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ORIGINAL PAPER

Picoliter droplet microfluidic immunosorbent platform for point-of-care diagnostics of tetanus

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Abstract We have developed a sensitive, specific, rapid and low cost picoliter microsphere-based platform for bioanalyte detection and quantification. In this method, a biological sample, biosensing microspheres, and fluorescently labeled detection (secondary) antibodies are co-encapsulated to capture the analyte (here: human anti-tetanus immunoglobulin G) on the surface of the microsphere in microfluidic pL-sized droplets. The absorption of the analyte and detecting antibodies on the microsphere concentrate the fluorescent signal in correlation with analyte concentration. Using our platform and commercially available antibodies, we were able to quantify anti-tetanus antibodies in human serum. In comparison to standard bulk immunosorbent assays, the microfluidic droplet platform presented here reduces the reagent volume by four orders of magnitude, while fast reagent mixing reduces the detection time from hours to minutes. We consider this platform to be a major leap forward in the miniaturization of immunosorbent assays and to provide a rapid and low cost tool for global point-of-care.

Keywords Droplet microfluidics · Point-of-care diagnostics · Biosensor microsphere · Tetanus immunization status

Introduction

Point-of-care (POC) diagnostics has attracted a lot of interest and investments for its promise to transform the global health [1]. Emerging POC applications have already improved the health care system at particular applications, for example infectious diseases diagnostics: syphilis, dengue and HIV [2–5]. Nevertheless, multiple barriers still exist for worldwide acceptance of the POC approach [1]. Today, enzyme-linked immunosorbent assay (ELISA) is the gold standard in the POC diagnostics for evaluation of biomarkers and detection of bio-analytes because of its sensitivity, accuracy and repeatability. Although widely used in clinical and basic biomedical research [6], standard ELISA diagnostics consumes significant labor, time, volumes of analyte and expensive reagents (>10 μL) per each reaction step. To reduce reagent volumes, a multiplex cytometric immunosorbent microsphere based array was introduced [6]. However, the device cost, a complex laser-based machine, and operator skills prevent worldwide practice of the microsphere-multiplex immunosorbent technique in POC diagnostics today. Therefore, a need exists in low cost, fast and sensitive techniques to analyze bio-analytes for global POC diagnostics.

In the last decades, microelectromechanical systems (MEMS) reduced the total bio-assaying costs by miniaturization of laboratory equipment and volumes of reagents used [7]. Droplet microfluidics is the recent emerging generation of MEMS with potential to revolutionize the miniaturization of bioassays [8, 9]. Droplet microfluidic devices use two phase system of water droplets formed in oil to reduce reaction volume, isolate and separate reaction, prevent absorption and evaporation, and provide temperature and gas permeability control [8, 10, 11]. Physical parameters of droplets: size, formation frequency, flow rat and mixing, are controlled by the microfluidic channel geometry, oil composition and fluids flow rates [8, 12–17]. Moreover, the flow of water droplets in the oil medium generates internal streamlines which cause to chaotic mixing, which presumably accelerates the reaction rates [18–21].

Droplet based MEMS have been successfully applied for digital polymerase chain reactions (PCR), synthetic biology and cell assaying [22]. In the digital PCR

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application, compartmentalization of reagents and effective mixing significantly, in addition to reagent absorption to channel walls of diffusion, increases amplification efficiency [23]. In synthetic biology, small volume of reaction and high-throughput of multiple individual reactions in droplet microfluidics enables fast screening of multiple engineered biological parts [22]. When assaying cells and microorganisms, droplets provide a closed controlled environment that prevents the diffusion of the molecules produced by cells; thus, enabling characterization of biochemical processing on a single-cell level [22]. Such a system was previously reported by our group for the detection of a single T cell secretion of IL-10 cytokine [24].

Droplet MEMS technology maturity, evident from advances in droplet generation, manipulation and control, provides an excellent basis for development of low cost, easy to use assays for POC diagnostics. Therefore, the goal of this work is to develop sensitive, specific, rapid and low cost droplet microfluidics based technology for bio-analyte detection and quantification with potential applications for the POC diagnostics. In our method, the biological analyte is captured by antibody-coated microspheres, and is detected with fluorescently labeled secondary antibodies in the picoliter droplet. Thus, the droplets function as high-density pico-volume immunoassay reactors. Such well-defined miniaturized reactors reduce the reagents volume by four orders of magnitude, prevent cross contamination and achieve fast mixing that enhances the binding reaction rates. To demonstrate the immediate potential of the developed platform, we choose to validate the technology for tetanus immunization diagnostics. Although vaccine preventable disease, tetanus still claims more than 300, 000 deaths each year, mostly in the developing countries [25]. We were able to detect anti-tetanus immunoglobulin G (IgG) in the human sera with sensitivity that promises a successful application of this platform for the POC diagnostics.

Experimental

Microfluidic device fabrication

Droplet microfluidic flow focusing devices were fabricated using soft lithography. Negative photo resist SU-8 2100 (MicroChem, Newton, MA, http://microchem.com/) was deposited onto clean silicon wafers to a thickness of 150 μm, and patterned by exposure to UV light through a transparency photomask (CAD/Art Services, Bandon, OR, http://www.outputcity.com). The Sylgard 184 poly(dimethylsiloxane) (PDMS) (Dow Corning, Midland, MI, http://www.dowcorning.com) was mixed with crosslinker (ratio 10:1), poured onto the photoresist patterns, degassed thoroughly and cured for 12 h at 75 °C. Next, we peeled the PDMS devices off the wafer and bonded them to the glass slides after oxygen-plasma activation of both surfaces. The microfluidic device was composed of two parts: a droplet forming nozzle (channel cross section $6.25\ 10^{-8}$ m^2, Fig. 1a section 1) and a 10^3 droplets storage array (channel cross section $3.13\ 10^{-7}$ m^2, Fig. 1a section 2). The multi droplet array used for this essay has an advantage as it provides simultaneous measurement of multiple reactions, thus it decreases the standard error of the mean. In the day of the experiment, the microfluidic channels were treated with PicoSur™ 2 (Dolomite Microfluidics, UK, http://www.dolomite-microfluidics.com/) by filling the channels with the solution as received and then flushing them with air. This treatment was done to improve the wetting of the channels with mineral oil in the presence (1 %, w/w) of the surfactant (span80). We used 1 mL syringes to load the fluids into the devices through Tygon Micro Bore PVC Tubing 100f, 0.010″ ID, 0.030″ OD, 0.010″ Wall (Small Parts Inc, FL, USA,). The individual syringe pumps (Areas *A,B,C* Fig. 1, Harvard Apparatus, USA, http://www.harvardapparatus.com) were used to control the flow rates of the oil and the reagents. The microspheres (0.5 mg mL^{-1}) conjugated with tetanus toxoid were injected through syringe *A*; the analyte and fluorescently labeled secondary antibodies were injected through syringe *C*. The oil phase was injected through syringe *B*. To form droplets, the flow-rate-ratio of water-to-oil was adjusted to $Q_w/Q_o=1$.

Microsphere sensor preparation

Clostridium difficile toxoid B protein (Abcam®, MA,USA, http://www.abcam.com/) was biotinylated with EZ-Link NHS-PEG4-biotin (Thermo Scientific, USA, http://www.thermoscientific.com) according to the manufacture protocol. ProActive® Streptavidin Coated Microspheres (10 μm, Bang Laboratories Inc., USA, http://www.bangslabs.com/) were conjugated with the biotinylated *Toxoid B protein* according to the manufacture protocol. Unbounded active sites were blocked with StarlingBlock™ (Thermo Scientific, USA, http://www.thermoscientific.com) for one hour. The microspheres were washed with the Phosphate Buffered Saline (PBS) (Sigma, USA, http://www.sigmaaldrich.com), diluted to the final concentration 0.5 mg mL^{-1}, and stored at 4 °C.

Tetanus IgG immunosorbent detection

A calibration curve, with four standard concentrations (0, 0.1, 0.5 and 1 IU mL^{-1}) available in ELISA tetanus commercial kit (Abcam®, MA, USA, http://www.abcam.com/), was constructed with our droplet microfluidics platform. For the human plasma experiments, a pool of EDTA plasma bank, created from discarded specimens from apparently 500 healthy men and women (kindly provided by Dr. Patrick

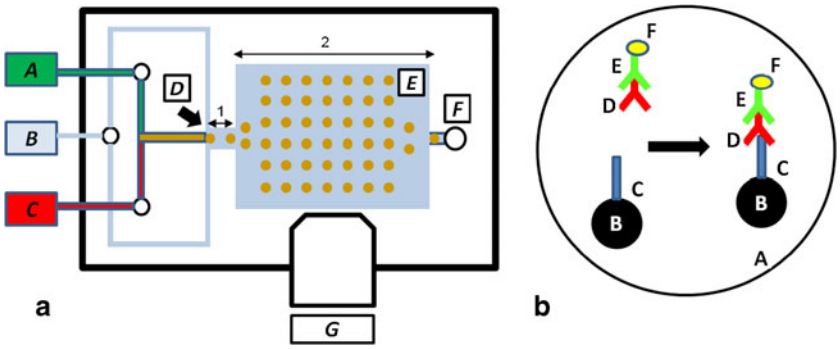

Fig. 1 Droplet microfluidics platform with encapsulated detecting microspheres. **a** Schematic view. *A*, *B* and *C* stand for the individual syringe pumps. *D* is the droplet forming nuzzle, *E* is the detecting array area, *G* is the microscope objective, *F* is the liquids outlet. **b** The developed assay components and immunosorbent reaction scheme. A is a solution with reagents, B is the detecting microsphere, C is tetanus toxoid, D is human anti-tetanus IgG analyte, E is secondary anti-human IgG antibody, and F is the fluorophore

Sluss from Massachusetts General Hospital), was used. In these experiments, the human plasma was diluted by 2 using dilution buffer provided by ELISA kit manufacture. In the first step, the analyzed IgG solution was mixed with IgG Goat Anti-Human pAb, Alexa Fluor®488 Conjugate secondary antibody (100:1 ratio) (Life Technologies, USA, http://www.lifetechnologies.com) and constantly mixed at the rotating disk for 15 min. In the second step, we co-encapsulated two solutions within the aqueous droplet using the platform previously described. First contained toxoid-conjugated microspheres at 0.5 mg mL^{-1} (syringe *A*, Fig. 1a), and second solution contained anti-tetenus IgG conjugated with IgG Goat Anti-Human pAb, Alexa Fluor®488 (syringe B, Fig. 1a). The flow rates were 20 μL min^{-1} for oil/surfactant mixture and 10 μL min^{-1} for the assay solution. When the array was filled with droplets with encapsulated reagents, the flow was stopped. Each droplet had a size of about 100 μm and traveled 2–5 mm on a chip to the specific location in the 1,000 well array. We detected the florescence signal from the microspheres up to 30 min post encapsulation.

Image analyses

The detection antibodies, present in droplet, were localized on microsphere captured analyte surface and generated a localized fluorescent detection signal. Fluorescence images were captured on a Zeiss 200 Axiovert microscope using a AxioCAM MRm digital camera and AxioVision 4.8 software. AlexaFluor488^{R} fluorescence (excitation 488 nm/emission 525 nm) was detected to evaluate the reagent concentration. To calculate the positive detection signal, we calculated the signal to noise (*S/N*) ratio as follows:

$$S/N = I/I_0 \tag{1}$$

where I is the average measured intensity from the microsphere center and I_0 is the average intensity from the background inside the droplet. We used ImageJ software for image processing and Microsoft Office Excel 2010 external statistical package for statistical analyses.

Statistics

Statistical analysis was performed using MATLAB Statistics toolbox (Mathworks, MA, USA, www.mathworks.com/).

Results and discussion

Microsphere assay for anti-tetanus IgG detection

Figure 1b illustrates the anti-tetanus IgG immunosorbent reaction in the droplet. The basic components for the detection of anti-tetanus IgG in the developed assay are the detecting surface (B), tetanus toxoid (C), human anti-tetanus IgG analyte (D) and secondary anti-human IgG antibody (E) with the conjugated fluorophore (F). In this assay, the tetanus toxoid was conjugated to the microsphere (B-C complex), and fluorophore labeled antibody was conjugated to the analyte (D-E-F complex) before the surface absorbance reaction. Therefore, the final absorption/dissociation reaction in this assay was:

$$B - C + D - E - F \underset{k_{off}}{\overset{k_{on}}{\Leftrightarrow}} B - C - D - E - F \tag{2}$$

where the B-C-D-E-F complex concentration was proportional to the signal emitted by the fluorophore molecule. k_{on} is the absorption kinetic constant and k_{off} is the dissociation kinetic constant

Droplet microfluidic device for bio-analyte assaying

In our platform, the reaction reagents were co-encapsulated in the droplet with the detecting microsphere in the droplet generation nuzzle as shown in Fig. 2a. Figure 2b shows the droplet with encapsulated biosensor inside the incubation array. In this study, we generated droplets with volume of ~520 pL,

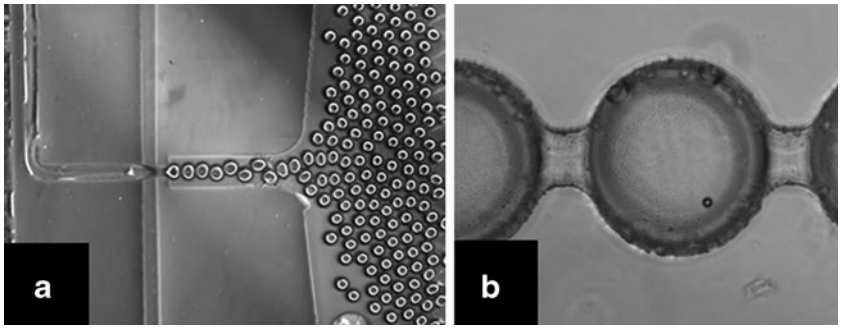

Fig. 2 Experimental droplet microfluidic system with detecting microspheres. **a** Droplet generation (2.5× magnification). **b** A single 520 pL droplet inside the array (10× magnification)

corresponding to a spherical drop diameter of 100 μm. In comparison, standard ELISA kits for anti-tetanus antibodies detection use 100 μL of analyte and reagents per each reaction well. An additional advantage of droplet assay is the small volume of individual reactions that reduces the reagents consumption from 10 μL in the standard ELISA kits to ~520 pL in the individual droplet—a four orders of magnitude reduction in reagents needed for a single reaction.

Fast reagent mixing inside droplets, suggested in the previous works [18–21], is visualized on Fig. 3. A stream of fluorescent reagents (anti-tetenus IgG conjugated with IgG Goat Anti-Human pAb, Alexa Fluor®488), indicated by the white arrows on Fig. 3a, b and c, is rapidly mixed with the stream of non-florescent reagents (toxoid-conjugated microspheres), indicated by the yellow arrows on Fig. 3a, b and c. The resulting droplets contain the mixed solutions, Fig. 3c, d. This rapid mixing is important as it can potentially explain the increase in reaction kinetics rates previously observed in biochemical reactions in the flowing droplets [18–21].

Immunosorbent assay for anti-tetanus IgG detection in droplet microfluidics format

Figure 4a, b and c show the images of the fluorescent signal on microsphere; the background is the unbounded fluorescently tagged secondary anti-human IgG antibody. The calibration curve of the droplet based immunosorbent assay appears in Fig. 4d. For each calibration point, we analyzed at least 40 individual droplets with detecting microspheres. We were able to detect 0.1 IU mL^{-1} of human anti-tetanus IgG using picoliter droplet microsphere system; thus, we demonstrated the required sensitivity shown by ELISA for detecting anti-tetanus IgG in human sera [26]. Furthermore, using mean square error method (R^2=0.99) we calculated the equation that describes *S/N* ratio at steady-state on a

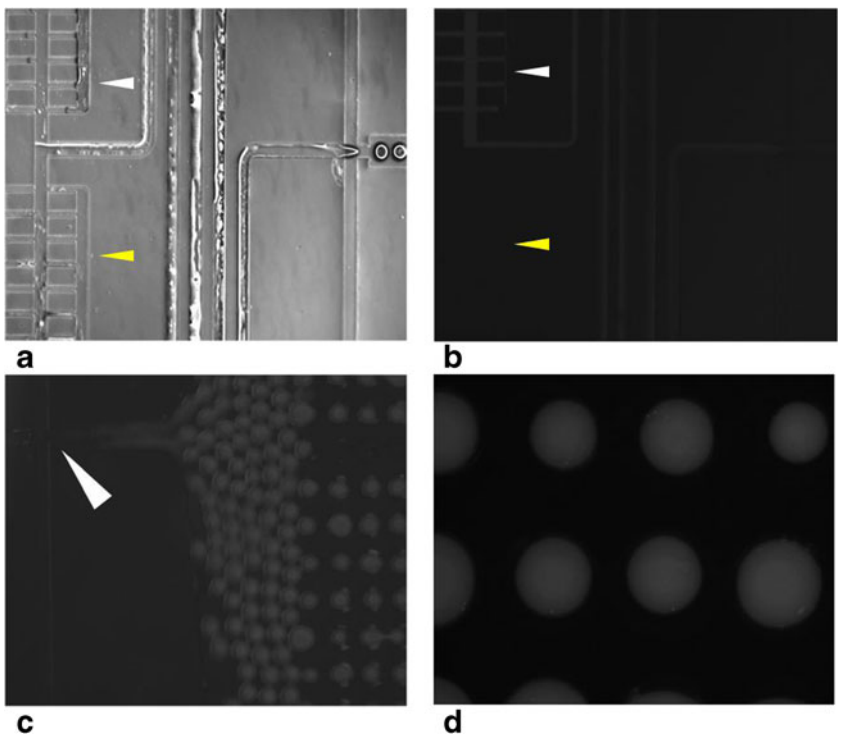

Fig. 3 Demonstration of reagents mixing on the chip. **a** Phase microscopy of a chip area. **b** Fluorescent microscopy of a chip area where tetanus toxoid conjugated microspheres (*yellow arrow*) and analyte and secondary Alexa Fluor®488 fluorescent labeled antibody (*white arrow*) are mixed (2.5× magnification). **c** Fluorescent microscopy of droplet generation (*white arrow* indicates the nuzzle for droplet generation) with reagents mixture (2.5× magnification). **d** Fluorescent microscopy image of droplet array with the mixed reagents and microspheres. *White arrow* indicates microspheres after immunosorbent reaction (10× magnification)

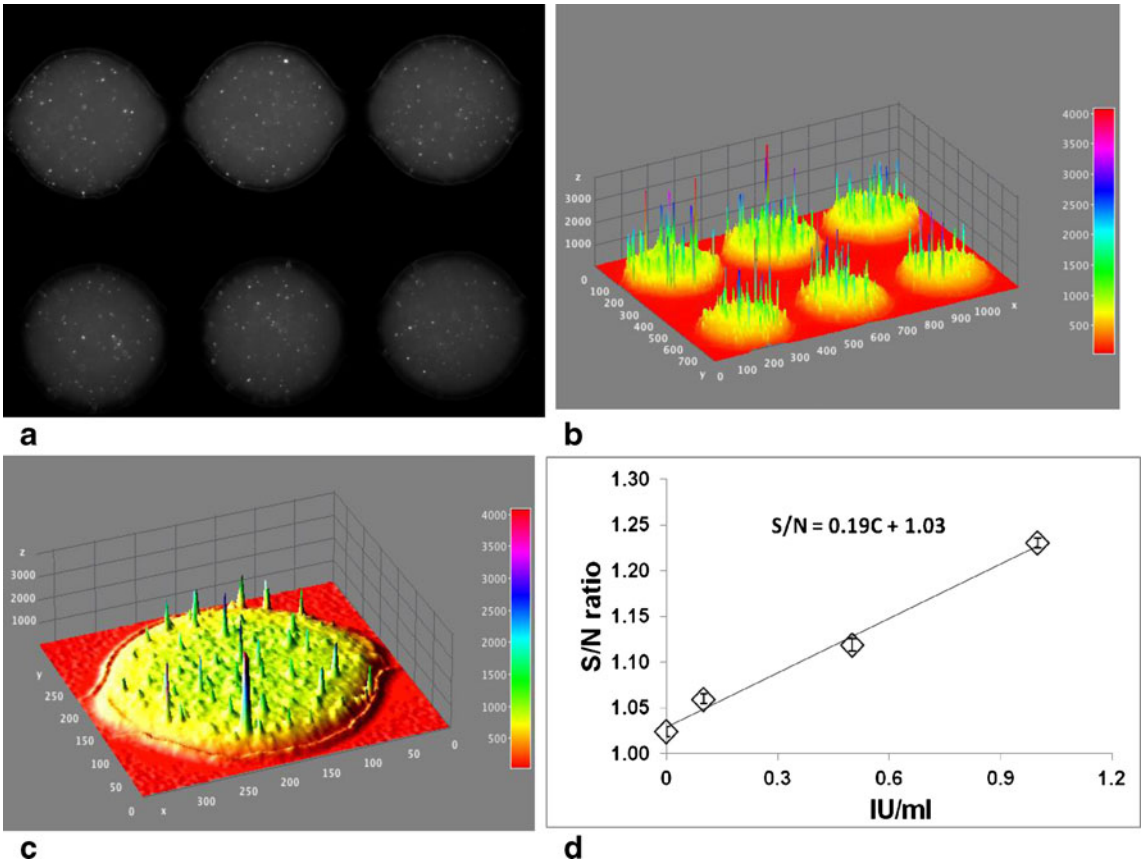

Fig. 4 Human anti-tetanus IgG detection with microspheres co-encapsulated in droplets. **a** Fluorescent microscopy detecting emission signal from microspheres, 15 min after the encapsulation (10× magnification). **b** Fluorescent emission signal analyses from droplet array. **c** Fluorescent emission signal analyses from a single droplet. **d** Calibration curve for human anti-tetanus IgG detection. Error bars show ± one standard deviation of the mean

detecting microsphere as a function of human anti-tetanus IgG concentration:

$$S/N = 0.19C + 1.03 \quad (3)$$

where C is the anti-tetanus IgG concentration in the sample.

We also validated the droplet detection platform using plasma sample collected from 500 adult immunized individuals. Using *qqplot* (Fig. 5), we showed that our measurements behave accordingly to Normal distribution with the following descriptive parameters: the mean (1.14), Std (0.03), coefficient of variance (0.03) and Confidence intervals for 5 % (1.0792, 1.1925). Using Eq. 3 and multiplying the result by 2 (dilution factor of sera inside the droplet), we calculated that the average concentration of anti-tetanus antibodies was ~1.12 IU mL^{-1}, which is in the range of the previously reported concentration of anti tetanus IgG in human sera [26].

Although this study is a proof of concept, focused on a single bio-analyte detection, this method can be easily applied to detect multiple analytes in the same sample by adding microspheres that bind other bio-analyte targets and different fluorophore tagged secondary antibodies. It is our believe that in combination with low cost optics and mechanics [4, 27, 28] the proposed microfluidic platform, can further reduce the total cost of POC diagnostics. An addition of the washing steps in the future device design may further increase the sensitivity of the assay.

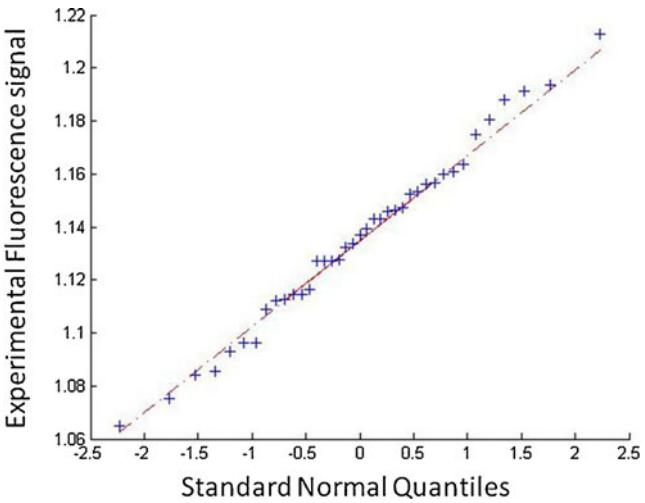

Fig. 5 Human sample florescent readout distribution. Testing the hypothesis that the measured values have a Normal Distribution profile. R=0.9924

Conclusions

We report on a droplet microfluidic-based and low volume microsphere-based immunosorbent assay with a sensitivity that is adequate to detect the anti-tetanus IgG in human serum samples, and which is six times faster than standard ELISA kits. Furthermore, our analysis showed that for the small volumes of droplets, we used four orders of magnitude less reagents volumes that currently used by available methods. This advantage leads to the lower cost of a single reaction that may play a significant role for the future application of this system for global POC diagnostics. Using different capturing proteins, conjugated to the detecting microsphere, this platform may be used for diagnostics of emerging infectious diseases, for example HIV, hepatitis B and C, syphilis, and dengue. Importantly, assays can be manufactured at the point-of -care site, using capturing antibodies specific for local diseases. We expect that this platform will pave the way to immunosorbent miniaturization and it represents a new tool for rapid and low cost point-of-care diagnostics.

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