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Rapid quantification of disease-marker proteins using continuous-flow immunoseparation in a nano-sieve fluidic device

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Abstract

Nanometer-scale fluidic devices offer an alternative to gels for separating biomolecules with better control and accuracy. Here we demonstrate the quantitative analysis of disease-marker proteins by continuously separating the antibody-protein immunocomplexes from the unbound antibodies, utilizing the anisotropically-patterned nano-sieve array (ANA) structures. The ANA structures, composed of periodically-patterned deep channels and shallow regions, allow the small antibodies to pass through the shallow regions easier than the large immunocomplex, when the flow-field is applied in an oblique direction. We examined two proteins used as disease markers, human C-reactive protein (CRP) and human chorionic gonadotropin (hCG), by using fluorescent-labeled polyclonal antibodies. We showed that the size of the immunocomplex and the field strength are the critical factors for the separation, and we successfully demonstrated the quantification of the proteins in the range of 0.05 to 10 $\mu\text{g/mL}$. Additionally, this device allows a convenient measurement of homogeneous binding kinetics, without the need for repeated binding experiments and immobilizing the molecules. The presented nanofluidic device will be a useful tool for the rapid quantification and the preparative immunoseparation of the target proteins.

INTRODUCTION

Immunoassay is an unrivalled analytical technique (both in conventional and miniaturized formats) for clinical diagnoses and biochemical studies, due to its extremely high specificity and sensitivity. One of the most-commonly adopted immunoassay formats is the heterogeneous immunoassay, which employs a solid-phase support to immobilize the antibody. The surface-bound antibodies selectively capture the target molecules ('pull down'), to be analyzed by additional rounds of secondary immuno-binding and chemical amplification. The heterogeneous immunoassay has been the platform of choice for many successful in-vitro diagnostics, and has also been implemented in micro/nanofluidic devices.^{1–5} Although the

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Supporting Information Available. Figures S1 and S2.

sensitivity is extremely high in general, immobilizing specific antibodies to the solid-phase support (either microbead or microchannel surface), while maintaining the activity and specificity of the antibody, is a non-trivial task, and often a source of error in detection.

An alternative immunoassay format is to utilize the (microchip) electrophoresis to separate immunocomplexes from the unbound antibody,^{6–12} for not only the quantitative analysis but also the preparatory separation/enrichment of the target molecules from a complex mixture. For example, a microchip electrophoresis device was used to detect MMP-8 in saliva, a marker protein for periodontal diseases, by combining upstream sample pretreatment processes.¹⁰ However, all previous implementations of immuno-separation assays were utilizing batch-mode separation, where a small sample plug is analyzed once at a time. In contrast, continuous separation schemes would be more appropriate for processing multiple analytes in series, and for increasing the sample throughput. As a method for continuous separation, free-flow electrophoresis (FFE) is often used for the preparative applications¹³ and has been demonstrated in miniaturized devices^{14,15}; however, to our knowledge, FFE has not been used for the immunological separation and purification of molecules. A microfluidic technique capable of continuously separating immunocomplex and the unbound antibody would be useful both for the rapid quantification and the affinity-based preparation of the target molecules out of a complex sample mixture.

Recently, precisely-engineered nanometer-scale structures have been developed as effective tools to accurately separate biomacromolecules or particulates with sizes similar to these structures.^{16–21} Compared to the conventional separation methods using disordered sieving matrix such as polymer gels or membranes, artificially-engineered sieving structures achieve a well-controlled biochemical separation and would provide a useful means for both clinical and biochemical research fields. In our previous study, we have developed microdevices incorporating 2-D patterned anisotropic nano-sieve array (ANA) structures, and demonstrated the rapid DNA or protein separation in a continuous manner.²¹ The alternation of the nanometer-scale deep channels and shallow regions works as a sieving structure, and molecules with different characteristics in size, flexibility, or electric charge flow along different trajectories inside the two-dimensional ANA region. For macromolecules with diameters smaller than the nanofilter constriction (shallow-region depth of d_s), the steric energy barrier allows molecules with a smaller size to pass through the constriction faster than larger molecules (Ogston sieving).¹⁷ On the other hand, for macromolecules with diameters larger than the nanofilter constriction size, passage requires the molecules to deform at the cost of their internal conformational entropy (entropic trapping),¹⁶ resulting in the greater passage rate for larger molecules. The most important advantage of the ANA devices is that we can separate molecules at their native conditions without employing sieving polymer matrixes or detergents, which would be suitable for the free-flow separation of the fragile immunocomplexes.

Here we demonstrate the rapid and quantitative immuno-separation assay of proteins, by utilizing the ANA structures along with fluorescence-labeled polyclonal antibodies. The target protein molecules form large immunological complexes with the presence of antibodies, resulting in a significant difference in size between the immunocomplexes and the unbound antibody. The ANA structures allow us to continuously separate the native molecular complexes and achieve the rapid quantification of the target proteins, by comparing the fluorescence signal intensities of the complexes and the unbound antibodies.

In the current study, we examine two proteins as model, human C-reactive protein (CRP) and human chorionic gonadotropin (hCG), both of which are used as markers for clinical diagnostics. CRP is a serum protein with a molecular mass of 115 kDa, and is composed of five identical subunits.^{22–24} The high level of CRP in serum indicates the presence of an acute

inflammation such as a cardiovascular disease, and the clinical cut-off value is 3~5 $\mu\text{g/mL}$. hCG is a glycoprotein hormone with a molecular mass of ~37.5 kDa, used as a marker for pregnancy as well as germ cell tumors.^{25–27} In addition to the quantitative immunoassay, we demonstrate the application of the ANA structure for studying the binding kinetics of protein and antibody, by continuously monitoring their separation behaviors.

EXPERIMENTAL SECTION

Fabrication and design of the ANA structures

Fabrication protocols of the ANA structures are described elsewhere.²¹ Briefly, the shallow regions and the deep channels of the ANA structures, as well as the inlet/outlet microchannels, were defined and etched stepwise on a Si wafer by means of photolithography and reactive ion etching. Next, through-wafer access holes were made by KOH etching for inlet and outlet reservoirs, and a 500-nm oxide layer was grown to render the channel surface non-conductive. The etched Si wafer was then bonded with a Pyrex wafer via anodic bonding, and finally the bonded wafers were cut into pieces by using a die-saw. The depths of the shallow region and the deep channels were measured by using a surface profilometer before bonding.

Fig. 1 (a) shows the schematic illustration of the ANA structure, and Fig. 1 (b) shows the design of the entire microdevice. The nano-sieve structure is composed of the periodically-patterned deep channels (depth of d_d) and the shallow regions (depth of d_s). The deep channels and the shallow regions alternate with a period of 1.0 μm , while there are silicon pillars of 0.5 $\mu\text{m} \times 1.0 \mu\text{m}$ in the shallow region, which prevent the collapse of the shallow region during the bonding process. Compared to the devices reported previously,²¹ the dimension of the pillar is decreased, to enhance the flow rate in the x -direction and therefore to improve the separation efficiency. We employed two types of devices with different values for d_d and d_s ; 300/60 nm devices and 500/120 nm devices. In this study, we mainly used the 300/60 nm devices which showed the better separation performance, so the results were obtained from the 300/60 nm devices, unless noted otherwise. The size of the entire ANA region is 5 mm $\times$ 5 mm, although we mainly observed the area near the sample inlet as defined in Fig. 1 (b). The width of the sample-inlet channel is 30 μm , and the x -position of the left-side of the inlet channel is 1000 μm .

Materials

Human C-reactive protein (CRP) and bovine serum albumin (BSA) were obtained from Sigma Aldrich Inc. (Saint Louis, MO). FITC-labeled goat anti-human CRP antibody was obtained from Bethyl Laboratories Inc. (Montgomery, TX). Human chorionic gonadotropin (hCG; >14000 IU/mg) was obtained from Scripps Laboratories Inc. (San Diego, CA). Biotin-conjugated chicken anti-hCG IgY and biotin-conjugated goat anti-human CRP antibody were obtained from Immunology Consultants Laboratory Inc. (Newberg, OR). Streptavidin conjugated with Alexa Fluor 488 was obtained from Invitrogen Corp. (Carlsbad, CA). CRP-free human serum was obtained from United States Biological Inc. (Swampscott, MA).

Sample preparation, separation, and quantitative analysis

We used 0.5 $\times$ Tris/Borate/EDTA (0.5 $\times$ TBE, composed of 44.5 mM Tris-borate and 1 mM EDTA, pH of ~8.3) buffer containing 0.5% (w/v) BSA. BSA prevents non-specific adhesion of molecules onto the channel surface. TBE 0.5 $\times$ buffer has an equivalent ionic strength of ~13 mM²⁸ with a corresponding Debye length λ_D of 2.6 nm, which is sufficiently smaller than the depth of the shallow region d_s . For the separation/analysis of CRP, CRP and antibody were mixed in the buffer solution (outside of the device), and then incubated for at least 2 h at a room temperature. The final concentration of CRP was varied from 0.023 to 11.5 $\mu\text{g/mL}$, while the antibody concentration was fixed at 50 $\mu\text{g/mL}$. For the analysis of CRP in serum, CRP

molecules were added into the CRP-free human serum, then the serum was diluted at 1/20 in the buffer solution, and finally, the antibody was added and incubated for 2 h. For the separation/analysis of hCG, we used biotin-conjugated polyclonal antibody to hCG (Ab) and Alexa Fluor 488 conjugated streptavidin (SA). At first, hCG and the antibody were mixed and reacted for 1 h, and then the labeled streptavidin was added and incubated for at least 2 h. The final concentrations of the antibody and streptavidin were fixed at 50 and 40 $\mu\text{g/mL}$, respectively, while the hCG concentration was varied from 0.02 to 10 $\mu\text{g/mL}$.

All the separation experiments were performed at a room temperature. Electroosmotic flow (EOF) was used to transport fluids in the ANA device. Initially, the ANA structure was completely filled with the buffer solution. After exchanging the buffer solution in the sample-inlet reservoir with the sample solution, voltages were applied to the inlet/outlet Pt electrodes, as shown in Fig. 1 (b), and the separation behavior was monitored by using a fluorescence microscope (IX-71, Olympus Corp.). The bottom (V_3) and right (V_4) sides of the ANA region were grounded for all experiments, while the applied voltages of the top (V_1 , including the sample inlet) and left (V_2) sides were controlled up to 100V. Fluorescence images were captured in the observation area shown in Fig. 1 (b) using a CCD camera (ORCA-ER, Hamamatsu Photonics K.K.), and the signal intensity was analyzed using an image processing software. The obtained fluorescence profiles were standardized by subtracting the background signal and calculating the total peak area.

RESULTS AND DISCUSSION

Separation of CRP-antibody complex and unbound antibody

Applying the horizontal flow-field (Flow- x) along with the vertical flow-field (Flow- y) is a prerequisite for the continuous separation of the immunocomplex and the unbound antibody. Fig. 2 (a) shows the micrographs of molecular streams near the entrance of the ANA region, with the fixed V_1 value of 100V and the CRP concentration of 0.46 $\mu\text{g/mL}$. When the voltage V_2 was changed from 20 to 60V, the stream angle changed from negative to positive. Upon the application of the Flow- x (V_2 of 20 or 60 V), the CRP-antibody complexes flowed along the deep channels (y -direction), while the unbound antibody flowed through the shallow region, resulting in the continuous separation of these molecules. When V_2 was 40 V, the entire flow direction was almost parallel to the deep channel, and separation did not take place. Fig. 2 (b) shows the fluorescence profiles obtained from the detection area shown in Fig. 2 (a), which was located just 1 mm downstream from the entrance of the ANA region. The angle between the flow of the unbound antibody and the y -axis was changed from -12.0° to 23.3° as V_2 was changed from 0 to 100 V, while the change was only $\sim 1.5^\circ$ in the case of immunocomplexes. The flow of the complexes did not show significant diffusion, keeping a constant width of about 30 μm throughout the 5 mm ANA region.

In this experiment, the concentration of the anti-CRP antibody (~ 330 pM) was ~ 83 times higher than that of CRP (4 pM), so we can safely assume that individual CRP molecules were completely covered with the multiple antibody molecules. CRP (115 kDa) is a disc-shaped molecule with diameter and thickness of 10 and 3 nm, respectively.²² On the other hand, goat IgG is a molecule with the mass of ~ 150 kDa and the hydrodynamic diameter of ~ 14 nm.²⁰ So the maximum size of the immunocomplex should be 30–40 nm in diameter, which is comparable to but slightly smaller than the depth of the shallow region (d_s of 60 nm).

In our previous study of DNA separation in the ANA structure, we observed the transition of separation behavior between the DNAs with sizes of 1000 and 2000 bp.²¹ In the case of DNAs shorter than 1000 bp (molecular mass lower than 660 kDa), the shape is rod-like and the configurational freedom is limited, so the smaller molecules flow through the shallow region faster than the larger molecules, due to the lower entropic barrier for smaller molecules (Ogston

sieving). On the other hand, DNAs longer than 2000 bp (molecular mass higher than 1300 kDa) are flexible in shape and larger than the shallow-region depth, so the separation efficiency is dependent on the chance of molecules to deform into the nano-sieve region, resulting in the longer DNAs moving faster to the lateral direction than shorter DNAs (entropic trapping). In this experiment of immunoseparation, the size of the immunocomplex is smaller than the long DNAs, and thus the separation mechanism would be based on the Ogston sieving rather than entropic trapping. Also, the migration distance of the immunocomplexes in the x -direction was extremely small, indicating their low flexibility in conformation.

Effect of field strength on separation

The field strength (i.e., flow speed) in the nanofilter separation systems affects the mobility of different-sized biomolecules with different magnitudes.²⁹ We thus examined how the flow speed and the complex size affect the separation efficiency, by changing the applied voltages and the concentration of the target CRP molecules. Fig. 3 (a) shows the separation behavior of the immunocomplex and the unbound antibody, when the CRP concentration was relatively high (4.6 $\mu\text{g/mL}$). The ratio of the voltages, $V_1:V_2$, was kept at 1:0.7, while V_1 was varied either at 100, 50, or 20 V. At the higher field-strength (V_1 of 100 V), we could not observe a clear separation. When the applied voltage was decreased, the fluorescent streamline of the immunocomplexes appeared along the deep channel, while the stream of the unbound antibody broadened due to the molecular diffusion. This result is consistent with our previous studies on Ogston sieving in nanofilters.²¹ Higher flow-field generates larger shear-stress to the molecules, forcing immunocomplexes to overcome the entropic barrier posed by the shallow regions.

In Fig. 3 (b–d), fluorescence profiles with two different CRP concentrations (4.6 and 0.46 $\mu\text{g/mL}$) at different field strengths are shown. When the CRP concentration was relatively low (0.46 $\mu\text{g/mL}$), the field strength did not affect the separation behavior of the immunocomplex, in contrast with the separation of high CRP concentration samples (4.6 $\mu\text{g/mL}$). This difference in the separation behaviors is likely attributed to the difference in the complex sizes. When the CRP concentration was 4.6 $\mu\text{g/mL}$, the molar ratio of CRP and antibody was $\sim 1:8.3$, which would be insufficient to form large complexes. These results show that the lower flow-field strength results in the clearer separation of the complexes, when the complex size is small.

Quantitative analysis of CRP

The peak area and height of the immunocomplex in the fluorescence profile depend on the concentration of the target molecules. We therefore conducted the quantitative analysis of CRP by using the peak of the unbound antibody as a reference, at the fixed values of V_1 and V_2 at 20 and 14 V, respectively. Fig. 4 (a) shows the comparison of the peak areas of the unbound antibody and the immunocomplex. Here Gaussian fittings were used to calculate the peak areas. Even for the control experiment without CRP, the relative peak area of the immunocomplex (S_1/S_2) was not absolutely zero, due to the asymmetric peak shape. The relative peak-area of the complex increased with the increase of the CRP concentration, up to 2.3 $\mu\text{g/mL}$. When CRP concentration was higher than 2.3 $\mu\text{g/mL}$, the peaks of the unbound antibody and the immunocomplex overlapped, resulted in the decrease of the peak-area of the immunocomplex, S_1 .

The relative peak- and valley-heights would provide the information on the size of the formed immunocomplexes. The comparison of these values is shown in Fig. 4 (b) and (c). The relative peak-height of the complex, h_{p1}/h_{p2} , which suggests the formation of large complexes, was maximum at the CRP concentration of 2.3 $\mu\text{g/mL}$. On the other hand, the lowest valley-height h_v indicates the presence of smaller complexes; this value increased with the increase of the

CRP concentration, when it is higher than 0.46 $\mu\text{g/mL}$. These measurements of the peaks enable the quantitative analysis in the relatively wide range of CRP concentration.

The lower limit of detection (LOD), defined as three times the standard deviation of the negative control, was ~ 50 ng/mL (equivalent to ~ 0.4 nM), inferred from Fig. 4 (a). This value is low enough to conduct the clinical diagnosis of CRP (cut-off value of 3–5 $\mu\text{g/mL}$) without any chemical amplification processes implemented. In comparison, the detection sensitivity of the conventional ELISA assay is higher, with usual LOD of less than 1 ng/mL. One factor affecting LOD of our system would be the absolute number of the target molecules in the nano-sieve region; when the CRP concentration is 20 ng/mL, only about 350 molecules are flowing in the detection area. The use of the vertical nano-sieve array with ultra-high aspect ratio structures and a larger optical length³⁰ would decrease LOD, and would increase the throughput for preparative applications. Also, techniques for amplifying the fluorescence signal would possibly be useful to obtain even higher sensitivity,⁴ unless they form immuno-precipitates.

In addition to the nano-sieve structures with depths of 300/60 nm, we examined 500/120 nm devices for the immunoseparation. In that case, we were able to separate the large complexes (CRP concentration of 0.23–1.15 $\mu\text{g/mL}$), but there was no significant difference between the peak shapes of high concentration conditions (>4.6 $\mu\text{g/mL}$) and control (0 $\mu\text{g/mL}$) (data shown in supplementary Figure S1). This result indicates that the threshold value of the molecular size to be separated in the 500/120 nm device is larger than that of the 300/60 nm device. In contrast, we expect that ANA structures shallower than 300/60 nm would have a potential to separate smaller molecules and/or molecular complexes.

Quantification of CRP in serum

The direct quantification of proteins in biological fluids is significant for providing a useful tool for the real clinical and research applications. The ANA devices would also be able to detect proteins in serum on the condition that large complexes are formed and they are separated from the unbound antibody. We therefore tried to detect CRP in serum; we initially prepared the standard serum containing CRP from the CRP-free human serum, and then, it was diluted at 1/20 in TBE 0.5 $\times$ buffer containing 0.5% (w/v) BSA and 50 $\mu\text{g/mL}$ of the fluorescent antibody.

Fig. 5 shows the fluorescence profiles of the separated unbound antibody and the CRP-antibody complex, when the applied voltages V_1 and V_2 were 100 and 60V, respectively. The ratio of the peak heights, h_{p1}/h_{p2} , was 0.07 ± 0.02 and 0.13 ± 0.04 for the initial CRP concentrations of 4 and 10 $\mu\text{g/mL}$, respectively, showing the applicability for the real biological sample. In this voltage condition, the flow speed inside the ANA region was 50–100 $\mu\text{m/sec}$, and thus, it took less than 30 sec for molecules flowing from the entrance of the nano-sieve region to the detection area. Also, the total time from the sample injection to the separation was a few minutes, showing its fast speed in separation and detection. Although the concentration of the serum was only 5%, the molecules in serum gradually adhered on the inner surface of the inlet channel, and the sample flow rate was decreased after 1 h of running, which is probably due to the change in the surface properties of the inlet channel. However, the devices made of glass and silicon substrates are re-usable; after exchanging the inner solution with distilled water, and drying and baking at 475 $^{\circ}\text{C}$ for 2 h on a hot plate, the surface property was completely recovered. There was no significant change in the separation performance, even after 20 times of usage.

Separation of hCG-antibody complex

The application of the presented ANA structure to the detection/analysis of other protein-antibody combinations is valuable to show its versatility. Human chorionic gonadotropin

(hCG) is therefore examined, along with the biotin-conjugated antibody to hCG (Ab) and Alexa Fluor 488 labeled streptavidin (SA). hCG is a glycoprotein with a molecular mass of 36.7 kDa and the approximate size of $\sim 7 \times 3.5 \times 3$ nm,²⁷ and it is considerably smaller than CRP.

Fig. 6 (a) shows the fluorescence profiles obtained by image-processing from the detection area shown in Fig. 2 (a). When Ab and SA were mixed and introduced, the peak position slightly shifted (~ 40 μm) to the negative x -direction, compared to the case of SA only. In addition, we did not observe the fluorescence signal along the deep channel, which shows that Ab and SA formed complexes but they were small enough to pass through the shallow region. In contrast, with the presence of hCG molecules, the fluorescence signal appeared along the deep channel region ($x = 1000$ – 1030 μm), indicating the formation of large complexes triggered by hCG molecules. Fig. 6 (b)–(d) shows the results of quantitative analysis of hCG, as in the case of CRP analysis shown in Fig. 4. When the hCG concentration was as low as 0.04 $\mu\text{g/mL}$, the peak of the complexes was sometimes undetectable, and LOD estimated from Fig. 6. (b) was ~ 0.1 $\mu\text{g/mL}$. This value was about twice as high as that of CRP detection, indicating that the required number of the target molecules for detection should be ~ 6 times higher. The smaller size of the hCG would result in the lower number of the attached antibodies and the decrease of the fluorescent intensity, even though we employed biotin-streptavidin bindings to enhance the sensitivity.

In this experiment, we used polyclonal antibody to hCG; however, the α -subunit of hCG is common for other hormone proteins, so specific antibodies to the β -subunit of hCG should be used for the clinical diagnosis.^{25,26} In that case, it might be better to initially use specific antibodies to β -subunit for identification, and then add other antibodies for the further increase of the complex sizes.

Additionally, it should be noted that some combinations of antibody and labeling dye cannot be employed for the protein quantification in the ANA structure. We tried to conduct CRP quantification by using biotin-conjugated polyclonal antibody to CRP and Alexa Fluor 488 conjugated streptavidin, at the same concentration conditions for hCG quantification. In that case, however, large complexes formed even without the presence of CRP molecules, and they flowed along the deep channel (data not shown). For the efficient immunoseparation and detection of proteins in the ANA structures, the change in the complex size should be triggered by the presence of the target protein.

Extracting immuno-binding kinetics

Various techniques are available for studying the reaction kinetics of antigen-antibody binding, including the conventional 96-well microtiter plate, micro gel-array format,³¹ surface plasmon resonance (SPR),^{32,33} cantilever-based mechanical sensors,^{34,35} and so forth. In most cases, however, a solid support should be used for immobilizing antigen or antibody, which possibly changes the molecular characteristics and usually decrease the binding activities due to the randomly-immobilized molecules. Specific techniques for oriented immobilization are available to keep the biological activities of antibodies,^{36,37} but they still have a limitation for studying molecular interactions at their native conditions. On the other hand, the ANA structure would function as a means for monitoring the dynamically changing molecular complexes, without employing immobilization processes or labeling techniques with FRET (fluorescence resonance energy transfer) probes.³⁸ We therefore examine how the time-course change of the formation of CRP-antibody complex occurs, when the protein and antibody are introduced into the ANA device (300/60 nm device) immediately after mixing. The applied voltages V_1 and V_2 were fixed at 20 and 14 V, respectively, and the CRP concentration was fixed at 0.46 $\mu\text{g/mL}$.

Figure 7 shows the time dependence of (a) the relative peak-area and (b) the relative peak-height of the immunocomplexes. In both cases, binding reaction was completed within 30 min. The relative peak-area increased more rapidly than the relative peak-height; the times that give 90% of the maximum height are 7.4 and 28.5 min for (a) and (b), respectively. This difference suggests that the formation of complexes that are large enough to flow along the deep channel (indicated by the peak height h_{p1} in (b)) is slower than that of smaller complexes (indicated by the peak area S_1 in (a)), which would be mainly due to the steric hindrance. In addition to the CRP-antibody complex formation, we conducted similar experiments for hCG-antibody-streptavidin complexes, as shown in Supplementary Figure S2. Further studies and applications would be possible, when a proper combination of the molecular complexes and the ANA geometries is employed.

CONCLUSIONS

We successfully demonstrated the continuous-flow separation-base immunoassay in the anisotropically-patterned nano-sieve array (ANA) devices, and achieved the direct and rapid quantification of target proteins at their native conditions. The laborious washing/mixing steps are dispensed with, making this system suitable for integrating with other operations such as sample pre-treatment or downstream concentration. The preparative application would also be possible, by increasing the throughput further. In the current device, the area of the ANA region is 5×5 mm; however, we actually need only a small portion of the channel region ($\sim 0.3 \times 1$ mm), suggesting that parallel analyses will be possible by making multiple inlets and introducing different samples simultaneously, as well as the multiple-sample processing in series. In addition, time-dependent homogeneous binding kinetics can be measured in a single experiment, which would provide uniqueness for general biochemical studies of protein/DNA interactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(a) Schematic illustration of the anisotropic nano-sieve array (ANA) structure and the separation mechanism of the immunocomplex and the unbound antibody. The fluid flow (EOF) is introduced both in x - and y -directions, while the mixture of the antibody and the target protein was introduced from a narrow inlet-channel. The small molecules (unbound antibody) flow through the shallow region easier than the large molecules (immunocomplex), and consequently, the unbound antibodies and the immunocomplexes flow along different trajectories inside the two-dimensional ANA region. (b) Schematic design of the microfabricated device incorporating the ANA structure. The small black circle shows the original point. The applied voltages are shown as indicated. This figure is not to scale.

Figure 2.

Separation behavior of the CRP-antibody complex and the unbound antibody, when the concentration of CRP was $0.46 \mu\text{g/mL}$. (a) Fluorescence micrographs taken in the observation area defined in Fig. 1 (b) when V_2 was varied as indicated. Dashed lines show the boundary of the ANA region and the inlet microchannels. (b) Fluorescence intensity profiles when the applied voltage V_2 was changed from 0 to 100 V. The data was obtained from the detection area shown as a red rectangle ($1150 \times 65 \mu\text{m}$) of Figure (a), and the intensities in the vertical direction (in y-axis) were averaged.

Figure 3.

Effect of the flow-field strength on separation performance. (a) Fluorescence images when the applied voltages were changed as indicated. Concentration of CRP was fixed at $4.6\text{ }\mu\text{g/mL}$ for all three conditions. (b–d) Fluorescence intensity profiles when the CRP concentration and the field strength were changed as indicated.

Figure 4.

Quantitative analysis of CRP, by comparing (a) peak areas calculated using Gaussian fitting, (b) peak-heights of the immunocomplex h_{p1} and the unbound antibody h_{p2} , and (c) peak-heights of the unbound antibody h_{p2} and the lowest valley-height h_v . Each data shows the mean $\pm$ s.d. of at least four experimental results.

Figure 5.

Fluorescence intensity profiles for the quantification of CRP in human serum, when the initial concentrations of CRP were 0, 4, and 10 $\mu\text{g/mL}$, respectively. The applied voltages V_1 and V_2 were 100 and 60V, respectively.

Figure 6.

Separation and quantification of hCG. (a) Fluorescence profiles showing the separation behavior of hCG-antibody (Ab)-streptavidin (SA) complex. V_1 and V_2 were 20 and 14V, respectively. (b)–(d) Quantitative analysis of hCG, by comparing (b) peak-areas divided by Gaussian fitting, (c) peakheights of the unbound antibody h_{p1} and the immunocomplex h_{p2} , and (d) peak-heights of the unbound antibody h_{p1} and the lowest valley height h_v . Each data shows the mean $\pm$ s.d. of at least three experimental results.

Figure 7.

Time-dependent immuno-binding by examining (a) the peak-area ratio and (b) the peak-height ratio of the CRP-antibody complex. The concentration of CRP was 0.46 $\mu\text{g/mL}$. The corresponding times at 90% of the maximum height are 7.4 and 28.5 min for (a) and (b), respectively. In (a), the yintercept is obtained from the value at 0 $\mu\text{g/mL}$ of Fig. 4 (a). Fitting curves were drawn based on reverse exponential equations with the least square. Each data shows the mean $\pm$ s.d. of four experimental results.