ABSTRACT

Single-molecule biomarkers captured on beads are successfully isolated and detected within femtoliter water-in-oil microdroplet array on hydrophilic-in-hydrophobic micropatterned surface in a flow cell. Enclosure of single bead into each droplet was efficiently achieved by sequentially injecting aqueous solution with beads and oil using micropipette. The feasibility of our device is demonstrated by detecting streptavidin-biotin interaction of which limit of detection was 78 zM. Such simple and efficient approach promises the development of highly sensitive and portable Enzyme-linked immunosorbent assay device by direct coupling with CMOS image sensor of which each pixel has comparable dimension with individual microdroplet.

KEYWORDS: Single-molecule assay, Microdroplet array, Streptavidin-biotin interaction, Enzyme-linked immunosorbent assay

INTRODUCTION

Enzyme-linked immunosorbent assay (ELISA) is widely used for detecting biomarkers of disease and infection. However, conventional ELISA with the volume scale of microliters has limit of detection (LOD) about 1 pM of target, because reaction product generated by reporter enzyme are subject to dilution. We previously showed that reaction of single enzyme can be easily measured by reducing the reaction volume down to femtoliter level [1]. Our strategy was recently applied to “digital ELISA” by Rissin et al., who enclosed a bead capturing single biomarker and enzyme in a femtoliter well [2]. However, in their approach relatively complex equipment is required for bead trapping and well sealing. Here, we propose a Lab-on-a-chip device realizing digital ELISA with simpler procedure. To achieve this, highly efficient bead trapping function was integrated into the surface-immobilized droplet array formed on hydrophilic-in-hydrophobic micropatterned surface [3]. With the device, we achieved LOD of 78 zM for streptavidin-biotin interaction.

PRINCIPLE OF ASSAY

Our approach utilizes more than 10^6 surface-immobilized femtoliter microdroplets to detect target biomarkers at the single-molecule level. Figure 1 shows the concept of the approach. In the first step, the target biomarkers are captured on beads with an enzymatic reporter in a test tube. When the samples contain extremely low concentrations of biomarkers, the beads carry either a single biomarkers or none according to the Poisson distribution. Then, each bead is effectively isolated within the femtoliter microdroplets arrayed on the surface of the device. Since fluorescent product molecules generated by the reporter enzyme are accumulated into the microdroplet, we can estimate the number of beads bound with single biomarkers by counting the droplets emitting fluorescence signal. The concentration of the biomarker in the assay sample is obtained from the ratio between the number of total trapped beads and that of beads with biomarkers.

EXPERIMENTAL

A hydrophobic polymer of carbon-fluorine (CYTOP, Asahi-glass) was spin-coated on a cleaned coverglass (Matsunami) at 2000 rpm for 60 s and then baked for 1 h at 180°C. This process was repeated 4 times to get appropriate thickness of the CYTOP layer (3 μm). Photolithography was carried out with a positive photoresist (AZP4903, AZ Electronic Materials). The resist-patterned substrate surface was dry-etched with O2 plasma by a reactive ion etching system (RIE-10NR, Samco) to expose a hydrophilic SiO2 glass surface. Figure 2 shows an assembled device which has more than 10^6 microwells (diameter: 5 μm, depth: 3 μm) in ~100 mm^2 area in a flow cell (wide: 15 mm, height: ~40 μm), which allows effective trapping of single
beads (diameter: 3 μm). For the detection of streptavidin-biotin interaction, amino-modified polystyrene beads (Micromod) were biotinylated by NHS-PEG-Biotin (Thermo scientific).

Our device ensured efficient preparation of large number of femtoliter water-in-oil microdroplet array containing single beads, by sequentially injecting aqueous solution with beads and oil using micropipette. Figure 3a shows schematic images of beads trapping and droplet formation process. Aqueous solution with beads was injected into the access port with a micropipette. Then injected beads were settled by gravity for 5 min and swept into microwells by injecting oil (Fluorinert FC40, Sigma) of which high density (1.85 g/mL) allowed efficient removal of the aqueous solution from the hydrophobic surface (CYTOP). On the other hand, the hydrophilic surfaces (SiO2) at the bottom of microwells retained the aqueous solution with trapped beads. The present setup showed that 61% of droplets contained single bead and 8.4% of introduced beads were trapped into the droplets.

RESULTS AND DISCUSSION

To demonstrate the feasibility of our device, LOD of streptavidin-biotin interaction was determined. Biotinylated beads (8×10⁶ particles) was mixed with various concentrations (0.1–1000 aM) of streptavidin-β-galactosidase (SβG) to capture on beads. Final sample volume was 1 mL, and 0.1 to 1000 aM corresponded to 60 to 6×10⁵ SβG molecules, which predicted that 0.0008% to 8% of beads carry the SβG molecule. After reaction, washed beads were injected into the device with a fluorogenic substrate, fluorescein-di-β-D-galactopyranoside, and then oil (Fluorinert FC40) was injected. After incubation for 30 min at room temperature, bright-field and fluorescence images were obtained to count total number of trapped beads without/with SβG (Figure 4). Fraction of SβG-beads proportionally changed with the SβG concentration and matched quite well with predicted value over the dynamic range of five digits (Figure 5). LOD of SβG determined from the nonspecific binding to non-biotinylated bead was calculated to be 78 zM and better than that (220 zM) of previous method [2].

CONCLUSION

In our device, LOD can be further improved by increasing number of microdroplet in an array and by suppressing nonspecific binding of target molecule to the bead. Furthermore, no necessity of a pump or manipulator for operation is prominent merit which allows development of a highly integrated device. Especially, our approach promises the development of portable digital ELISA device by direct coupling with CMOS image sensor of which each pixel has comparable dimension with individual microdroplet.
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Figure 4. Digital detection of streptavidin-biotin interaction. Bright-field (top) and fluorescence (bottom) images of droplet array. Certain number of droplets contains a bead bound with single-molecule streptavidin-β-galactosidase (SβG) and emits fluorescence.

Figure 5. Dependence of fraction of beads bound with single streptavidin-β-galactosidase (SβG) in the total number of trapped beads on the SβG concentration in the sample. The limit of detection (LOD) was calculated to be 78 zM.