A highly efficient bead extraction technique with low bead number for digital microfluidic immunoassay

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Here, we describe a technique to manipulate a low number of beads to achieve high washing efficiency with zero bead loss in the washing process of a digital microfluidic (DMF) immunoassay. Previously, two magnetic bead extraction methods were reported in the DMF platform: (1) single-side electrowetting method and (2) double-side electrowetting method. The first approach could provide high washing efficiency, but it required a large number of beads. The second approach could reduce the required number of beads, but it was inefficient where multiple washes were required. More importantly, bead loss during the washing process was unavoidable in both methods. Here, an improved double-side electrowetting method is proposed for bead extraction by utilizing a series of unequal electrodes. It is shown that, with proper electrode size ratio, only one wash step is required to achieve 98% washing rate without any bead loss at bead number less than 100 in a droplet. It allows using only about 25 magnetic beads in DMF immunoassay to increase the number of captured analytes on each bead effectively. In our human soluble tumor necrosis factor receptor I (sTNF-RI) model immunoassay, the experimental results show that, comparing to our previous results without using the proposed bead extraction technique, the immunoassay with low bead number significantly enhances the fluorescence signal to provide a better limit of detection (3.14 pg/ml) with smaller reagent volumes (200 nl) and shorter analysis time (<1 h). This improved bead extraction technique not only can be used in the DMF immunoassay but also has great potential to be used in any other bead-based DMF systems for different applications. © 2016 AIP Publishing LLC. [<http://dx.doi.org/10.1063/1.4939942>]

I. INTRODUCTION

Immunoassay is a popular technique for immunodiagnosis that exploits the specific interaction and complexation of antibody and antigen to quantify desirable analytes in a physiological liquid, e.g., blood,^{1–3} serum,^{4,5} or urine.^{6,7} Sandwich immunoassay is the most commonly used assay format for protein analysis. In such assay, two types of antibodies, which are often called capture antibody and detection antibody, respectively, are used to bind to different sites of the

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antigen. Typically, the capture antibody, which is highly specific for the antigen, is first immobilized to a solid surface. The antigen is then added, followed by the addition of the detection antibody. As a result, the antigen is “sandwiched” between the two antibodies, forming an antibody-antigen-antibody complex. When the antigen concentration increases, the amount of detection antibody increases accordingly, leading to a stronger measured response.

Bead-based immunoassay employs the microbead as a three-dimensional (3D) solid carrier, which is superior to a planar two-dimensional (2D) format in providing an increased surface-to-volume ratio and minimizing spatial hindrance.^{8–10} Hence, the bead-based immunoassay increases the sensitivity of detection and requires fewer samples per measurement. Furthermore, decreasing the starting number of antibody coupled beads was shown to effectively elevate the responding signal,⁸ since less starting bead number would allow more antigens to bind to the surface of each bead, as illustrated in Fig. 1. Direct evidence from the scanning electron microscope (SEM) images has shown that the surfaces of the functionalized beads were covered with matrix-like exogenous substances.⁹ Therefore, even at the low level of antigen, signal can be amplified by using fewer beads in immunoassay. However, in previous bead-based immunoassay report, lots of the beads were lost in the washing process that would affect the accuracy of the responding signal.⁸ Also, large amount of sample volumes were needed.

Digital microfluidic (DMF) is an emerging technology that electrostatically manipulates discrete droplets on an array of insulated electrodes.^{10–13} Electrowetting is the most popular mechanism to control the small droplets precisely. To date, most available examples^{14–18} of DMF immunoassay are based on the use of magnetic beads as solid carries to execute the complex, multistep washing protocols. The magnetic beads suspended in the droplet can be extracted by means of magnetic fields in washing process.^{19,20} In general, washing protocol involves several steps to remove unwanted materials (e.g., unbound antibodies, antigens, or reporters) suspended in the droplet: (1) immobilizing the beads physically or magnetically; (2) extracting beads by separating the mother droplet into two daughter droplets (one droplet with beads and the other droplet without beads) with proper droplet operations; (3) removing the droplet without beads and merging the droplet with beads to another droplet of fresh buffer; and (4) repeating steps (1)–(3) as needed to achieve the desired removal of unwanted materials.

To date, two magnetic bead extraction methods coupling with magnetic fields for microbead washing were developed in DMF: single-side electrowetting method^{14–16} and double-side electrowetting method.^{17,18,21–24} These two methods employed the 1D array of electrodes with equal size. As illustrated in Fig. 2(a1), the first method used an external magnet to immobilize the beads on the right electrode, thus the beads remained at the right electrode while the electrowetting force was applied on the opposite side of the droplet (Fig. 2(a2)). This method was capable of removing unbound reagents in two washes which was quite efficient.¹⁴

FIG. 1. Effect of starting number of antibody coupled bead. (a) Few antigens were captured per bead as more beads were used. (b) More antigens were captured per bead as fewer beads were used.

FIG. 2. Illustrations of different methods to wash magnetic beads on DMF platform: (a1)–(a3) Single-side electrowetting method.^{14–16} (b1)–(b3) Double-side electrowetting method.^{17,18,21–24} (c1)–(c3) Proposed method.

However, lots of beads were required to achieve the bead immobilization in bead extraction, and bead loss was unavoidable during washing process. Figure 2(a3) shows the force diagram of the magnetic beads in droplet under the influence of magnetic force and surface tension. Surface tension on the droplet intends to move the bead toward left, while magnetic force will try to immobilize beads. Therefore, large bead volume (i.e., high bead number) is required to have sufficient magnetic force to resist the surface tension from electrowetting to move the beads.^{25,26} In the second method, the beads were first immobilized on one side of the middle electrode due to the attraction of external magnet (Fig. 2(b1)). Then the electrowetting force was applied to both sides of the droplet to split the mother droplet into two daughter droplets (Fig. 2(b2)), where one droplet with most beads would be moved to the right and the droplet with the waste would be moved to the left. This method could perform bead extraction with low bead number, however, it was inherently inefficient where 10–18 washes were required,¹⁸ and bead loss was unavoidable.

Here, we propose an improved double-side electrowetting method to achieve highly efficient bead extraction for separating magnetic beads in DMF immunoassay with low bead number. This new magnetic bead extraction protocol employs a series of unequal electrodes consisting of one regular electrode and two smaller electrodes, and the mother droplet is initially located at the left electrode with regular size (Fig. 2(c1)). The droplet is stretched along the series of electrodes by electrowetting force, followed by turning off the regular (left) electrode while keeping the small electrodes on, as shown in Fig. 2(c2). Due to the external magnet, the magnetic beads will be attracted to the right and immobilized. Then, turning on the right and left electrodes can split the mother droplet into two unequal daughter droplets by electrowetting force (as shown in Fig. 2(c3)). According to fluid mechanics,²⁷ the surface tension is proportional to the length of contact, i.e., circumference of droplet contacted with the solid surface.²⁸ Therefore, with a smaller electrode, the surface tension on the droplet becomes smaller.^{29,30} Then fewer magnetic beads are enough to provide sufficient magnetic force to resist the surface tension. Also, the left daughter droplet is bigger which can take away more waste volume in one washing step than the conventional equal-electrode design, it is expected to have better washing efficiency. In this study, the effects due to different electrode size ratios will be investigated, including bead number, bead loss, and washing efficiency. The proposed bead extraction technique is then further integrated with a DMF immunoassay to demonstrate the performance enhancement as an example. This technique has great potential to be used in any other bead-based systems for different applications such as cell purification,^{31–33} bacteria separation,³⁴ DNA capture, and purification.^{35–38}

II. MATERIALS AND METHODS

A. Reagents and materials

The commercially available soluble tumor necrosis factor receptor I (sTNF-RI) enzyme-linked immunosorbent assay (ELISA) kit was purchased from R&D Systems. Recombinant human sTNF-RI (Cat. DY225, R&D Systems) and antibody pairs, including capture antibody and detection antibody, are directed against varied epitopes of individual soluble receptors. Activation buffer (Na_2HPO_4 100 mM, pH 6.2, J. T. Baker), coupling buffer (MES 50 mM, pH 5.0, J.T. Baker), and washing buffer (Tween 20, 0.1% v/v, Sigma) in phosphate-buffered saline (PBS, pH 7.2–7.4, Biowest) were prepared for bead coupling. Coupling controls were examined to confirm the efficiency of immobilization on staining 1500 beads with R-phycoerythrin (PE)-conjugated goat anti-mouse IgG (Cat. 115-116-146, Jackson Immuno-Research Laboratories, Inc.). The absorption spectrum of R-PE has three maxima of absorbance at 490, 545, and 566 nm; the maximum fluorescent emission occurs at 580 nm. Pluronic F127 is from Sigma Chemical. De-ionized (DI) water had a resistivity of $18 \text{ M}\Omega\cdot\text{cm}$ at 25°C .

B. Covalent coupling of antibodies to COOH-beads

Superparamagnetic carboxylated polystyrene beads of diameter $6 \mu\text{m}$ (QuantumPlex™ M Carboxyl, Cat. 250, Bangs Laboratories, Inc.) were activated and coated with capture antibodies according to a modified protocol.³⁹ Briefly, each bead set ($10 \mu\text{l}$, 1.25×10^6 microspheres) was magnetically applied to remove the supernatant. Beads were washed twice with activation buffer, and resuspended with activation mix ($100 \mu\text{l}$), containing N-hydroxysulfosuccinimide sodium salt (10 mg/ml, Sulfo-NHS; Thermo Scientific) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (10 mg/ml, EDC; Thermo Scientific) in an activation buffer. The reaction mixture was incubated for 20 min near 23°C in darkness in an end-over-end rotator followed on washing twice with coupling buffer ($500 \mu\text{l}$). The activated beads were then incubated with antibody solution ($80 \mu\text{l}$, with final concentration $40 \mu\text{g/ml}$ in a coupling buffer) for at least 2 h near 23°C in darkness in an end-over-end rotator. The beads were eventually washed twice with washing buffer ($500 \mu\text{l}$) and resuspended in PBS ($100 \mu\text{l}$, containing bovine serum albumin 1% w/v, and NaN_3 , 0.05% w/v). Coated beads were stored in darkness at $2\text{--}8^\circ\text{C}$.

C. DMF device fabrication and actuation

DMF devices were fabricated in the clean room facilities of Nano Facility Center (National Chiao Tung University, Taiwan) using standard photolithography and metal etching, as described previously.⁴⁰ Briefly, the top glass plate (thickness 0.7 mm) containing a blank ITO electrode covered with Teflon (thickness 55 nm) as a hydrophobic layer was prepared. Driving electrodes were patterned on the bottom glass plate and coated with SU-8 (thickness $1.6 \mu\text{m}$) and Teflon (thickness 55 nm) as the dielectric and the hydrophobic layers, respectively. The gap between the top and bottom plates was controlled by the stainless-steel spacer (thickness $25 \mu\text{m}$, MiSUMi).

Droplet actuation was achieved by applying voltage $50 \text{ V}_{\text{rms}}$ and 1 kHz to driving electrodes. Electronic signal for the actuation sequence was controlled using LabVIEW (National Instruments) with the help of a digital I/O device (USB-6251, National Instruments). All electrodes were kept grounded by default. Higher voltages can increase the transporting speed but also may increase the likelihood of dielectric breakdown across the dielectric and hydrophobic layers. Magnetic force was provided by an external neodymium magnet (diameter 10 mm, thickness 20 mm, 0.5 T, N52 grade, Asia Magnets Co., Ltd., Taiwan). For washing test, the DMF device was mounted on the stereo microscope (SZX16, Olympus) equipped with a color CCD camera (Model DFK 51AU02, Imaging Source) for image capture. For DMF immunoassay, the DMF device was mounted on an inverted fluorescence microscope (IX73, Olympus) for visualization. A cooled CCD camera (DP80, Olympus, 1360×1024 pixels) was used to capture the fluorescence images.

D. Washing test

To quantify the loss of the bead during the washing process, a bead loss test was developed for different magnetic bead extraction methods. An 125-nl mother droplet containing certain number of magnetic beads was positioned on the initial electrode and then split into two daughter droplets, waste droplet and the droplet with most beads. The bead loss rate is defined as the ratio between the bead number in the waste droplet after bead extraction step and the starting bead number in mother droplet initially.

Since the number of washing cycles to achieve an acceptable purification would seriously influence the total time to result in immunoassays, a washing test was developed to investigate the washing efficiency on the proposed magnetic bead extraction scheme. An 125-nl droplet containing fluorescence dye (R-Phycoerythrin-conjugated streptavidin, Cat. 016-110-084, Jackson Immuno-Research Laboratories, Inc.) was prepared as the test droplet to simulate a droplet containing unwanted fluorescence molecular to be removed. The washing rate is defined as the reduction percentage in measured fluorescence signal after the washing process relative to the initial signal before the washing process, and 98% has been regarded as an acceptable rate for a successful washing process.¹⁴ Therefore, in each washing test, once the measured fluorescence signal after wash was lower than 2% of the initial signal, the washing process was considered to be successful. Fewer washing steps to achieve at least 98% washing rate represent a better washing efficiency.

E. On-chip immunoassay protocol

An on-chip protocol was developed to implement on-chip reaction and on-chip detection on a DMF immunoassay chip, as shown in Fig. 3. The size of right manipulation electrodes is $2828\ \mu\text{m} \times 2828\ \mu\text{m}$ for antigen droplet and buffer droplet with antibody coupled beads, and the size of left manipulation electrodes is $2236\ \mu\text{m} \times 2236\ \mu\text{m}$ for detection antibody droplet and reporter droplet. Three $707\ \mu\text{m} \times 707\ \mu\text{m}$ manipulation electrodes were designed at middle to separate beads with the proposed improved double-side electrowetting method. The magnet needs to be manually positioned according to the direction of bead extraction during the immunoassay procedure. For example, while extracting the magnetic beads to the left is needed in washing step, the magnet is placed on the top of the left small electrode manually. In contrast, while extracting the magnetic beads to the right side, the magnet is changed to place on the top of the right small electrode.

The on-chip reaction was accomplished by a series of repeating processes, including mixing, incubation, and washing processes on droplets. First, a 200-nl droplet containing antibody coupled beads was created from the reservoir using $2828\ \mu\text{m} \times 2828\ \mu\text{m}$ electrodes. Then the

FIG. 3. Layout of DMF immunoassay, including six reservoirs and three different sizes of actuation electrodes.

excess liquid was split from beads using $2828\ \mu\text{m} \times 2828\ \mu\text{m}$ and $707\ \mu\text{m} \times 707\ \mu\text{m}$ electrodes with proposed bead extraction method illustrated in Fig. 2(c). Another 200-nl droplet (created by $2828\ \mu\text{m} \times 2828\ \mu\text{m}$ electrode) containing sample was then mixed with the antibody coupled beads and incubated at near $23\ ^\circ\text{C}$ for 10 min. To wash the magnetic beads, the magnetic beads were immobilized by an external magnet and the excess liquid was separated from beads by the proposed bead extraction method. An 125-nl droplet (created by $2236\ \mu\text{m} \times 2236\ \mu\text{m}$ electrode) containing biotinylated detection antibody ($1\ \mu\text{g}/\text{ml}$ in PBS) was dispensed and mixed with the immobilized beads and incubated at near $23\ ^\circ\text{C}$ for 5 min. Magnetic beads were then separated by proposed bead extraction method. Another 125-nl droplet (created by $2236\ \mu\text{m} \times 2236\ \mu\text{m}$ electrode) containing R-PE-labeled streptavidin ($2\ \mu\text{g}/\text{ml}$ in PBS) was then dispensed and mixed with beads and incubated at near $23\ ^\circ\text{C}$ for 3 min. Magnetic beads were separated again by proposed bead extraction method and merged with another fresh washing droplet for fluid replacement. Then, an 12.5-nl daughter droplet (created by $707\ \mu\text{m} \times 707\ \mu\text{m}$ electrode) with aggregated beads was split from the 125-nl mother droplet for fluorescence detection. The on-chip detection method is the same as our previous method.⁴⁰

F. DMF immunoassay on sTNF-RI

Using the on-chip protocol, a calibration curve was established from standard solutions of sTNF-RI (0, 0.1, 1, 5, 20, 80, and $160\ \text{pg}/\text{ml}$). Duplicate measurements at each concentration were averaged and fitted with a four-parameter logistic equation to determine the limit of detection (LOD).³⁹ The LOD was the concentration corresponding to the position on the curve of the average signal generated from blank measurements plus three times the standard deviation of the blank measurements. The error bars represented the standard deviation between two measurements on the DMF immunoassay.

In our previous work,⁴⁰ an off-chip protocol utilizing the conventional bead-based immunoassay coupled with an on-chip detection method was developed, where the reaction process and the washing process were manually carried out in the Eppendorf tube. Only the detection process was executed on chip. The results from both off-chip and on-chip protocols will be compared.

III. RESULTS AND DISCUSSION

In this section, bead loss test with different bead number, especially low number of beads, is first implemented for both single-side and double-side electrowetting methods. Then the bead loss and washing efficiency of proposed method are investigated at different electrode size ratios. The performance of on-chip immunoassays by using the proposed magnetic bead extraction method will also be examined and compared to the results from the off-chip protocol without using this new bead extraction technique.

A. Single-side electrowetting method for magnetic bead extraction

To investigate the bead loss rate in using the single-side electrowetting washing method, a bead loss test is first performed with different starting numbers of beads. As shown in Fig. 4(a), there are two equal electrodes, and the size of each electrode is $2236\ \mu\text{m} \times 2236\ \mu\text{m}$, which will be about the area of initial droplet. The droplet with beads is first introduced to the surface of left electrode, as shown in Figs. 4(b1) and 4(c1). Then the droplet is moved to the right electrode by electrowetting force, as shown in Figs. 4(b2) and 4(c2). While the external magnet tries to immobilize the beads on the right electrode, the droplet is moved back to the left electrode by electrowetting force. It is found that two different outcomes are possible, depending on the starting number of beads. As shown in Figs. 4(b3), when 4350 beads are used as the starting bead number, most of the magnetic beads are successfully extracted from the droplet with a low bead loss rate 6% in the waste (left) droplet. For a lower starting bead number, 1866 beads, the magnetic force is insufficient to overcome the surface tension force to

FIG. 4. Single-side electro-wetting method for magnetic bead extraction. (a) The pattern of electrode for single-side electro-wetting method. (b1) A droplet with 4350 beads. (b2) Droplet with 4350 beads is moved to right electrode close to the magnet. (b3) Droplet is transported away from the magnet for bead extraction. (c1) A droplet with 1866 beads. (c2) Droplet with 1866 beads is moved to right electrode close to the magnet. (c3) Droplet is transported away from the magnet for bead extraction (scale bar: $500 \mu\text{m}$). (d) The relationship between bead loss rate and bead number. At least 2550 beads are required to achieve single-side electro-wetting method. Bead loss is unavoidable.

immobilize any bead, as shown in Fig. 4(c3), i.e., beads are all in the waste droplet, leading to 100% bead loss rate.

Figure 4(d) summarizes the results of bead loss test with different starting bead numbers by the single-side electro-wetting washing method. The ordinate axis represents the bead loss rate which is defined as the ratio between the bead number in the waste droplet and the mother droplet. The abscissa represents the starting number of beads. It shows that it needs at least 3000 beads to have low bead loss rate. When the number of beads is fewer than 2550, magnetic beads cannot be retained by the external magnet at all, i.e., 100% bead loss rate. As illustrated in Fig. 2(a3), the magnetic force is proportional to the amount of magnetic beads; whereas the surface tension is proportional to its diameter. Hence, bead extraction can only be achieved with large bead number to provide sufficient magnetic force to resist the surface tension from electro-wetting force to move the waste droplet.

B. Double-side electro-wetting method with equal electrodes for magnetic bead extraction

In a typical double-side electro-wetting method, it uses three equal electrodes, and a mother droplet containing magnetic beads is initially placed on the middle electrode. The size of each electrode is also $2236 \mu\text{m} \times 2236 \mu\text{m}$, which will be the area of initial droplet, as shown in Fig. 5(a). To generate two asymmetric daughter droplets for a higher dilution ratio (volume ratio), the right electrode is turned on for about 1 s to stretch the droplet from center electrode to the right electrode a little and then turned it off. Therefore, the beads are attracted to the right edge of the droplet, as shown in Fig. 5(b1). Then applying the electro-wetting force on the right and left electrodes can split the mother droplet into two unequal daughter droplets, as shown in Fig. 5(b2), where the small droplet with most beads would be moved further to the right and the big droplet with the waste would be moved to the left. It is found that the bead extraction with low bead loss rate is possible by using this double-side electro-wetting technique at bead number less than 100; however, bead loss problem is still inevitable, as shown in Fig. 5(c). The loss of any amount of beads may affect the accuracy of the responding signal, especially for a low number of starting beads. Previous study also showed that multiple washing steps up to 10–18 were required for the typical double-side electro-wetting method with equal electrodes.¹⁸

FIG. 5. Double-side electrowetting method with equal electrodes for magnetic bead extraction. (a) The pattern of three equal electrodes. (b1) A droplet with 199 beads is stretched to the right electrode a little. (b2) Droplet is split into two unequal daughter droplets, where magnetic beads are extracted in the small droplet (scale bar: 1 mm). (c) The relationship between bead loss rate and starting bead number.

C. Improved double-side electrowetting method with unequal electrodes for magnetic bead extraction

The proposed improved design uses three electrodes with two different sizes in bead extraction process. Figure 6(a) shows the layout of electrode pattern. The size of left electrode is $2236 \mu\text{m} \times 2236 \mu\text{m}$, which is about the area of initial mother droplet. To have unequal droplet sizes after splitting, the dimensions of small electrodes are $1000 \mu\text{m} \times 1000 \mu\text{m}$ for volume ratio 5: 1 and $707 \mu\text{m} \times 707 \mu\text{m}$ for volume ratio 10: 1, respectively. The mother droplet is first positioned on the left electrode and then stretched along the small electrodes to the right by activating these two small electrodes to have electrowetting force. Due to the magnet close to the right electrode, magnetic beads are attracted to the right edge of the elongated droplet, as shown in Fig. 6(b1) with starting bead number of 17 in volume ratio 10:1 design. Then by activating the left electrode and the right electrode, as shown in Fig. 6(b2), the droplet is split into two unequal daughter droplets, and the attraction by the magnet is sufficient to retain all 17 beads in the small droplet to have zero bead loss.

The measured bead loss rates for different starting bead numbers at two different electrode area ratios are shown in Fig. 6(c), where the ordinate axis represents the bead loss rate and the abscissa represents the starting number of beads. It shows that the proposed bead extraction method with unequal electrodes (volume ratio of 5:1 or 10:1) significantly minimizes the loss of beads during washing step. In particular, for the bead number less than 100, zero bead loss can be achieved stably by the proposed method.

To further compare the washing efficiency, washing tests are performed for both volume ratio of 5:1 and 10:1. As shown in Fig. 6(d), a larger volume ratio provides a better washing efficiency. Only one wash is required for 10:1 design to reach acceptable levels of washing (2% residual fluorescence signal) while two washes are required for 5:1 design, since a larger volume-ratio design can take away more waste volume in one washing step. For all three bead extraction methods, the required time to extract beads in a single washing step was similar; however, the proposed bead extraction method can reduce the required washing steps as well as the total washing time. Improving washing efficiency may not help to reduce total analysis time too much, comparing to the other two bead extraction methods, but fewer washing steps can also reduce the amount of required washing buffer.

FIG. 6. Proposed unequal-electrode design for magnetic bead extraction. (a) The pattern of three electrodes with two different sizes. (b1) A droplet with 17 beads initially. (b2) Droplet is split into two unequal daughter droplets by the proposed bead extraction technique. Inset shows enlarged image of the right daughter droplet edge with all 17 beads (scale bar: 500 μm ; inset scale bar: 100 μm). (c) The measured results on bead loss rate at different starting bead numbers in volume ratios of 5: 1 (red squares) and 10: 1 (green triangles). Zero bead loss can be achieved with bead number less than 100. (d) Comparison of the washing efficiency for volume ratios of 5: 1 (red squares) and 10: 1 (green triangles) by measuring the residual fluorescence after each wash step. Only one wash is required for 10: 1 design to reach 98% washing rate.

D. DMF immunoassay with the new bead extraction technique

To validate the benefit of using the proposed new bead extraction technique for immunoassay with high washing efficiency at low bead number, a DMF immunoassay is carried out for human sTNF-RI at concentrations 1 pg/ml with various starting bead numbers by the on-chip immunoassay protocol described in Section II. The washing steps are executed after incubation steps to remove all the unwanted materials (e.g., unbounded antibodies, antigens, or reporters) suspended in the droplet. The proposed washing protocol mainly involves three steps: (1) immobilizing the beads magnetically; (2) extracting beads by separating the mother droplet into two daughter droplets (one droplet with beads and the other droplet without beads) by the proposed bead extraction technique; and (3) removing the droplet not containing beads and merging the droplet with beads to another droplet of fresh buffer. Bead number ranges from 59 to 4058 beads. As shown in Fig. 7(a), no signal is detected as bead number more than 1000, and the signal increases with less starting bead number. The results verify that low bead number is helpful to enhance responding signal, especially at low sample concentration, since more antigens are allowed to bind to the surface of each bead. Therefore, even at the low level of antigen, signal can be amplified by using fewer beads.

Further, the calibration curve from standard solution of sTNF-RI with various concentrations is carried out by the on-chip protocol, as shown in Fig. 7(b). Bead number in immunoassay is controlled near 25 beads. Among many performance characteristics of an immunoassays protocol, the required sample volume, time to result, and LOD are of great interest. In previously reported

FIG. 7. Quantitative analysis of sTNF-RI. (a) Bead number effect on the immunoassay at 1 pg/ml concentration of human sTNF-RI. (b) Calibration curve obtained from the DMF chip with varied concentration.

results⁴⁰ using off-chip protocol, the reaction process and the washing process were manually carried out in the Eppendorf tube, therefore, it required large amount of samples, 80 μ l. The total time to result was over 4 h. The LOD from the on-chip detection method and the off-chip detection method (cytometry-based method) were 15 pg/ml and 217 pg/ml, respectively.⁴⁰ In this work, the proposed on-chip protocol is shown to achieve better performance. The required sample volume is reduced to 200 nl per immunoassay, the total time to result is within 1 h, and LOD is 3.14 pg/ml.

IV. CONCLUSIONS

In previously reported two magnetic bead extraction methods for DMF platform, the single-side electrowetting approach could provide high washing efficiency, but it required a large number of beads. The double-side electrowetting method with equal-electrode design could reduce the required number of beads, but multiple washes were needed. Furthermore, bead loss during washing process was unavoidable in both methods. Here, an improved double-side electrowetting method with unequal-electrode design is proposed. It is shown that, with proper electrode size ratio, only one wash step is required to achieve 98% washing rate without any bead loss at bead number less than 100 in a droplet. Since less starting bead number in a droplet allows more antigens to bind to the surface of each bead, signal can be amplified even at the low level of antigen. An on-chip protocol is then developed on a DMF immunoassay by using the new bead extraction technique. It is shown that, in our sTNF-RI model immunoassay, LOD 3.14 pg/ml is achieved, only 200 nl sample volume and less than 1 h of analysis time are needed. Comparing to previous off-chip protocol in immunoassays with on-chip detection, a better LOD and reduced reagent volumes with shorter analysis time are demonstrated. This highly efficient bead extraction technique not only can be used in the DMF immunoassay for the immunodiagnosis but also has great potential to be used in any other bead-based DMF applications with light-based detection mechanisms.

There are two key factors to improve the bead loss and washing efficiency in our electrode design. First, the splitting point should be away from the beads to minimize the loss of beads into the waste droplet. Second, a higher electrode size ratio increases the washing efficiency. Any electrode design based on these principles may be helpful. Moreover, since 0.5 T field was found enough to trap the beads, it would be meaningful to miniaturize the system by integrating a microfabricated electromagnet (i.e., microcoil)^{41–45} to replace the magnet in the future.

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