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Design of a Compact Disk-like Microfluidic Platform for Enzyme-Linked Immunosorbent Assay

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This paper presents an integrated microfluidic device on a compact disk (CD) that performs an enzyme-linked immunosorbent assay (ELISA) for rat IgG from a hybridoma cell culture. Centrifugal and capillary forces were used to control the flow sequence of different solutions involved in the ELISA process. The microfluidic device was fabricated on a plastic CD. Each step of the ELISA process was carried out automatically by controlling the rotation speed of the CD. The work on analysis of rat IgG from hybridoma culture showed that the microchip-based ELISA has the same detection range as the conventional method on the 96-well microtiter plate but has advantages such as less reagent consumption and shorter assay time over the conventional method.

The use and development of immunoassay technology have drawn a great deal of interest because of the high selectivity and sensitivity.[1] Enzyme-linked immunosorbent assay (ELISA), typically carried out in a 96-well microtiter plate, is the most commonly used method among various immunoassays. It has been widely used for detection and quantification of biological agents (mainly proteins and polypeptides) in the biotechnology industry and is becoming increasingly important in clinical, food safety, and environmental applications. However, conventional ELISA involves a tedious and labor-intensive protocol that often results in large errors and inconsistent results. The process requires a series of mixing (reaction/incubation) and washing steps. It often takes many hours to 2 days to perform one assay due to the long incubation times during each step. These long incubation times are mostly attributed to inefficient mass transport of the antigen/antibody from the solution to the surface, whereas the immunoreaction itself is a rapid process.[2]

Microfabrication technology enables the integration of a number of fluid-handling components within a single device by using a network of microchannels. Due to their small scale, microdevices have advantages such as large specific volume, very low reagent consumption, and short diffusion length. Such microdevices can enhance the reaction efficiency, simplify procedures, reduce the assay time and sample or reagent consumptions, and provide highly portable systems.

Several developmental advances have been realized in miniaturization of capillary electrophoresis-based immunoassays[3−8] and microchip-based ELISA.[9−11] The capillary electrophoresis-based immunoassays utilized an electric field to separate the antigen−antibody complex from the rest of the molecules in the assay solutions. Reagent mixing and incubation, which dominates the total assay time, were carried out off-chip[6,7] and on-chip.[8] However, difficulties were encountered in the detection of proteins in the microchannel because of the small volume of the microchannel (usually in the nanoliter range) and the low concentration of molecules being detected in the assay solution.

ELISA, on the other hand, uses an enzymatic reaction to convert substrates into products having a detectable signal (e.g., fluorescence). Each enzyme in the conjugates can covert hundreds of substrates into products, thereby amplifying the detectable signal and enhancing the sensitivity of the assay. Several ELISA microchips are developed based on an immunoreaction on the surfaces of a single microchannel[9,10] or on microbeads that are trapped in the microchannel.[11] A sandwich immunoassay of D-Dimer in plasma was carried out in a disposable plastic microchip. The time required for assay was only 5−15 min,[9] whereas a few hours are usually required for a similar ELISA. Sato and co-workers[11] integrated an immunosorbent assay system into a glass microchip for immunoassays of human secetory immunoglobulin A and determination of carcinoembryonic antigen for cancer diagnosis. The integration reduced the time necessary for the antigen−antibody reaction and shortened the overall analysis time from 45 h to 35 min. These devices take advantage of the high surface-to-volume ratio of the microchannel for fast immunoreaction and short length for fast diffusion. However, each step of the ELISA was still carried out manually.

This paper presents an integrated microfluidic device on a compact disk (CD) to perform ELISA for rat IgG from hybridoma

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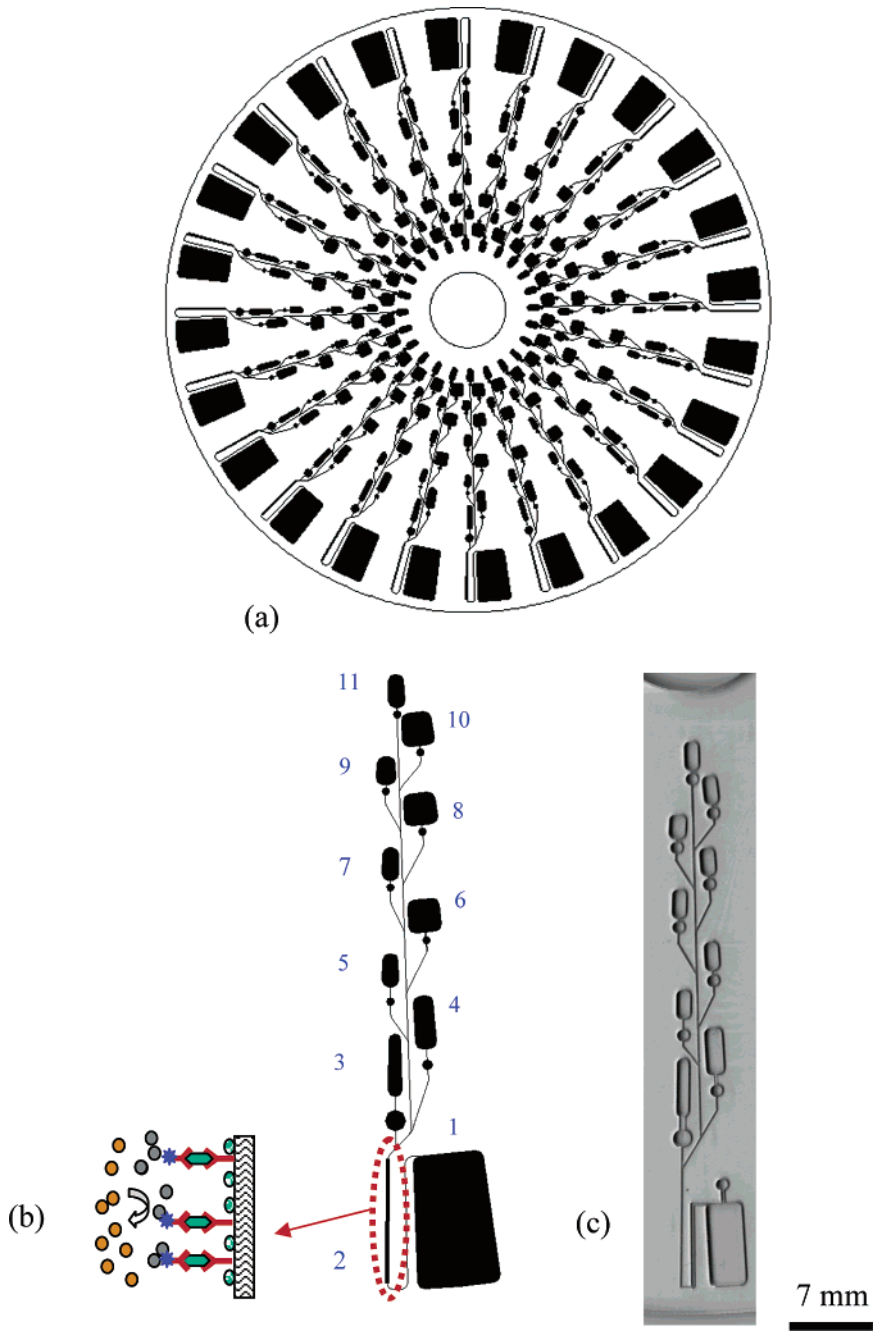

Figure 1. Schematics of (a) a CD-ELISA design with 24 sets of assays, (b) a single assay, (1, waste; 2, detection; 3, first antibody; 4, 6, 8, 10, washing; 5, blocking protein; 7, antigen/sample; 9, second antibody; and 11, substrate), and (c) photo of a single assay.

cell culture. The microfluidic platform combined several microfluidic functions (e.g., capillary valving, centrifugal pumping, and flow sequencing). Centrifugal and capillary forces were used to control the flow sequence of different solutions involved in the ELISA process. Each step of the ELISA process was carried out automatically by controlling the rotation speed of the CD.

EXPERIMENTAL SECTION

Microchip Design. The design of a CD-ELISA with 24 sets of ELISA microdevices on a 12.7-cm disk is shown in Figure 1a. The schematic of a single assay is explained in Figure 1b, while an actual assay on a plastic CD is shown in Figure 1c. The substrate, conjugate, washing, primary antibody, blocking protein, and antigen solution can be preloaded into corresponding reservoirs before the test. The centrifugal and capillary forces are used to control the flow sequence of different solutions involved in the ELISA process. The basic principles of the centrifugal pumping and capillary valving[12] are briefly described below.

In the CD microfluidic platform, the centrifugal force provides the pumping pressure. The pumping force per unit area (P_C) due to the centrifugal force is given by

$$dP_C/dr = \rho\omega^2 r \quad (1)$$

where ρ is the density of the liquid, ω is the angular velocity of

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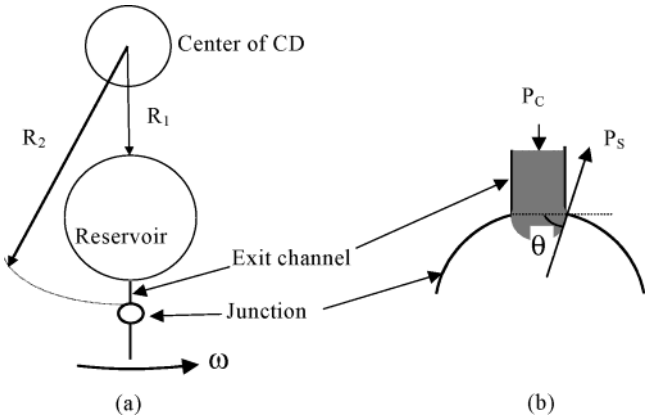

Figure 2. (a) Schematic illustration of fluid propulsion and (b) close-up view of the liquid front at the junction where the liquid is held by capillary force.

the CD platform, and r is the distance of a liquid element from the center of the CD.

Integration of eq 1 from $r = R_1$ to $r = R_2$ gives

$$\Delta P_C = \rho\omega^2(R_2 - R_1)((R_1 + R_2)/2) = \rho\omega^2\Delta R\bar{R} \qquad (2)$$

where $\bar{R}$ is equal to $(R_1 + R_2)/2$ and R_1 and R_2 are the two distances of the liquid elements from the center of the CD as shown in Figure 2a.

It is very important for a CD microfluidic platform to deliver the solution from each reservoir in a prespecified manner. The delivery of solution from a single reservoir allows the measuring reservoir to be filled without releasing solutions in other reservoirs. Capillary burst valves were incorporated into the microfluidic platform design for this purpose. When the fluid reaches the junction through the microchannel, the capillary force at the end of the microchannel due to a change in geometry tends to hold the fluid as illustrated in Figure 2b. The capillary force per unit area (P_s) due to surface tension is given by

$$\Delta P_s = (C\gamma \sin\theta)/A \qquad (3)$$

where γ is the surface tension of the fluid, θ is the contact angle, A is the cross-section area of the microchannel, and C is the associated contact line length.

The burst frequency is defined as the angular frequency at which ΔP_C is greater than or equal to ΔP_s. At this rotation speed, the liquid overcomes the pressure generated by the capillary force ΔP_s and flows through the capillary valve, releasing liquid from the reservoir. The burst frequency, f_b, calculated from eqs 2 and 3 is given by

$$f_b = \left(\frac{\gamma \sin\theta}{\pi^2\rho\Delta R\bar{R}d_H}\right)^{1/2} \qquad (4)$$

where d_H (equal to $4A/C$) is the hydrodynamic diameter of the channel connected to the junction. The capillary burst valve is a passive valve that requires no moving parts. It is controlled by the angular speed of rotation, fluid density, surface tension, and geometry and location of the channels and reservoirs.

The flow sequence is designed in such a way that the antigen solution is released into the measurement site first at a low rotation speed. This action allows the first antibody to bind onto the microchannel surface. The solid surface at the measurement site needs to be modified so that it has a high protein affinity. After incubation, the washing solution is released to wash out the unbounded antibodies into the waste reservoir. Then the blocking protein, the washing solution, the antigen (sample or standard), the washing solution, the conjugate solution, the washing solution, and finally the enzyme substrate are delivered to the measurement site, one by one sequentially at increasing rotation speeds.

Microdevice Fabrication. The designed microfluidic patterns were drawn using commercial AutoCAD software (AutoCAD 2000, AutoDesk, Inc). Channels and reservoirs (with depths ranging from 60 to 800 μm and widths ranging from 127 to 762 μm) were generated on a poly(methyl methacrylate) plate (12 cm in diameter) by a computer numerically controlled (CNC) machine (Dynapath Delta CNC, Chevalier). The end mills (single-ended, two-flute subminiature end mills) were purchased from McMaster-Carr (Chicago, IL), and the diameter of the mills ranged from 127 to 762 μm.

CD Microfluidic Testing Setup. The same experimental setup described previously [12] was used for CD-ELISA testing.

Reagents. The first antibody (affinity purified antibody goat anti-rat IgG (H+L)), the antigen (rat IgG), and the second antibody (affinity purified antibody horseradish peroxidase labeled goat anti-rat IgG (H+L)) were purchased from Kirkegaard & Perry Laboratories (KPL, Gaithersburg, MD). Dulbecco's phosphate-buffered saline (without calcium chloride, without magnesium chloride) was purchased from Invitrogen Life Technologies (Carlsbad, CA). All other reagents were purchased from Sigma Aldrich. All reagents were used as received.

The buffer solutions, Tris assay buffer (TAB, 50 mM Tris, 0.5 M NaCl, 1% BSA, and 0.1% Tween 20, pH 7.5), Tris wash buffer (TWB, 20 mM Tris, 0.5 M NaCl, 0.1% Tween 20, pH 7.5), and Tris-HCl buffer solution (0.15 M, pH 8.5), were prepared in our laboratory. The substrate HPPA (3 mg/mL) was prepared in a 0.15 M Tris-HCl buffer solution (pH 8.5). One microliter of 30% hydrogen peroxide was added to every 7.5 mL of HPPA solution and mixed thoroughly immediately prior to use.

Procedures. For prototyping, a five-step flow sequencing CD (see Figure 3) was used. The first-antibody and the BSA blocking were carried out off-chip by applying the first antibody (2.5 μg/mL) to the detection reservoir (reservoir 2). The antibody was allowed to adsorb onto the surface of this reservoir. After a 30-min incubation, the excess antibody was removed by washing solution (TWB solution). The TAB solution was then added to block all excess protein-binding sites on the surface of the microchip.

After a 15-min incubation and washing off the excess protein, the antigen/sample, washing, second antibody, and substrate solutions were loaded into their corresponding reservoirs. The CD was mounted onto the motor plate. The rotation speed of the CD was set to 360 rpm (±15 rpm) to release the sample solution from reservoir 3 (containing to-be-assayed antigen) into reservoir 2 for the binding process of antigen−antibody. According to the literature, several minutes of incubation are sufficient to reach equilibrium of the immunoreaction in a microchannel having a

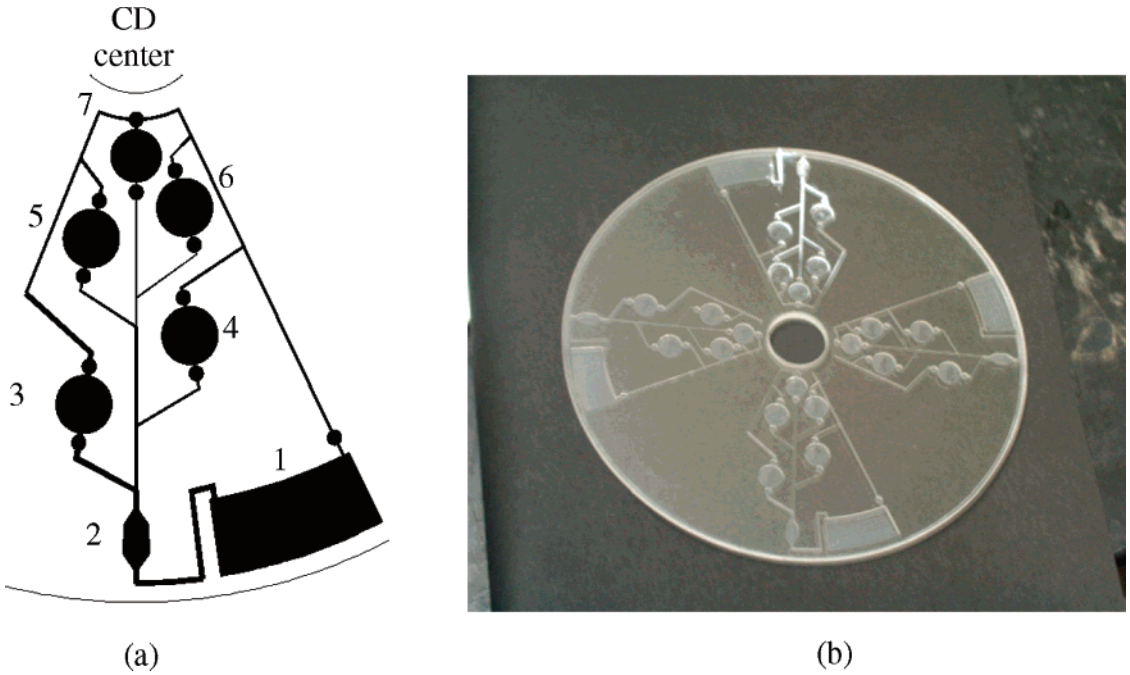

Figure 3. (a) Schematic of five-step flow sequencing CD (1, waste; 2, detection; 7, antigen/sample; 8, 9, washing; 10, second antibody; and 11, substrate) and (b) a CNC-machined CD.

dimension similar to that of reservoir 2.[2] To ensure the immunoreaction reached equilibrium, a 15-min incubation was chosen between two adjacent assay steps. After the incubation, reservoir 2 was washed with washing solution (from reservoir 4) at a rotation speed of 560 rpm (±30 rpm). Based on previous experience,[12] 3 times the volume of the washing solution is sufficient to displace the existing water-based solution in reservoir 2. The quantity of washing solution was therefore set at ~3 times the volume of reservoir 2 in the CD. The conjugate solution (in reservoir 5) was released into reservoir 2 at a rotation speed of 790 rpm (±35 rpm) to let the enzyme-labeled secondary antibody bond to the primary antibody. After incubation, reservoir 2 was washed with washing solution (in reservoir 6) at a rotation speed of 1190 rpm (±55 rpm). The substrate solution (in reservoir 7) was released at a rotation speed of 1280 rpm (±65 rpm) into reservoir 2.

Immediately after release of the substrate, the disk was stopped and detection was carried out using an inverted fluorescence microscope (Nikon Eclipse TE2000-U). A 100-W mercury light source with a 335/20-nm filter and a dichroic mirror was used as the excitation source. The fluorescence signal was obtained through a dichroic mirror and a 405/40-nm filter. Images were recorded with a 12-bit high-resolution monochrome digital camera system (CoolSnap HQ). The intensity of the fluorescence was analyzed using the Fryer Metamorph Image Analysis System.

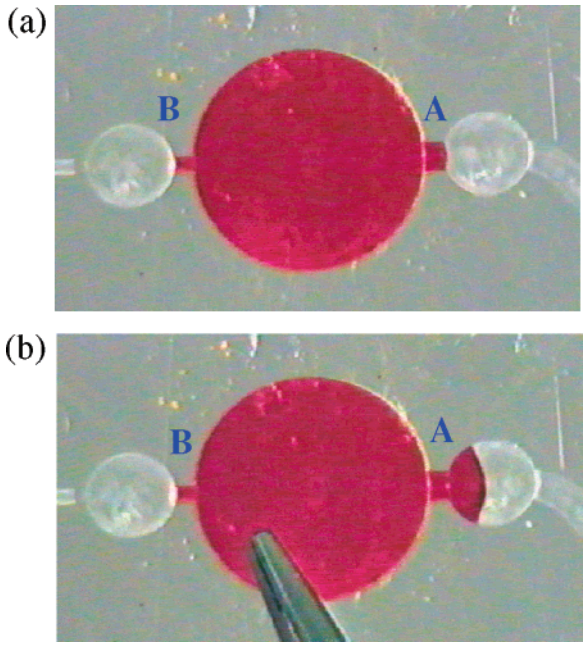

Figure 4. Capillary valving experiment. (a) Reservoir was filled with food dye and liquid was held by capillary valving at the end of channels A and B; (b) food dye was released through channel A but not through channel B. (Channel A was 508 μm wide and 508 μm deep; channel B was 127 μm wide and 63.5 μm deep.)

RESULTS AND DISCUSSION

Capillary Valve. A simple experiment was carried out to demonstrate the valving function by the capillary force as shown in Figure 4. In this experiment, a reservoir (2.62 mm in diameter and 610 μm in depth) was connected to a larger microchannel A (508 μm wide and 508 μm deep) on one side and a smaller microchannel B (127 μm wide and 63.5 μm deep) on the other side and sealed with adhesive tape. The reservoir was filled with food dye, and the fluid was held at the end of channels A and B by capillary force as shown in Figure 4a. By applying a gentle pressure by pressing the adhesive tape on the top, the food dye burst off the capillary valve at the end of channel A but not channel B (see Figure 5b). According to eq 2, the capillary force per area of channel B is ~6 times of that of channel A. Therefore, liquids burst through channel A, but not channel B.

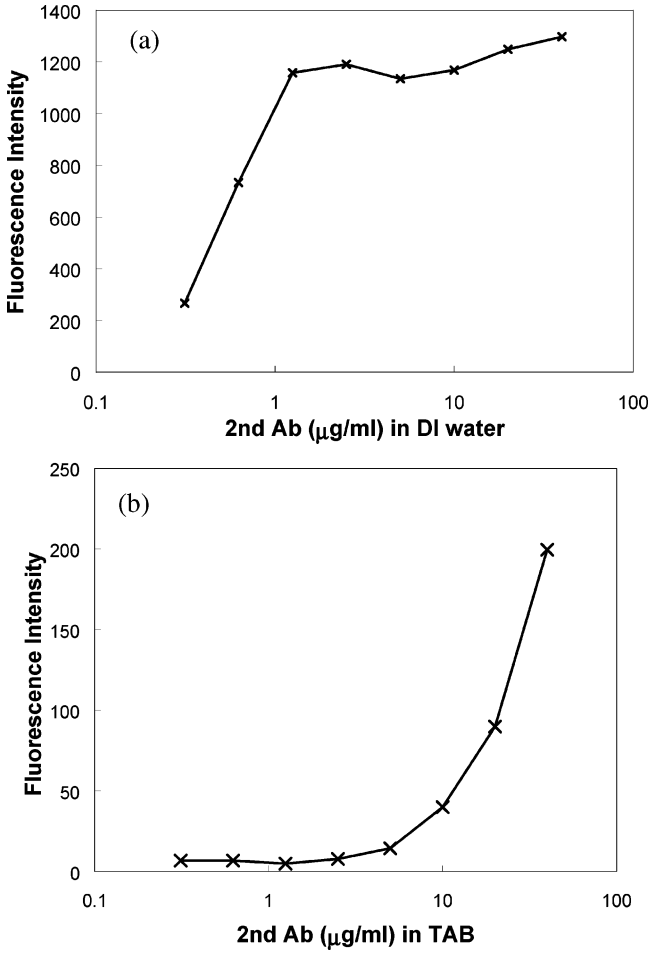

Figure 5. Effect of medium on the antibody adsorption on 96-well microtiter plate (HPPA, 3 mg/mL; enzymatic reaction time 30 min): (a) antibody diluted in DI water and (b) antibody diluted in TAB buffer.

Medium Effect. The effect of medium on antibody adsorption onto the solid surface was also examined. In this experiment, antibody adsorption was carried out by adsorbing the second antibody (diluted in the TAB and the DI water, respectively) onto the substrate surface in the 96-well microtiter plate. The substrate (HPPA, 3 mg/mL) was added after the incubation and washing. The fluorescence intensity of the enzymatic reaction product was used as an indicator of the amount of antibody adsorption. As shown in Figure 5a, for antibody diluted in DI water, the fluorescence intensity of the product increases with increase of antibody concentration in the low antibody concentration range (<2 μg/mL) but reaches a maximum at a higher antibody concentration (>2.5 μg/mL), indicating that antibody adsorption onto the polymer surface reaches equilibrium (or the surface is saturated). A further increase of the antibody concentration would waste the expensive reagent. For antibody diluted in TAB (see Figure 5b), the higher the concentration of the antibody, the stronger the florescence intensity of the product detected. It should also be noted that even at a very high antibody concentration (40 μg/mL), the fluorescence intensity is much weaker than the results from antibody diluted in DI water (Figure 5a). This implies that much less antibody in the TAB solution was adsorbed onto the solid surface than in the DI water. This is because the TAB solution contains 10 mg/mL BSA, which competes strongly with the antibody for the protein-binding sites on the solid surface.

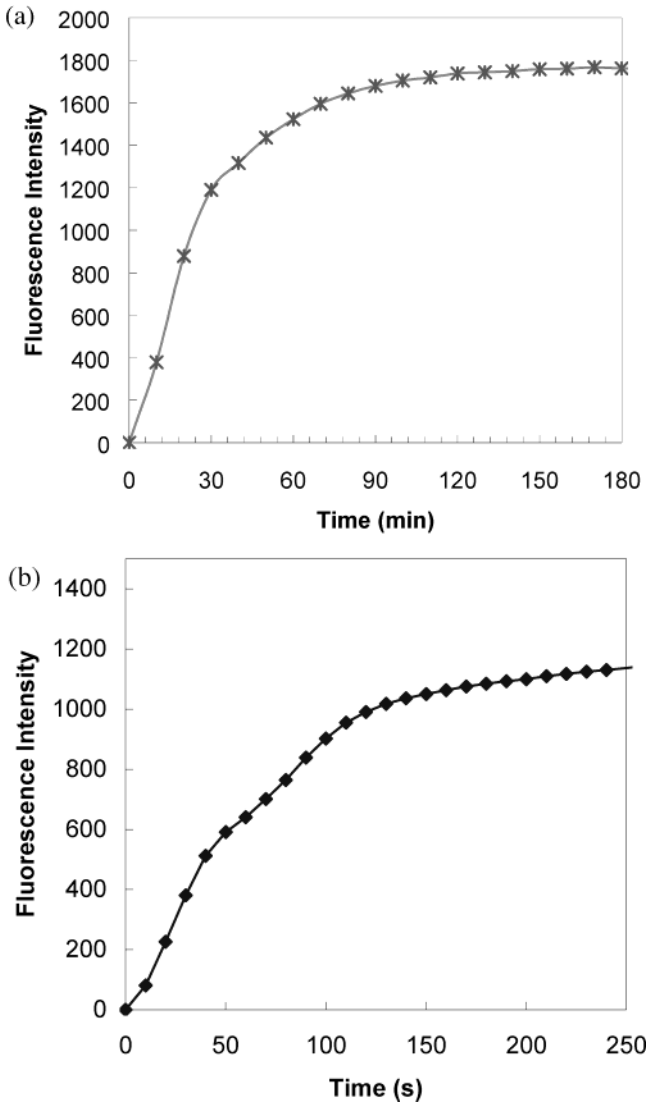

Figure 6. Enzymatic reaction of HPPA (a) in the 96-well microtiter plate and (b) inside a microchannel.

This illustrates the excellent ability of BSA to block the protein-binding site on the solid surface, therefore limiting the nonspecific binding.

Enzymatic Reaction. The time response of the enzymatic reaction of HPPA was examined. The fluorescence intensity of the enzymatic reaction product was used as an indicator of the extent of the enzymatic reaction and was monitored as a function of time. A comparison of the enzymatic reaction rate between the microchip and the microtiter plate is shown in Figure 6. The enzymatic reaction in a microtiter plate took almost 120 min to complete or reach equilibrium (see Figure 6a), while only ~200 s was required in the microchannel (see Figure 6b).

In a 96-well microtiter plate, the specific surface area of 100 μL of solution in each well (6.5 mm in diameter and 3 mm in height) is ~944 m^2/m^3. A microchannel with dimensions of 140 μm ×100 μm × 2 mm has a specific surface area of 34 300 m^2/m^3, which is ~36 times larger than that of the microtiter plate. This provides more reaction area for the substrate (in unit volume) to react with the enzyme on the solid surface. The diffusion length in the microtiter plate is 3 mm (the height for 100 μL of liquid in each well), whereas that of the microchannel is only 50 μm. The

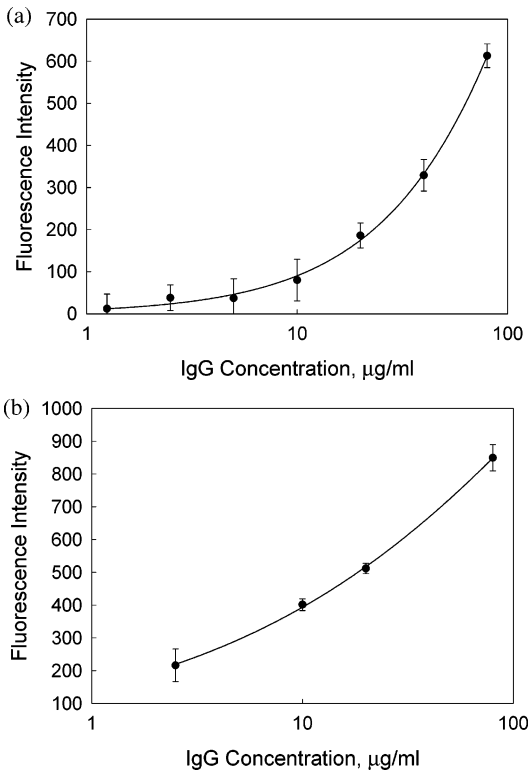

Figure 7. Calibration curves of rat IgG from (a) 96-well microtiter plate and (b) microchip.

characteristic time required for a molecule to diffuse is proportional to the square of the diffusion length. Therefore, the diffusion time of the substrate to the enzyme on the microchannel surface can be much faster than that in the 96-well microtiter plate. The larger surface-to-volume ratio and the shorter diffusion length contribute to the fast enzymatic reaction.

Analysis of Rat IgG from Hybridoma Cell Culture. During the detection, a special holder was used so that images were captured at the same region for each microchannel. The signal (intensity of the fluorescence) variation in the same microchannel is within 5%. Figure 7 shows the calibration curves of the standard rat IgG from the 96-well microtiter plate and the microchip with fluorescence signal. The data were obtained from three independent measurements with different microtiter wells or microchannels. The result in the microchip has a signal similar to that of the microtiter plate within the same detection range. The detection limit is 5 mg/L (31 nM) of the rat IgG (MW ~160 000). Since the concentration of the rat IgG from the hybridoma culture is typically in the range of 1−100 mg/L, this microfluidic platform is expected to be suitable for practical measurement.

For microchip ELISA, 10 μL of reagent was consumed in each assay step and each step took 15 min for incubation. Therefore, consumption of reagents (the most expensive ones are antigens and antibodies) on the microchip was 30 μL, which is one-tenth that of the 96-well microtiter plate (300 μL). The total assay time on the microchip was a little more than 1 h, which is much less than that required for the microtiter plate. The microchip assay conditions chosen in this paper were not fully optimized. The ultimate detection limit is determined by many factors such as the choice of the reagents, immobilization of the first component on the solid surface, etc. Through improving the binding capability of the first reagent and optimizing the assay conditions, the reagent consumption can be further reduced to less than 1 μL for each step and each assay can be finished within 0.5 h.

CONCLUSION

We have successfully demonstrated that flow sequencing can be achieved on a CD-like microfluidic platform by integrating the necessary microfluidic functions such as centrifuge pumping and capillary valving. Burst valves with well-defined and well-separated critical frequencies can be constructed on the disk by varying channel dimensions and the distance of the burst valve from the center of rotation. A preliminary analysis of rat IgG from hybridoma culture shows that the microchip-based ELISA has the same detection range as the conventional method on the 96-well microtiter plate, while having advantages over the conventional method such as less reagent consumption and shorter assay time. To increase the detection sensitivity, surface modification would be very valuable to improve the protein's adsorption and immobilization efficiency. The application of this novel CD-ELISA can be extended for fast detection of food-borne pathogens and toxins.

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