

INTRODUCTION

Immunoassay has been widely used in food safety, environmental monitoring, vitro diagnostics, and so on. After years of development, the most sensitive assays have allowed protein detection down to fg ml^{-1} concentration.¹ However, these high-sensitivity assays are always realized using heterogeneous assays which generally require several washing steps, resulting in increased complexity, making the analytical protocols time-consuming and less robust. In contrast, in homogeneous assays, no washing or physical separation is needed. Recently, tremendous progresses have been made in the homogeneous assay because it is relatively simple, robust, and easy to implement,^{2–6} which are of particular importance for point-of-care applications.

Current homogeneous assays are mostly based on detection of fluorescence,⁷ chemiluminescence,⁸ or scattered light. Fluorescence detection always requires special fluorescent labels and is likely to have issues due to fluorescent bleaching and autofluorescence of interfering compounds and/or biological samples.⁹ In addition, the fluorescence detection generally involves complicated instruments because of the weak signals. In chemiluminescence protocols, at least two reagents, chemiluminescent labels and reaction substrates, are needed, and these agents must be preserved and added separately. Light scattering assays involve immunoaffinity interaction induced particle agglutination and generally need only one reagent, antibody

^{a)} Author to whom correspondence should be addressed. Electronic mail: xyye@mail.tsinghua.edu.cn

conjugated particle. In addition, scattered light intensity is much stronger than fluorescence and the issues corresponding to fluorescence no longer exist, which greatly reduce the complexity on detection system.

Turbidity and nephelometry are two widely used methods to determine the extent of particle aggregation in medical diagnostic and environmental monitoring for biomolecules, microbes, heavy metal ions, and so on.¹⁰ In these two assays, the intensity of light scattered from or transmitted through an assay solution is recorded and correlated to analyte concentration. Nevertheless, these two assays are mostly used for high concentration analyte analysis because of their low sensitivity. Dynamic light scattering (DLS), a technique used routinely for determination of particle size and size distribution, has also been adopted for particle agglutination-based assays.^{11–13} Recently, a washing-free one-step homogeneous assay protocol was implemented using gold nanoparticles by measuring the hydrodynamic diameter of the nanoparticle aggregates with a DLS instrument, and a detection limit of 0.5 ng/ml for mouse IgG was achieved.¹² However, DLS instruments are relatively delicate and sensitive to temperature, which may limit its applications in point-of-care testing and hinder its integration with microfluidic chip. Moser *et al.* implemented on-chip agglutination detection based on the swelling of the released magnetic beads (MBs) plug under microscope, and about 3 pM biotinylated bovine serum albumin (bBSA) could be detected.¹⁴ The same group improved the detection method by counting singlets and agglutinated doublets using microscope.¹⁵ The magnetic beads were focused by magnetic force and a sheath flow to make sure all particles in the same focal plane, and the detection limit stayed at 6 pM for bBSA. However, a microscope is not suitable for point-of-care applications.

Flow cytometer is a great choice for discriminating agglutinated particles from monomers because of its ability to distinguish and count particles of different sizes. Commercial flow cytometer normally requires bulky instruments and consumes large amount of reagents. To solve this problem, many efforts have been made to miniaturize the flow cytometer. Up to now, several chip-based microflow cytometers have been reported. A microfluidic device using hydrodynamically focusing for counting and sizing particles and agglomerates has been demonstrated, and 100 ng C-reactive protein per ml could be detected.¹⁶ The scattered light was collected at 15° and 45°, and the standard deviation (SD) from the average scattering intensity was about 25%–30%. To reduce signal variance, four-sided sheath flow was realized on microfluidic chip using rugged¹⁷ or chevron-shaped¹⁸ microstructure in the microchannel. However, at least two inlets, sample inlet and sheath flow inlet, and in general two driving pumps are needed, which will complicate the microfluidic system. The sheath flow makes a lot of waste liquid, which is bad for point-of-care applications. In addition, the need for sheath flow has hindered the integration of these chip-based flow cytometer into total analysis systems. Approaches focusing particles to the center of the flow channel using dielectrophoresis¹⁹ or standing surface acoustic wave²⁰ have been reported too, which avoid the use of sheath flow. These peripheral assisted setups still make the flow cytometers relatively complicated. A microflow cytometer without particle focusing has been reported for fluorescent dye assay.²¹ This sheathless microflow cytometer only utilized fluorescence from target analytes and did not use scattered light, which could ignore the scattered light from the microchannel walls. By narrowing the microchannel down to the size fairly smaller than the laser spot, the light intensity of laser spot and induced fluorescence in the interrogation region was quite uniform. However, this optical configuration cannot be directly used for the detection of scattered light because of the strong scattering from microchannel walls. For the detection of agglutination, the precision determination of particle diameter is not necessary. Provided that the aggregates can be distinguished from the monomers, the extents of agglutination can be determined. From this point, a micro-chip for the detection of bead agglutination may leave out the sheath flow because of the weak demands for signal variances.

In addition to the detection method, particle is another key element for agglutination-based assay. MBs have the advantage of ease of manipulation and can promote inter-particle agglutination in presence of magnetic field.^{14,15,22} Herein, we present a method of the homogeneous assay based on bead agglutination using a micro-chip sheathless flow cytometric device with

scattered light detection. The relationship between the extents of agglutination and the analyte concentrations was verified using streptavidin-coated MBs and bBSA as a model assay with a commercial flow cytometer. Here, bBSA is just a representative of target antigen, while streptavidin represents capture antibody. Based on the detection results in which the forward scattered light of dimers was much more stronger than that of monomers, a benchtop detection system collecting forward scattered light near 0° is designed and built up. The extents of agglutination, that is, the analyte concentrations were determined by counting the numbers of monomers and aggregates using the micro-chip sheathless flow cytometric device. The experimental results with the micro-chip sheathless flow cytometric device were compared with those of the commercial flow cytometer.

MATERIALS AND METHODS

Detection setup

A schematic of the micro-chip sheathless cytometric device is shown in Fig. 1. As shown in Fig. 1(a), a semiconductor laser (Aunion Tech Co., China) was used as the light source at 660 nm. The light intensity of the laser beam was adjusted with a polarizer. The attenuated laser beam was focused using a plan-convex lens ($f = 60$ mm, Daheng Optics, China) onto the micro-channel of the microchip. The focused laser spot was $140\text{ }\mu\text{m}$ in diameter and has an intensity distribution of Gaussian profile. An aperture with diameter of 1 mm was placed in front of the microchip to spatially filter the stray light. The laser beam after the microchip was blocked using a light stop, so only the scattered light beyond the light stop was collected by a $10\times$ microscope objective (working distance 15 mm, NA 0.25, Daheng Optics, China). The scattered light in the range of 4° to 14° with respect to the incident laser beam was detected using a photodiode (PD) (BPW21, Siemens, Germany). The objective and photodiode were carefully adjusted to make sure that the image of the light spot in the microchannel was just on the photodiode. Another aperture with diameter of 1 mm was attached onto the photodiode to spatially reject scattered light from the edge of the laser spot. This design facilitated the selective collection of light from the center of the laser spot, where is referred to as interrogation region (Fig. 1(b)), thus weakening the influence of the uneven distribution of light intensity of Gaussian beam and reducing the signal variance of the scattered light. A photograph of the benchtop detection system of the micro-chip sheathless flow cytometric device was shown in the supplementary material (Fig. S1).²⁶ Fig. 1(b) shows the schematic of the microchip having

FIG. 1. (a) Schematic of forward scattered light detection system for the micro-chip sheathless flow cytometric device. P, polarizer; M, mirror; L, plan-convex lens; A1, aperture; C, microchip; LS, light stop; MO, microscope objective; A2, aperture; PD, photodiode; Current Amp, variable gain current amplifier; DAQ, data acquisition device; and PC, personal computer. (b) Schematic of the microchip with laser beam focused onto the microchannel (not to scale). The interrogation region is referred to as the region where scattered light can go through aperture A2 and hit on PD.

a microchannel with width of 2 mm and height of 60 μm . The fabrication process of the microchip is shown in the supplementary material.²⁶ Sample was perfused into the microchannel through the inlet tube using a syringe pump (TJ-2A, Longer Precision Pump Co., China) equipped with a 1 ml syringe. The outlet tube was directly connected to a waste reservoir. The diameter of the laser spot is smaller than the width of the microchannel to avoid light scattering from the microchannel walls.

Beads flow randomly in the microchannel and only those flowing through the interrogation region are detected and counted. However, two or more beads flowing through together may lead to misjudging monomers as aggregates. The coincidence of n beads flowing through the interrogation region together, $P(n)$, can be estimated by a Poisson process as follows:²¹

$$P(n) = (c\Delta V)^n e^{-(c\Delta V)} / n!, \quad n = 0, 1, 2, \dots, \quad (1)$$

where c is the number of beads per volume, ΔV is the volume of interrogation region. Therefore, the possibility of two or more beads flowing through together, P , which is referred as coincidence error, can be expressed as follows:

$$P = 1 - P(0) - P(1) = 1 - (1 + c\Delta V)e^{-(c\Delta V)}. \quad (2)$$

The coincidence error of the detection system limits the accuracy of the discrimination between monomers and aggregates. In this study, the interrogation region has a diameter of 100 μm and is 60 μm deep. For samples with a concentration of 5×10^2 beads/ μl , the estimated coincidence error is 2.4%. The coincidence error can be further reduced by decreasing the interrogation region volume ΔV , or diluting the sample to lower the bead concentration c .

The output current of the PD was amplified using a variable gain current amplifier (57-988, Edmund Optics Inc., USA), and the gain was set at 10^7 during experiments. A data acquisition card (USB-6009, National Instruments, USA) was used to collect and convert the voltage signals from the current amplifier into digital signals and was interfaced to a personal computer via a USB cable. Data acquisition and processing are programmed in LabVIEW (National Instruments, USA). The data acquisition rate was set at 1 kHz.

Protocol for agglutination reaction

Taking advantage of the strong affinity of streptavidin for biotin, we use the model assay with streptavidin and biotin for principle proving. In our verification experiments, bBSA (Biosynthesis biotechnology Co., China) was used as the analyte (or target Ag). Every BSA molecule can bond about 8 biotin molecules, so each bBSA molecule may easily link to two streptavidin-coated beads, thus forming doublets or larger aggregates. Streptavidin-coated Myone Dynabeads (Invitrogen, Switzerland) with diameter of 1 μm were used throughout the experiments, and the nominal concentration is about 10^{10} beads/ml. Before experiments, the MBs were washed three times according to the user manual and were diluted by a factor of 100 with 12 mM PBS (Phosphate Buffered Saline). To reduce non-specific agglutination, the diluted MBs suspension was sonicated for 5 min. The bBSA with an original concentration of 1 mg/ml was serially diluted with PBS by 10^{-3} to 10^{-8} , resulting in a concentration series from 1 $\mu\text{g/ml}$ (15 nM) to 10 pg/ml (0.15 pM).

The beadagglutination assay reaction was performed in a 0.6 ml centrifuge tube. In brief, the procedure was as follows: 40 μl MBs suspension was mixed with 160 μl bBSA, and the resulting 200 μl reaction mixture was gently shaken for 20 min to avoid MBs settling, allowing the MBs to capture bBSA sufficiently; subsequently, the centrifuge tube was put on a permanent magnet in order to attract all MBs to the tube wall for 3 min, prompting agglutination between MBs; then, the centrifuge tube was once again shaken for 5 min to disperse the non-specific agglutination. Finally, the reaction mixture was diluted further to a proper concentration for particle counting detection. The negative sample was made by mixing 40 μl MBs suspension with 160 μl PBS and applied the same operations mentioned above. All operations mentioned

above were conducted at room temperature. The buffer solution used in all experiments, which was only referred to as PBS before, was 12 mM PBS with 0.1% Tween 20 added to reduce non-specific agglutination. Three batches of independent experiments were conducted at different days, and for every analyte concentration the bead agglutination assays were performed with at least 3 repeats.

Protocol for agglutination detection

The commercial flow cytometer used in our study was BD FACSCalibur (BD, USA), and the sample flow rate was set at low rate (12 $\mu\text{l}/\text{min}$) for all experiments. The reaction mixture was diluted 1:1 by adding 200 μl of PBS for detection using Calibur. The signal of forward scatter (FSC) and side scatter (SSC) was recorded. Detection of 40 s or 40 000 events was set as the record ending conditions. The measurements were analyzed using FlowJo 7.6.1 (Treestar Inc., USA).

To test with the micro-chip sheathless flow cytometric device, the reaction mixture was diluted 1:20 by taking an aliquot of 50 μl and adding 950 μl PBS. The flow rate of the syringe pump was set at 50 $\mu\text{l}/\text{min}$. Different signal sampling time ranging from 3 s to 120 s was tested to determine the required number of counted events.

RESULTS AND DISCUSSION

Characterization of bead agglutination using Calibur

The scatter plots of a negative sample and a sample containing 100 ng/ml bBSA detected by BD Calibur were shown in Fig. 2. The number of events counted in Fig. 3(a) is 40 000, and the elapsed time was 35 s indicating the real bead concentration of $\sim 1.2 \times 10^4$ beads/ μl in the original reaction mixture, while the number of events in Fig. 2(b) is 17 505 during 40 s of detection time. Several subpopulations can be clearly distinguished in the scattering plot of Fig. 2(b) and are indicated as R1, R2, R3, R4, and R5, respectively. We concluded that these 5 regions indicate monomer, dimer, trimer, tetramer, and larger aggregates. In Fig. 2(a), which shows the scattering plot of the negative sample, nearly 90% of events are in R1, indicating a 10% non-specific agglutination. The proportion of monomers was higher than 95% without permanent magnets attraction (data not shown). Thus, about half of the non-specific agglutination in the negative sample may result from magnet attracting operation and non-zero remnant magnetization of some MBs. In the scatter plot of the sample containing 100 ng/ml bBSA, the proportion of monomer decreases to 36.6% (Fig. 2(b)). This result illustrates that significant bead agglutination would occur in presence of bBSA. The proportion of aggregates with the operation of permanent magnets attraction was 50% higher than that without permanent magnets attraction (data not shown), which demonstrated that the application of permanent

FIG. 2. Scatter plots showing the light intensity of FSC and SSC obtained from Calibur. Data from (a) a negative sample and (b) a sample containing 100 ng/ml bBSA.

FIG. 3. The relationships between the proportions of monomers (a), dimers, trimers, and larger aggregates (b) and bBSA concentrations.

magnets significantly facilitates the formation of aggregates. From Fig. 2, we could also conclude that FSC was a better signal for discriminating dimers and multimers from monomers. The light intensity of FSC and SSC of R1–R4 is included in the supplementary material.²⁶

As shown in Fig. 3, the proportions of monomers, dimers, trimers, and larger aggregates change with bBSA concentration. The proportion of monomers decreases when bBSA concentration increases from 0.15 pM to 1.5 nM. Saturation occurs for bBSA concentrations higher than 1.5 nM, where the streptavidin on MBs is almost fully linked with free bBSA and few spots are available for further forming aggregates, thus the proportion of monomers increases. For dimers, the proportion remains nearly constant when bBSA concentration is higher than 1.5 pM. The linear range was only from 0.15 pM to 1.5 pM. This phenomenon can be explained using kinetics of bead agglutination.²³ Dimers were formed from monomers aggregating; meanwhile, dimers were depleted by the formation of trimers and larger aggregates. When the rates of formation and depletion of dimers approached equilibrium, the concentration of dimers would become stable. However, the total particle numbers kept decreasing during aggregation. Thus, only when forming rate of dimers was lower than their depleting rate, the proportion of dimers would remain stable with the increase of bBSA concentrations. This trend can also be observed for trimers except that saturation occurs for bBSA concentrations higher than 150 pM. The maximum proportion of larger aggregates shows up at 1.5 nM bBSA, while at the same concentration the proportion of monomers decreases to minimum.

Figure 3 shows that the proportion of monomers is the best representative for bBSA concentration. To build up relationships between the analyte concentration and the extent of agglutination, a normalized signal S was defined as

$$S = 1 - \frac{\eta_{m,C}}{\eta_{m,blank}}. \quad (3)$$

$\eta_{m,C}$ is the proportion of monomer in the sample with an analyte concentration of C , $\eta_{m,blank}$ is the proportion of monomer in the negative sample. In the statistical process, only events in the selected regions were counted, and others were taken as noise signals and abandoned.

Three batches of experiments were conducted individually in different days, and the reaction mixture was analyzed using BD Calibur. As shown in Fig. 4, the dose-response curves of the agglutination assays were plotted in terms of the normalized signal. The error bars represent SD of four measurements. The SD of the negative sample in the three batches was 0.017, 0.011, and 0.007, respectively. The detection limit was as low as 0.15 pM where the levels of S were larger than 3 times as the SD of the negative samples. Saturation occurs for analytes with concentrations higher than 1.5 nM, where few spots on MBs are available for forming aggregates. The

FIG. 4. Dose-response curves of the agglutination assays. Three batches were conducted individually in different days. Each dot is the average result of four measurements, and the error bars represent standard deviation of the four measurements.

dose-response curve roughly displays a linear behavior in the ranges of 0.15 pM to 1.5 nM. The data show good consistency between different batches of experiments.

Agglutination detection based on micro-chip sheathless flow cytometry

The reaction mixtures were also detected using the micro-chip sheathless flow cytometric device. The microchannel is much wider than the laser spot, which can eliminate the scattering of channel walls. Counting all particles is unnecessary because the proportion of monomers is used for characterization of bead agglutination. Fig. 5 shows the scattered signals of a sample containing 1 $\mu\text{g/ml}$ bBSA obtained using the micro-chip sheathless flow cytometric device. A segment of 1 s out of 120 s sampling time is presented here. Each peak relates to a particle passing through the interrogation region. The background signal was about 0.7 V, and a threshold value of 1.2 V was set to distinguish particle scattered signals from noise. The peaks of scattered signals were recognized and labeled with red dot. The duration time of each scattered signal is about 0.013 s, and the average particle throughput in this case is 38 particles/s.

FIG. 5. A data plot showing the scattered signals of a sample containing 1 $\mu\text{g/ml}$ bBSA obtained using the micro-chip sheathless flow cytometric device (blue solid line: waveforms of the signals; red solid line: the threshold value set to distinguish scattered signals from noise; and red dot: the recognized peaks of the scattered signals).

Figure 6 shows the forward scattering intensity distribution histograms obtained from typical runs of a negative sample (Fig. 6(a)) and of a sample containing 100 ng/ml bBSA (Fig. 6(b)). A rough comparison of these two histograms shows that the proportion of signals lower than 2.7 V in Fig. 6(a) is higher than that in Fig. 6(b). Thus, the signals lower than 2.7 V are a representative of monomer, while higher signals represent aggregates. The details about selecting the threshold voltages for discriminating monomers and aggregates are included in the supplementary material.²⁶ The signals higher than 2.7 V shown in Fig. 6(a) mostly result from the non-specific agglutination, which can also be seen in the results of Calibur. In the histogram with 100 ng/ml bBSA (Fig. 6(b)), the proportion of signals higher than 2.7 V is much higher than that with negative sample (Fig. 6(a)). This is attributed to the formation of aggregates due to beads agglutination. Not like the results shown in Fig. 2, it was impossible to distinguish between different sizes of aggregates, such as dimers, trimers, or larger aggregates. This is mainly because of the Gaussian profile of the laser spot and the non-spherical nature of these aggregates.¹⁶ The same particles would pass through different parts of the laser spot thus causing different scattered signals, because the particles should disperse uniformly in the microchannel without sheath flow. In addition, the irregular aggregates with different sides towards the laser beam would also scatter light with different intensities. However, as long as the scattering intensity of aggregates was much stronger than that of a monomer, the aggregates can be distinguished from the monomers, which is enough for our bead agglutination assay method.

Figure 7 shows the proportion of monomers at different sampling time with different bBSA concentrations. The proportion of monomers fluctuates obviously when sampling time is less than 40 s and becomes stable with sampling time longer than 60 s. When sampling a short period of time, the number of counted events is not enough, and the statistical results will vary with sampling time. In the subsequent experiments, the sampling time was set as 120 s to make sure enough events be collected. At least 1600 events were counted during 120 s sampling for all samples.

Dose-response curve using the micro-chip sheathless flow cytometric device

The dose-response curve was also obtained using the micro-chip sheathless flow cytometric device. The scattered signals lower than 2.7 V were considered as monomers, and the normalized signals were calculated using Equation (1). Fig. 8 shows the dose-response curve of the proportion of monomers and the normalized signals. These curves are similar to Figs. 3 and 4, and saturation occurs for analyte concentrations higher than 1.5 nM, too. The normalized signal is 0.052 for bBSA with concentration of 1.5 pM, which is 3 times higher than 0.0139, the SD of the negative sample. The detection limit is 1.5 pM (100 pg/ml), which is an order of magnitude higher than that using Calibur. The values of the normalized signals shown here are lower than that shown in Fig. 4. This is mainly because of the sheathless mode, in which the position and the orientation of particles relative to the laser beam are random. The scattering intensity

FIG. 6. Histograms of the forward scattering intensity obtained from a negative sample (a) and a sample containing 100 ng/ml bBSA (b).

FIG. 7. The relationships between the proportion of monomers and sampling time with different bBSA concentrations.

of a dimer changes with its orientation to the incident light, but is at least 2 times stronger than that of a monomer (details in the supplementary material²⁶), indicating that dimers can be distinguished from monomers at the same incident light intensity. But the light intensity distribution in the laser beam is Gaussian profile, the small aggregates passing through the edge of the interrogation region in the laser spot would be misread as monomers, leading to overestimation of the proportion of monomers. However, the proportion of dimers was relatively stable when bBSA concentration was higher than 1.5 pM as shown in Fig. 3. Thus, misreading dimers as monomers did not confuse the relationship between the normalized signals and the bBSA concentrations, but it makes the detection limit and sensitivity worse. Details are discussed in the supplementary material.²⁶

The detection limit shown here is still lower than or similar to other bead-agglutination based homogeneous assay,^{14–16,24} which mainly benefited from the use of the forward scattered light and only counting the particles passing through the interrogation region near the center of the laser spot. The ratio of the scattered light intensity of aggregates to that of monomer was higher in FSC compared with other scattering angles. This allowed a better discrimination of monomers and aggregates in the micro-chip sheathless flow cytometric device than the use of scattered light in other angles. In addition, the light intensity of FSC was much stronger than that of SSC or scatter in other angles, making the detection system presenting a good signal-to-noise ratio and improved robustness. With the use of microscope objective and the aperture attached on the PD, only particles passing through a region relatively close to the center of the

FIG. 8. Dose-response curve of the agglutination assays detected by the micro-chip sheathless flow cytometric device. (a) The proportion of monomers vs bBSA concentration and (b) the normalized signal vs bBSA concentration. Each dot is the average result of three measurements, and the error bars represent SD of the three measurements. The SD of the normalized signal for the negative sample is 0.0139.

laser spot, in which the distribution of the laser intensity was relatively uniform, were detected, reducing the signal variance to some extent. The assay presented here uses the proportion of monomers to characterize the extents of agglutination. This kind of processing method neglects the variances of scattering intensity from monomers or aggregates, leading to the decrease of the signal variance and the increase of detection sensitivity. Except for the high detection sensitivity, the micro-chip sheathless flow cytometric device has the simplest chip structure, which needs only a main microchannel for sample flow. The assay protocol consists of only two steps: first, mixing MBs and analyte solution for analyte capture, and second, driving the reaction mixture through the micro-chip sheathless flow cytometric device for optical detection. Before detection magnet attraction can be performed to promote bead agglutination. No washing or separation is needed. These procedures can all be implemented on microfluidic chip using circular mixing²⁵ and electromagnets^{14,15} or movable permanent magnets.²² All in all, the bead agglutination based assay protocol and the micro-chip sheathless flow cytometric device specially designed for bead agglutination assay provide with potential solution for point-of-care applications.

CONCLUSIONS

A micro-chip sheathless flow cytometric device designed for homogeneous agglutination assay was developed. A micro-chip with only one inlet and one outlet was used in the sheathless flow cytometric device. Only particles flowing near the center of the laser spot were detected to reduce signal variance by using objective imaging and aperture filtration. The bead-agglutination based homogeneous assay protocol was verified using commercial Calibur first and then using the developed micro-chip sheathless flow cytometric device, and detection limits of 0.15 pM and 1.5 pM were achieved, respectively. The linear ranges were from detection limits to 1.5 nM. This sensitivity benefited from the use of forward scatter to distinguish aggregates from monomers. The setup for the micro-chip needs no sheath flow, which is of great importance for integration with microfluidic components or devices; meanwhile, the system has a relatively high sensitivity. By modifying the magnetic beads with special capture antibody, the developed method can be easily adjusted for the detection of various original antigens. So the developed micro-chip sheathless flow cytometric device provides with a promising tool for bead-based on-chip agglutination assays that can be implemented on versatile microfluidic platforms.

The bead-based agglutination assays take advantage of homogeneous assay which are free of separation steps and provide with a more amenable strategy in the development of a near instantaneous assay protocol for point-of-care applications. The developed micro-chip sheathless flow cytometric device can form the basis of a fully integrated microfluidic agglutination detection system comprising pumping, mixing, agglutination module, and subsequent particle-counting module based on optical detection of aggregates in a flow. The simplicity of the protocol of the homogeneous assay based on bead agglutination using the micro-chip sheathless flow cytometric device makes it a very competitive tool for use in resource-limited environments or for point-of-care diagnostics.

ACKNOWLEDGMENTS

This work was supported by National Instrumentation Program (Grant No. 2013YQ19046701).

¹H. C. Tekin and M. A. M. Gijs, *Lab Chip* **13**, 4711 (2013).

²L. R. Hirsch, J. B. Jackson, A. Lee, N. J. Halas, and J. L. West, *Anal. Chem.* **75**, 2377 (2003).

³A. Ranzoni, G. Sabatte, L. J. Van Ijzendoorn, and M. W. J. Prins, *ACS Nano* **6**, 3134 (2012).

⁴R. Pacheco-Gomez, J. Kraemer, S. Stokoe, H. J. England, C. W. Penn, E. Stanley, A. Rodger, J. Ward, M. R. Hicks, and T. R. Dafforn, *Anal. Chem.* **84**, 91 (2012).

⁵K. Aurich, S. Nagel, G. Glöckl, and W. Weitschies, *Anal. Chem.* **79**, 580 (2007).

⁶K. Karns and A. E. Herr, *Anal. Chem.* **83**, 8115 (2011).

⁷F. Degorce, A. Card, S. Soh, E. Trinquet, G. P. Knapik, and B. Xie, *Curr. Chem. Genomics* **3**, 22 (2009).

⁸H. Akhavan-Tafti, D. G. Binger, J. J. Blackwood, Y. Chen, R. S. Creager, R. De Silva, R. A. Eickholt, J. E. Gaibor, R. S. Handley, K. P. Kapsner, S. K. Lopac, M. E. Mazelis, T. L. McLernon, J. D. Mendoza, B. H. Odegaard, S. G. Reddy, M.

- Salvati, B. A. Schoenfelner, N. Shapir, K. R. Shelly, J. C. Todtleben, G. Wang, and W. Xie, *J. Am. Chem. Soc.* **135**, 4191 (2013).
- ⁹C. Williams, *Nat. Rev. Drug Discovery* **3**, 125 (2004).
- ¹⁰L. B. Bangs, *Pure Appl. Chem.* **68**, 1873 (1996).
- ¹¹Q. Dai, X. Liu, J. Coutts, L. Austin, and Q. Huo, *J. Am. Chem. Soc.* **130**, 8138 (2008).
- ¹²X. Liu and Q. Huo, *J. Immunol. Methods* **349**, 38 (2009).
- ¹³X. Liu, Q. Dai, L. Austin, J. Coutts, G. Knowles, J. Zou, H. Chen, and Q. Huo, *J. Am. Chem. Soc.* **130**, 2780 (2008).
- ¹⁴M. A. M. Gijs, Y. Moser, and T. Lehnert, *Lab Chip* **9**, 3261 (2009).
- ¹⁵R. Afshar, Y. Moser, T. Lehnert, and M. A. M. Gijs, *Anal. Chem.* **83**, 1022 (2011).
- ¹⁶N. Pamme, R. Koyama, and A. Manz, *Lab Chip* **3**, 187 (2003).
- ¹⁷P. B. Howell, J. P. Golden, L. R. Hilliard, J. S. Erickson, D. R. Mott, and F. S. Ligler, *Lab Chip* **8**, 1097 (2008).
- ¹⁸J. P. Golden, J. S. Kim, J. S. Erickson, L. R. Hilliard, P. B. Howell, G. P. Anderson, M. Nasir, and F. S. Ligler, *Lab Chip* **9**, 1942 (2009).
- ¹⁹D. Holmes, J. K. She, P. L. Roach, and H. Morgan, *Lab Chip* **7**, 1048 (2007).
- ²⁰Y. Chen, A. A. Nawaz, Y. Zhao, P.-H. Huang, J. P. McCoy, S. J. Levine, L. Wang, and T. J. Huang, *Lab Chip* **14**, 916 (2014).
- ²¹W. Shi, L. Guo, H. Kasdan, and Y.-C. Tai, *Lab Chip* **13**, 1257 (2013).
- ²²G. Degré, E. Brunet, A. Dodge, and P. Tabeling, *Lab Chip* **5**, 691 (2005).
- ²³I. V. Surovtsev, M. A. Yurkin, A. N. Shvalov, V. M. Nekrasov, G. F. Sivolobova, A. A. Grazhdantseva, V. P. Maltsev, and A. V. Chernyshev, *Colloids Surf., B* **32**, 245 (2003).
- ²⁴A. Ranzoni, J. J. H. B. Schleipen, L. J. Van Ijzendoorn, and M. W. J. Prins, *Nano Lett.* **11**, 2017 (2011).
- ²⁵M. Du, Z. Ma, X. Ye, and Z. Zhou, *Sci. China Technol. Sci.* **56**, 1047 (2013).
- ²⁶See supplementary material at <http://dx.doi.org/10.1063/1.4936926> for a photograph of the optical setup, microchip fabrication, light intensity of FSC and SSC, determination of threshold voltage for discriminating aggregates, simulation of scattering intensity, and monomers identification in the micro-chip sheathless flow cytometric device.