One-step Target Protein Detection from Whole Blood in a Lab-on-a-Disc

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ABSTRACT

We report a fully integrated, one-step target protein detection utilizing centrifugal microfluidics on a polymer based disc (Lab-on-a-Disc). The design principle is based on microbead-based immunoassay. Since microbeads with high surface-to-volume ratio (50 times higher than a microtiter well) are efficiently mixed in reaction chamber by clockwise and counter-clockwise rotation repeatedly, antibody-antigen reaction is facilitated. So, total reaction time is dramatically reduced from 90 min to 20 min and the final detection value is obtained within 38 min. In addition, the required volume of sample and reagents are reduced 2 ~ 5 times. As model studies, Hepatitis virus B surface antibody(Anti-HBs) detection from whole blood were conducted using Lab-on-a-Disc and compared to commercial ELISA kit. The dynamic range of our method is 6 times wider than the commercial ELISA with the same functional sensitivity (10 mIU/mL).

Keywords: Lab-on-a-Disc, immunoassay, microbead, Anti-HBs

1 INTRODUCTION

There have been significant advances in the lab-on-a-chip development for biomedical applications recently [1-4]. However, the practical applications in clinical diagnostics, such as immunoassay, require processing of complex fluids; e.g. whole blood, conjugate buffer, substrate etc. Due to the complex nature of the sample and many operational steps, most of the sample detection steps still rely on the time-consuming traditional bench top methods. As a result, development of rapid and efficient on-chip specific biomolecule detection, for example protein detection, in "real" sample analysis remains as a major bottleneck for the realization of the true lab-on-a-chip.

Many diagnostic tests and assays use micro-size uniform polystyrene bead, or microspheres, as substrates or supports for immunologically based reaction, such as ELISA [5-16]. One reason is that ELISA and other immunoassay techniques have been miniaturized onto microchip platforms and require very small sample volume. By using the microbead that has large surface-to-volume ratio, detection sensitivity and analysis time have been further improved [9]. In this report, the polystyrene (PS) microbead with the mean diameter of 117 μm is used and its surface is modified by carboxyl functional group (-COOH) to conjugate Hepatitis B surface antigen (HBsAg) covalently on the surface as shown in Fig. 1.

Recently, we reported a novel microvalve system, Laser Irradiated Ferrowax Microvalve (LIFM) [17,18]. Addition of 10 nm-size ferromagnetic particles to wax medium can make very fast and robust microvalves with response time of 12 msec by single laser irradiation (808 nm, 1.5W). Through the LIFMs, fully automated and more complex processing, such as, mixing, target binding, washing, separation, is possible without any external operation.

Here, we report a fully integrated and automated target specific protein detection device utilizing centrifugal microfluidics on a polymer based disc. The design principle

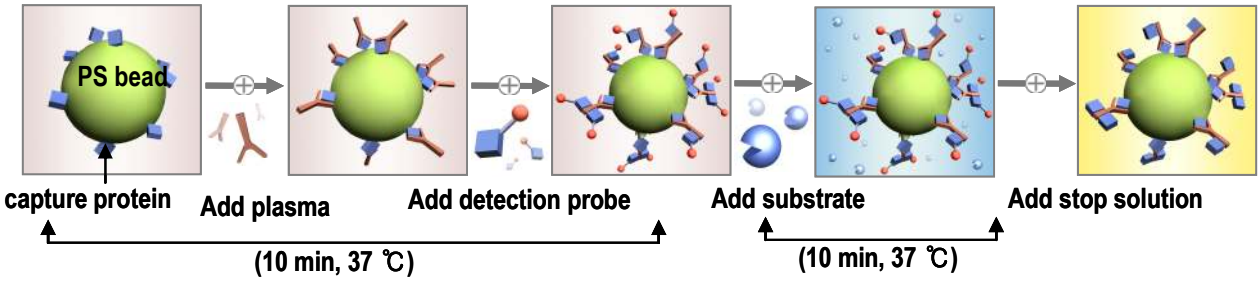

Figure 1. Schematic diagram of reaction principle. Carboxyl modified polystyrene bead (diameter 117 um) is covalently binded by HBsAg. When target sample (whole blood) with Anti-HBs and detection probe are loaded together, the Anti-HBs are captured on the bead, where the detection probes are subsequently binded on the bead. Free Anti-HBs and detection probes are washed out. After addition of substrate and stopping solution, the absorbance is measured at 450 nm and 630 nm as a reference.

is schematically shown in Fig. 1. As a model study, Hepatitis virus B surface antibody (Anti-HBs) detection from whole blood were conducted using Lab-on-a-Disc fully loaded with PS bead, detection probe, washing buffer, substrate, and stopping solution. Total process is finished within 38 min with only one manual input of 120 µl of whole blood.

2 MATERIALS AND METHODS

2.1 Protein Conjugation on Microbead

Our model is to detect Anti-HBs as a target in human whole blood. The first step is to conjugate target specific protein (HBsAg) on the surface of microbeads. As shown in Fig. 2, the microbead (BeadTech, Korea) is carboxylated polystyrene bead with the mean diameter of 117 µm with 1% divinyl benzene crosslinked and its carboxylation is 3.0 mmol/g. The most commonly used bead is sub-micrometer to a few micrometer because of its very high surface-to-volume ratio. However, we used relatively large microbeads for these reasons: (1) one is the confinement in the mixing chamber. Our microchannel has the depth of 100 µm. If the bead diameter is over 100 µm, the bead is easily confined in the mixing chamber. (2) another is easy mixing. Our relatively big bead has heavier than the small bead. Thus, the beads in the mixing chamber are easily mixed by slight vibration. This mixing makes effective binding between the beads and targets. Further more, after washing, the beads are easily settled down and separated from the residue with the low centrifugal force.

Covalent conjugation was employed for the immobilization of HBsAg on the surface of the bead. By means of the covalent conjugation, higher activity and stability are obtained rather than physisorption [13]. HBsAg (GreenCross Co., Korea) as a capture protein is recombinant IgG molecule with a molecular weight of 140 kDa.

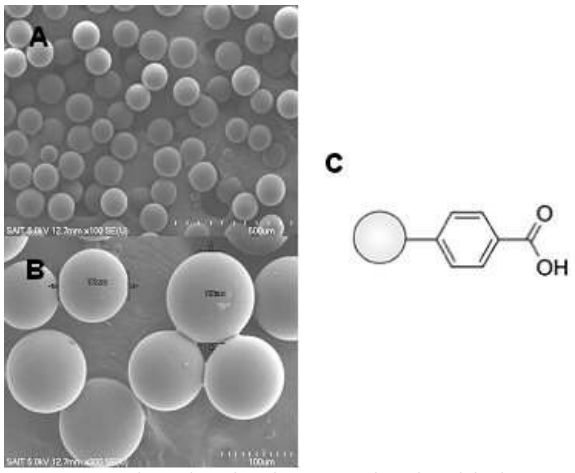

Figure 2. Carboxylated polystyrene beads with the mean diameter of 117 µm measured by HELOS particle size analyzer. The bead is crosslinked by 1% divinyl benzene and its carboxylation is 3.0 mmol/g. (A) SEM image. Scale bar, 500 µm. (B) SEM image. Scale bar, 100 µm. (C) Molecular structure of microbead.

Covalent conjugation protocols have historically focused on the surface capacity of protein being used, for example, the surface saturation capacity of polystyrene for bovine IgG was calculated at ~2.5 mg/m^2 [14]. The specific surface area to mass ratio for a sphere can be calculated: A/M (m^2/g) = 6/ρD, where ρ is density in g/mL and D is diameter in µm. For polystyrene bead, where ρ = 1.05 g/mL, A/M = 5.7/D. Thus, if D = 117 µm, then A/M = 0.049 m^2/g. Therefore, the maximum binding amount of HBsAg is 0.1218 mg HBsAg/g bead. Usually, the protein concentration should be substantially higher than the calculated value up to 10X. Our optimal concentration is 10 mg HBsAg/g bead (8X).

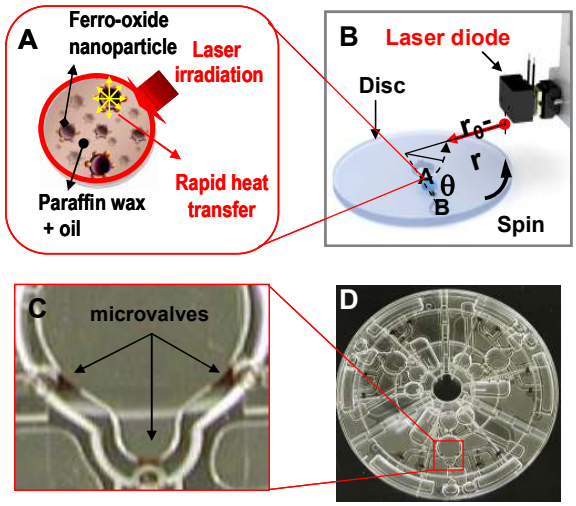

Figure 3. Brand-new microvalves in Lab-on-a-Disc. (A)~(B) Working principle and composition of microvalve. (C) 3 real fabricated microvalve with the dimension of 1 x 0.5 x 0.1 mm. (D) 42 Microvalves are integrated for 3 sample tests in one disc.

2.2 Lab-on-a-Disc Fabrication

One of the main components in our Lab-on-a-Disc is the ferrowax microvalve. The key concept of the microvalve operation mechanism is to use metal nanoparticles as nanoheaters and heat by laser irradiation in order to obtain rapid melting of paraffin wax (Fig. 3A). Once if it is implemented in a Lab-on-a-Disc, it is easily controlled by laser beam (808 nm, 1.5W) in a polar coordinate. For example, in order to transfer 100 µL of solution in reservoir A to reservoir B, as soon as the ferrowax valve is melted by irradiation of laser for 1 sec, the disc is spinning up to 30 Hz (revolution/sec) with an acceleration of 30 Hzsec^{-1}. These basic valve operation steps are repeated many times to control multiple valves on the disc (Fig. 3B). In addition, because the dimension of the microvalve is very small, multiple microvalves

can be implemented together in one disc. As a model study, our disc with the diameter of 120 mm has 42 microvalves (Fig. 3C and 3D).

3 RESULTS AND DISCUSSION

Microfluidic layout in Fig. 4 is designed to detect a target protein by means of one step ELISA method as shown in Fig. 1. "One step" means binding reaction among capture protein, target protein, and detection protein occurs simultaneously. Thus, capture protein conjugated beads and detection protein can be loaded together in the reaction chamber. The reaction chamber has a design for bead volume and bead separation. Our bead surface area is 3 times more than that in common ELISA kit. The corresponding bead bed volume is 25 µl that is equal to the volume of the lower region of the reaction chamber. When the beads are settled down to remove reagent residue, the beads are packed in the lower region of the reaction chamber and then, the reagent residue is removed easily through the side microchannels.

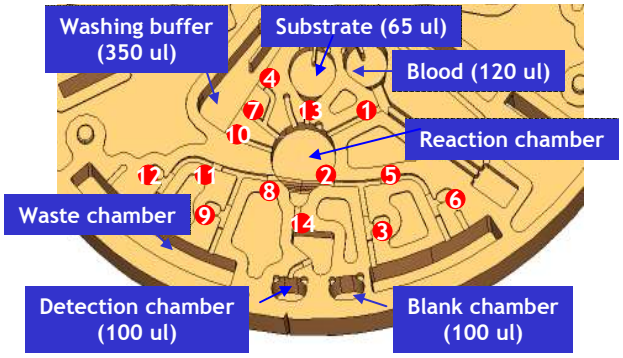

Figure 4. Microfluidic layout and functions in one third of Lab-on-a-Disc. The number in red filled circle shows the position of microvalve. The numbers of 3, 6, 9, 12 are normally opened microvalves and the other numbers are normally close microvalves.

Total process is summarized as follows: After plasma separation in the blood chamber at high rotational speed of 60 Hz, plasma is transferred into the reaction chamber. Then binding reaction proceeds during 10 min (60 min in common ELISA) while continuous mixing occurs inside the reaction chamber by clockwise and counter-clockwise rotation repeatedly.

Mixed residue and washing residue are removed by microchannels connected by the reaction chamber and the two waste chambers. To remove the mixed residue, the normally-closed microvalve (2nd in Fig. 4) is opened by laser irradiation and the residue is transferred into the waste chamber by centrifugal force (40 Hz). After then, the normally-opened valve (3rd in Fig. 4) is closed to enclose the reaction chamber. In the same manner, washing steps (3 times) are done sequentially. During each washing step inside the reaction chamber, efficient mixing is done to remove unbounded proteins from the beads rapidly.

After washing, substrate (TMB) is transferred to the reaction chamber and enzyme (HRP) reaction proceeds for 10 min (30 min in common ELISA). Finally, the final product, whose color is blue, is transferred to the detection chamber. The enzyme reaction is terminated by the stopping solution previously loaded in the detection chamber.

Table 1. Protocol comparision between our method(Immuno LOD) and common ELISA(Genedia Anti-HBs ELISA 3.0, Korea). Required volume of target(plasma) and reagents are 2 ~ 5 times less than the common ELISA. Total reaction time is 20 min (ELISA 90 min) due to highly efficient mixing and large reactive surface area.

Assay Protocol		Immuno LOD	Common ELISA
Surface	Type	**PS bead**	**Well plate**
	Area	3X	1X
Capture protein	Binding	**Covalent**	**Adsorption**
Target (plasma)	Volume (µl)	**50**	**100**
Detection protein	Volume (µl)	25	25
Incubation	Time (min)	**10**	**60**
Washing	Volume (µl)	350	1750
	Times	3X	5X
Substrate	Volume (µl)	65	100
	Time (min)	**10**	**30**
Stopping	Volume (µl)	55	100

Hepatitis virus B surface antibody (Anti-HBs) is one the most frequent test item as a clinical blood analysis after Anti-Hepatitis inoculation, or, before surgery, or at health inspection. So, we chose Anti-HBs as a study model.

Immunoassay protocol for detection of Anti-HBs on Lab-on-a-Disc is optimized for a fully automated analyzer. Table 1 shows the difference between our Lab-on-a-Disc and common ELISA kit. As mentioned earlier, the conjugation method of capture protein (HBsAg) on bead surface in our study is covalent coupling, but adsorption in the common ELISA kit. First of all, reaction time is dramatically reduced from 90 min to 20 min except others' extra operations, such as plasma separation, washing, detection, and valve operation. It is mainly due to highly efficient mixing based on the microbead reaction system. If we apply centrifugal force both in clockwise and counter clockwise direction repeatedly, the microbeads are instantaneously mixed within 1 sec as shown in Fig. 5. Centrifugal force induces large vorticity inside the chamber that makes fluidic rotation. Right at the change of the direction of disc rotation, inertial force still acting on the microbeads dominates. These two forces afford the efficient mixing of microbeads and solution inside the reaction chamber.

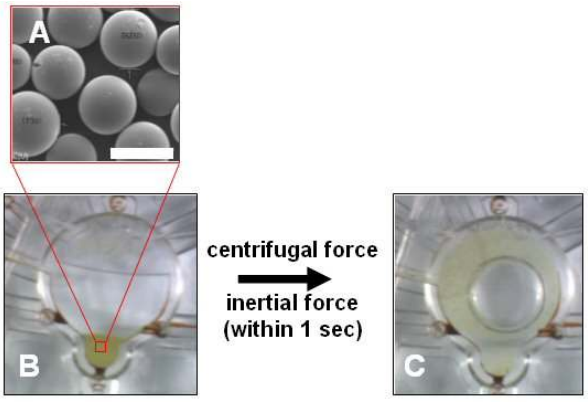

Figure 5. Highly efficient mixing between microbeads and reagents. (A) SEM image of polystyrene microbeads with average of diameter, 117 μm (scale bar 100 μm). (B) Separate state of microbeads. (C) Mixed state of microbeads.

Characterisitics	Immuno LOD	common ELISA
Dynamic Range	0 ~ 1000 mIU/mL	0 ~ 150 mIU/mL
Linearity (R^2)	0.995	0.998
Functional Sensitivity	10 mIU/mL	10 mIU/mL
Precision (CV%)	4.5%	3.3%

Table 2. Performance characteristics for Anti-HBs. Compared to the common ELISA(Genedia Anti-HBs ELISA 3.0, Korea), dynamic range is about 6 times wider with the same functional sensitiviy.

Furthermore, the amount of target sample in the Lab-on-a-Disc is half times less than in the common ELISA kit and other reagents' volume is reduced and optimized for the small volume of the chamber in the disc.

Table 2 shows the performance characteristics for Anti-HBs. Its dynamic range is 0 ~ 1000 mIU/ml with the linearity of R^2 = 0.995, which is 6 times wider than the common ELISA kit. This dynamic range is equivalent to commercial large blood analyzer, such as, 0 ~ 1000 mIU/ml for AxSYM AUSAB (Abbott), 2 ~ 1000 mIU/ml for Elecsys Anti-HBs (Roche), and 1 ~ 1000 mIU/ml for ADVIA Centaur Anti-HBs (Bayer) [19]. The functional sensitivity is 10 mIU/mL (0.69 ng/ml), which is the lower limit of a positive value of Anti-HBs.

4 CONCLUSION

With microbead-based immunoassay on Lab-on-a-Disc, a rapid and sensitive assay of a protein could be realized. By using the fully integrated Lab-on-a-Disc, laborious tasks were eliminated, and assay time was remarkably reduced from 3 hr to 38 min. Additionally, no special skills are needed for the assay.

Even though Anti-HBs detection was demonstrated here, other proteins, such as HBsAg, Anti-HCV etc, can be applied easily to the same Lab-on-a-Disc. It means any other proteins are applicable to the Lab-on-a-Disc if their assay protocols are based on one step ELISA.

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