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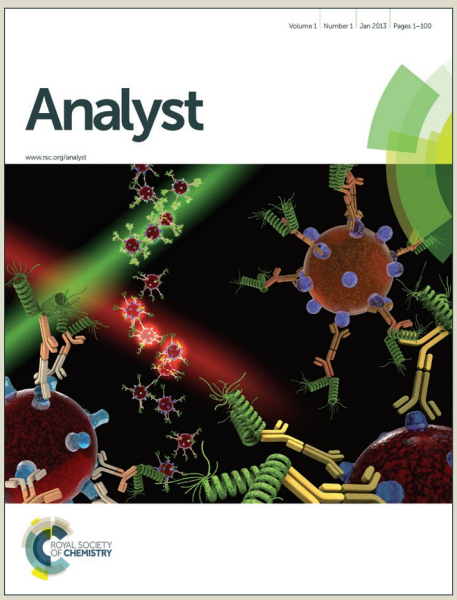

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A single-step enzyme immunoassay capillary sensor composed of functional multilayer coatings for diagnosis marker proteins†

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A single-step, easy-to-use enzyme immunoassay capillary sensor, composed of functional multilayer coatings, was developed in this study. The coatings were composed of substrate-immobilized hydrophobic coating, hydrogel coating, and soluble coating containing enzyme-labeled antibody. The response mechanism involved a spontaneous immunoreaction triggered by capillary action-mediated-introduction of sample antigen solution and subsequent separation of unreacted enzyme-labeled antibodies and antigen-enzyme-labeled antibody complexes by the molecular sieving effect of the hydrogel. An enzyme reaction at the substrate-immobilized hydrophobic coating/hydrogel coating interface resulted in a protein-selective fluorescence response. An antigen concentration-dependent response was obtained for diagnostic marker protein samples (hemoglobin A1c (HbA1c), 7.14–16.7 mg/mL; alpha-fetoprotein (AFP), 1.4–140 ng/mL; C-reactive protein (CRP), 0.5–10 μg/mL) that cover a clinically important concentration range. Successful measurement of CRP in diluted serum samples demonstrated the application of this capillary sensor.

Introduction

Enzyme immunoassay (EIA) is an analytical method for antigen quantification using enzymes based on antigen-antibody reactions.[1,2] It is utilized in a variety of analytical tests both in research and in medical fields.[3–5] However, since the methodology involves complicated solution exchanges and washing/rinsing operations, the analysis time is lengthy.[6,7]

Recently, several researchers have developed various types of microfluidic immunoassay devices that utilize microspace characteristics.[8–13] Microdevices can process small sample amounts[14,15] and have a relatively faster analysis time.[15–20]

Previous research on microfluidic EIAs have mainly focused on only improving sensitivity[21–24] and shortening assay time.[15–20] On the other hand, recent developments in immunochromatography[25–29] and paper-based fluidic devices[30–33] provides simpler and easier EIAs than that of conventional one mainly because the process involves only a simple dropping of the sample solution. However, these methods are not suitable for quantitative analysis.[34,35]

Our laboratory has been active in developing various sensor capillaries that use functional molecules immobilized on the square-capillary inner wall for the detection of ions, sugars, and proteins.[14,36–40] The characteristic of the capillary sensor is the free arrangement of multiple sensor capillaries in parallel, which enables a simultaneous analysis of different types of analytes.[41–43] Furthermore, since capillary action can be used for sample introduction, a mere contact of the sample solution with the capillary edge allows an easy introduction of the sample into the device and its subsequent assay.[44–46]

Here, we attempted to develop a single-step capillary-based EIA. Simultaneous measurement of several analytes, by placing capillaries in parallel, as well as quantitative analysis by acquiring fluorescence or colored images of the sensor coating or solutions in capillaries are the advantages of this method.

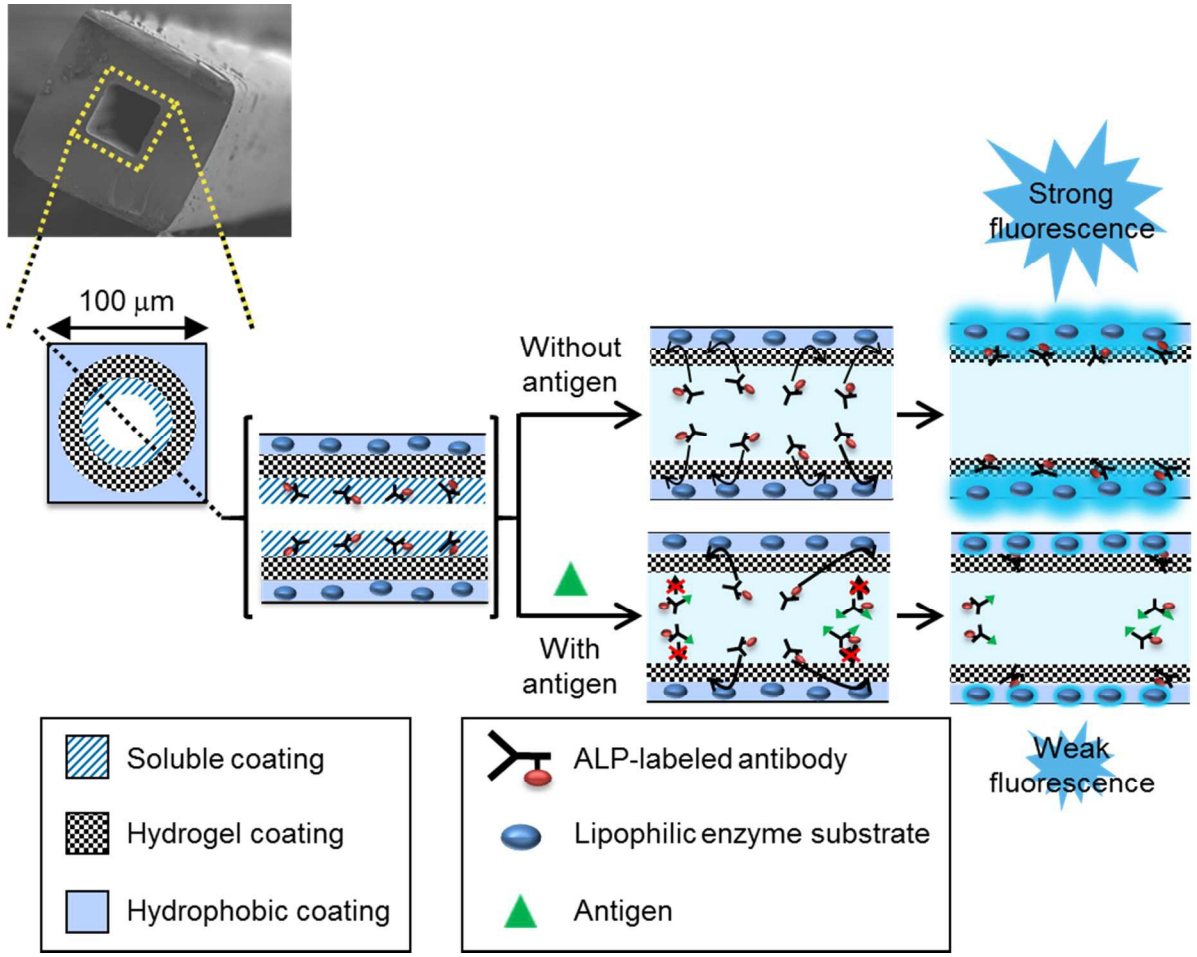

Figure 1 Concept of single-step capillary-based EIA.

One of the reasons for complicating operations in conventional EIA are the reagent solution exchanges and washing/rinsing procedures. In order to solve this problem in a capillary-based system, immobilization of all required reagents within the capillary is necessary. Furthermore, in order to eliminate the washing step, it is necessary to separate unreacted antibody species from immunoreaction complexes. For this purpose, we focused on the molecular sieving effects of hydrogels. Compared with unreacted antibody species such as enzyme-labeled antibodies, the immunoreaction complexes possess increased-molecular sizes. Thus, separating these species using the molecular sieving effect is theoretically possible.

In order to implement these concepts, we designed functional multilayer coatings within the capillary (Figure 1). The layers, in order from the capillary inner wall, were a substrate-immobilized hydrophobic coating, a hydrogel coating, and an enzyme-labeled antibody-immobilized soluble coating. The substrate-immobilized hydrophobic coating includes a lipophilic substrate that is insoluble in an aqueous sample solution. The hydrogel coating is made of polyacrylamide typically used for molecular size separation. The enzyme-labeled antibody-immobilized soluble coating is water-soluble and dissolves in the sample solution when a sample solution is introduced into the capillary. Therefore, introduction of sample antigens by capillary action would allow for a spontaneous dissolution of soluble coating and releases enzyme-labeled antibodies for subsequent reaction with antigen. In this case, chemical species reactive with the substrates are unreacted-enzyme-labeled antibodies and antigen-enzyme-labeled antibody complexes. As stated above, these complexes possess a larger molecular size. Thus it was expected that the unreacted enzyme-labeled antibodies penetrate through the hydrogel coating and react with the lipophilic substrate at the surface of the hydrophobic coating to give fluorescence response. In contrast, the antigen-enzyme-labeled antibody complexes are not expected to penetrate the hydrogel coating or in the case that they did, they were expected to penetrate through the hydrogel coating very slowly. In this case, no fluorescence response is expected.

Based on these concepts, we designed and developed a single-step, easy-to-use EIA capillary sensor and report its basic characteristics and provide examples of its application.

Experimental

Materials

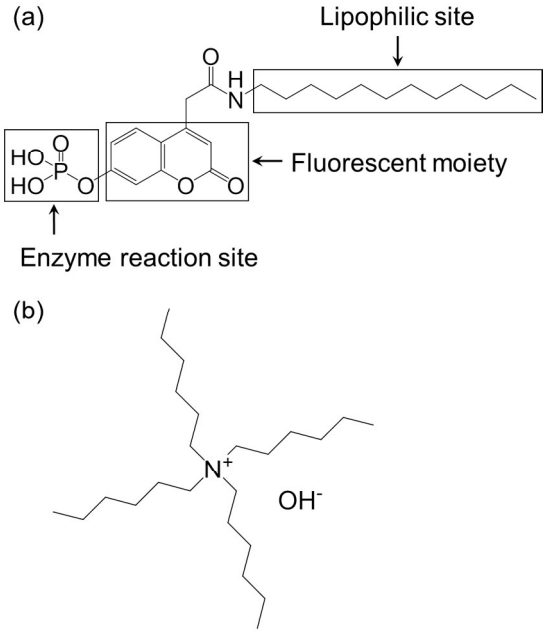

Figure 2 Chemical structures of the lipophilic fluorescent substrate (a) and the lipophilic base (b).

Square capillaries with 300-μm outer widths (flat to flat) and 100-μm inner widths were purchased from Polymicro Technologies (Phoenix, AZ, USA). The polyimide coating of these capillaries was removed by heating before use. Poly(vinyl chloride-co-vinyl acetate-co-vinyl alcohol) (PVC), methyl cellulose, rabbit IgG, and goat IgG were purchased from Sigma-Aldrich (St. Louis, MO, USA). Di-n-octyl phthalate and tetrahexylammonium hydroxide (10% methanol solution) were purchased from Tokyo Chemical Industry (Tokyo, Japan). Tetrahydrofuran (THF) was purchased from KANTO CHEMICAL (Tokyo, Japan). Acrylamide, *N*,*N*′-methylenebisacrylamide, diammonium peroxydisulfate (APS), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), and polyethyleneglycol (PEG) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Anti-human C-reactive protein (CRP) antibody was purchased from Abcam (Cambridge, UK). Human CRP was purchased from Acris Antibodies (Herford, Germany). Anti-human hemoglobin A1c (HbA1c) antibody and anti-human alpha-fetoprotein (AFP) antibody were purchased from Abnova (Taipei, Taiwan). Human HbA1c standard solution (7.14–16.7 mg/mL) was purchased from Reference Material Institute for Clinical Chemistry Standards (Kanagawa, Japan). Alkaline phosphatase (ALP) labeling kit was purchased from DOJINDO (Kumamoto, Japan). ALP-labeled anti-rabbit IgG antibody was purchased from Rockland (Gilbertsville, PA, USA). Control serum was purchased from NISSUI PHARMACEUTICAL (Tokyo, Japan). All reagents were used without further purification. The distilled and deionized water used had resistivity values of more than 1.8×10^7 Ωcm at 25 °C.

Preparation of single-step immunoassay capillary

A lipophilic substrate was synthesized by a procedure similar to that described in a previous report.[47] Details are described in the supporting information (see ESI†). All fluid handling that was necessary for modification of the capillary inner surface was carried out using a glass syringe. Firstly, a PVC-coated capillary was prepared. The typical procedure used was as follows. The capillary was filled with THF solution containing 2 mg of lipophilic substrate, 32 mg of PVC, 64 mg of di-n-octyl phthalate, 16 mg of tetrahexylammonium hydroxide methanol solution, and 750 mg of THF, after which it was immediately flushed with air and dried at 70 °C.

Subsequently, a hydrogel layer was modified on the PVC coating. The PVC-coated capillary was filled with a prepolymer solution composed of 50 μL of 80% acrylamide (acrylamide : *N*,*N*′-methylenebisacrylamide = 37.5 : 1), 30 μL of 2% methyl cellulose, 50 μL of 1 M Tris-HCl buffer (pH 8), 1 μL of 10% APS, 2 μL of 10% TEMED, and 67 μL of water, and then immediately flushed with air, and heated at 40 °C for 10 min.

Finally, a PEG layer containing ALP-labeled antibody was modified on the hydrogel layer. In order to avoid a spontaneous reaction of ALP-labeled antibody with the substrate in plasticized PVC during the immobilization process, a highly viscous PEG solution was used for immobilization. The capillary was filled with a PEG solution composed of 3 μL of 50 μg/mL ALP-labeled antibody, 4 μL of 30% PEG (100 mM Tris-HCl buffer (pH 8) solution), and 8 μL of water, then immediately flushed with air. This capillary was stored in deep freezer (-80 °C).

Operational procedure

The coated capillary was divided into 15 mm-long pieces, and sample solution was introduced into each capillary by capillary action. Fluorescence was detected by a fluorescence microscope equipped with a charge-coupled device (CCD) camera (VB-7000, VB-7010, VB-L10, Keyence, Osaka, Japan) (24-bit RGB color; excitation filter: 330/80 nm [bandpass]; emission filter: 400 nm [longpass]). Fluorescence images were converted into a numerical response by using ImageJ software.

Results and discussion

Enzyme reaction at the interface of aqueous sample solution and plasticized PVC containing lipophilic substrate

For implementation of the concept shown in Figure 1, it is necessary that an enzyme reaction at the organic-aqueous interface, in which the fluorescent substrate is located in the organic phase and the enzyme is located in the aqueous phase, should be investigated. We have been developing ion sensors or biosensors based on plasticized PVC membranes or coatings containing lipophilic ionophores or lipophilic dyes.[48,49] Therefore, for this study, we started by using plasticized PVC. With respect to the molecular design of the lipophilic fluorescent substrate, a lipophilic derivative of a commercially available fluorescent substrate of coumarin was synthesized and used. However, since the pK_a value of the enzyme reaction

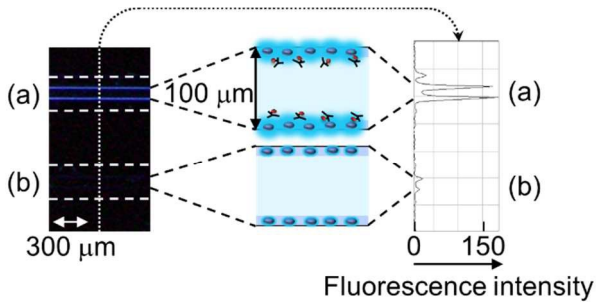

Figure 3 Typical fluorescence image analysis of capillaries coated with plasticized PVC. (a) ALP-labeled antibody in buffer solution (1 μg/mL), (b) buffer solution, only. The right profile of fluorescence intensity was obtained from the left image by ImageJ software.

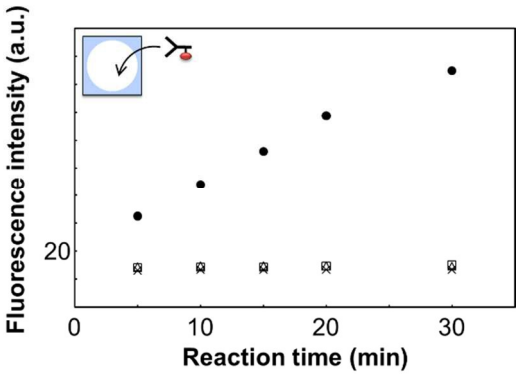

Figure 4 Effect of the lipophilic base within plasticized PVC coating on fluorescence response toward ALP-labeled antibody solution (1 μg/mL).

product is relatively high (pK_a of the reference compound: 7.79),[50] we tested a tetrahexylammonium hydroxide as an additional lipophilic base (Figure 2) to maintain the plasticized PVC phase as slightly basic, which was expected to obtain a strong fluorescence response.

Initially, we examined a simple enzyme reaction on a substrate-immobilized hydrophobic coating-aqueous solution interface by using plasticized PVC containing lipophilic substrate and lipophilic base. Typical fluorescence images are shown in Figure 3. A fluorescence response was successfully obtained from PVC coating, when the sample solution contained ALP-labeled antibody (ALP-anti rabbit IgG). However, the introduction of a buffer solution without ALP-labeled antibody provided very weak background fluorescence as shown in Figure 3b. In order to investigate this response in detail, substrate-immobilized plasticized PVC with or without lipophilic base was prepared on the capillary inner wall and a buffer solution with or without ALP-labeled antibody was introduced; the results are shown in Figure 4. When the plasticized PVC contained a lipophilic base and an ALP-labeled antibody buffer solution, fluorescence intensity increased significantly. However, other experimental conditions did not result in any fluorescence response. These results suggested that the enzyme reaction proceeded smoothly at the organic

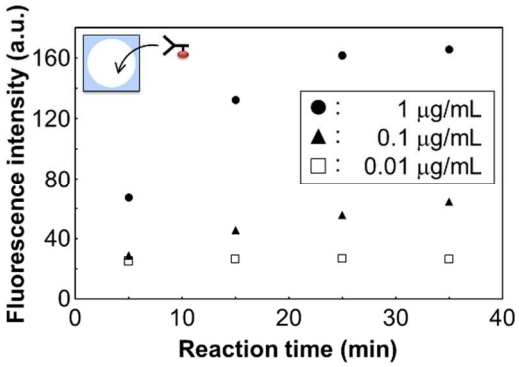

Figure 5 Response profiles of plasticized PVC coatings towards different concentrations of ALP-labeled antibody solutions.

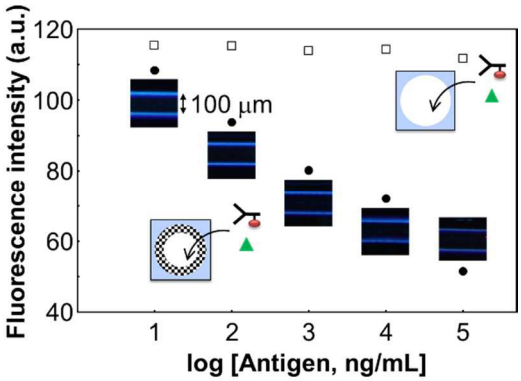

Figure 6 Effect of the hydrogel layer on the fluorescence response of plasticized PVC coatings containing fluorescent ALP substrate toward ALP-labeled antibody solutions containing different antigen concentrations.

(PVC-coating)-aqueous interface in the presence of the lipophilic base tetrahexylammonium hydroxide. Since the lipophilic base played an important role in keeping the PVC phase slightly basic, a strong fluorescence response was obtained. It should be noted that there was minimum fluorescence increase owing to spontaneous decomposition of the substrate, mediated by the lipophilic base (see Figure 4). The neutralization of lipophilic base and "acidic" lipophilic substrate in PVC phase may have avoided the decomposition of phosphate group in substrate molecule. Furthermore, the concentration of ALP-labeled antibody was optimized. For this purpose, sample solutions containing different concentrations of ALP-labeled antibody (ALP-anti rabbit IgG) were introduced into a capillary with substrate-immobilized hydrophobic coating; the fluorescence response profiles are shown in Figure 5. Increasing concentration of antibody resulted in a stronger fluorescence response, and 95% response time was approximately 25 min in each case. Among the tested conditions, 1 μg/mL ALP-labeled antibody gave the strongest signal within a reasonable reaction time. Thus, this condition was used for further experiments.

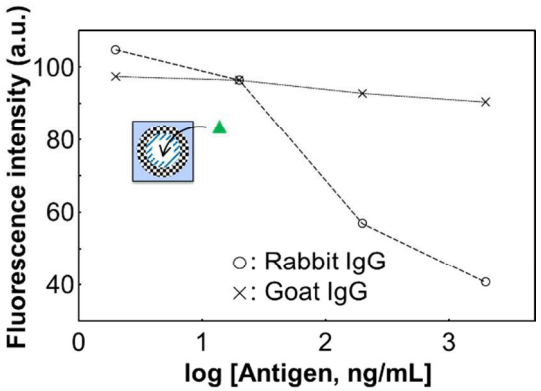

Figure 7 Response curve of a single-step capillary sensor for rabbit IgG.

Effect of hydrogel coating

The effect of hydrogel coating on the substrate-immobilized hydrophobic coating was examined. For this purpose, two capillaries were prepared. The first capillary contained both, a substrate-immobilized hydrophobic coating, and a hydrogel coating on the inner wall. The second capillary contained only a substrate-immobilized hydrophobic coating. Sample solutions containing varying antigen concentrations and constant ALP-labeled antibody concentrations were introduced into these capillaries (Figure 6). In case of the capillary without hydrogel coating (□), fluorescence intensities remained constant even if the antigen concentrations were varied. This suggested that fluorescence response was governed by the concentration of ALP-labeled antibody, and immunoreaction or antigen concentration did not affect the enzyme reaction at the organic-aqueous interface. On the other hand, in case of the capillary with a hydrogel coating (●), fluorescence intensity decreased as antigen concentration increased. This suggested that the amount of ALP-labeled antibodies that reached the substrate-immobilized hydrophobic coating surface decreased with increasing antigen concentration. Although the composition of the immunocomplex between the ALP-labeled antibodies and antigens was not clear, we could successfully implement the concept of immunocomplex separation by hydrogel.

Preparation of single-step EIA capillary and evaluation of antigen-antibody reaction selectivity

A single-step EIA capillary was prepared by immobilizing a third layer containing ALP-labeled anti-rabbit IgG antibody, and the selectivity of antigen-antibody reactions was examined. Rabbit IgG or goat IgG solutions were introduced into each capillary, and the fluorescence intensity was measured (Figure 7). When rabbit IgG (○) was introduced, fluorescence intensity decreased with increase in antigen concentrations. In contrast, introduction of goat IgG solutions (×) resulted in fairly constant fluorescence signals even when the antigen concentrations varied. These results suggested that the single-step EIA, which included a simple introduction of the sample by capillary action, and the selective detection of analyte was quite successful.

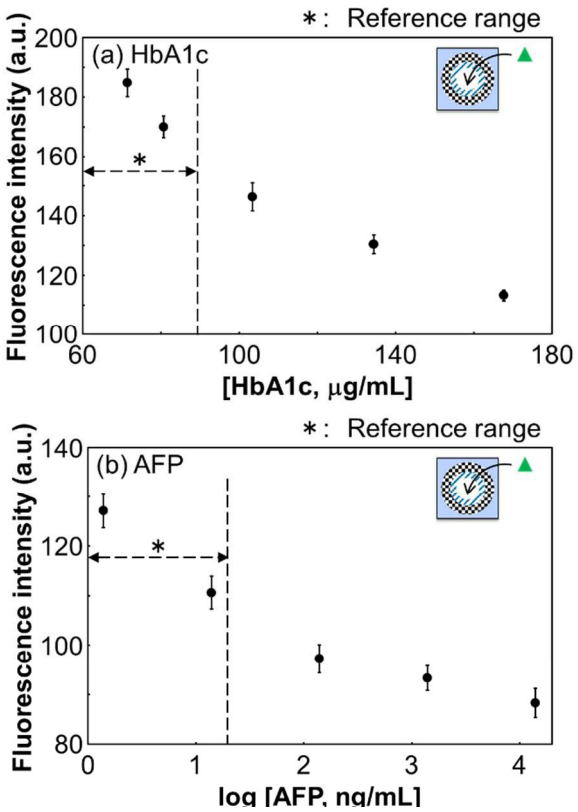

Figure 8 Response curve of various single-step capillary sensors immobilizing different ALP-labeled antibodies. (a) HbA1c, (b) AFP

Measurements of diagnostic marker proteins (HbA1c and AFP)

In order to expand the present method towards detection of other proteins, we examined the feasibility of measuring HbA1c and AFP, proteins important in medical diagnostics. We introduced standard sample solutions into each capillary, which immobilized the corresponding ALP-labeled antibodies within soluble coating. In case of HbA1c, commercial standard sample solutions were diluted (100-fold) in buffer solution (Tris-buffered saline; 50 mM of Tris-HCl solution, 150 mM of NaCl solution, pH 7.4) to adjust the response range (Figure 8). As expected, fluorescence intensity decreased as antigen concentration increased. Interestingly, although the same amount of ALP-labeled antibodies were immobilized within the soluble coatings, the dynamic ranges for HbA1c and AFP differed. Since the molecular weights of HbA1c (ca. 68,000 Da)[51] and AFP (ca. 70,000 Da)[52] are almost similar, and since monoclonal antibodies were used in these experiments, most likely the composition of antigen-antibody complexes formed in capillary and their molecular sizes was the same. One explanation might be the difference in antibody binding affinity/avidity to each antigen. When sandwich ELISA of AFP was carried out, calibration curve was almost saturated above 10–100 ng/mL range.[53] However in case of HbA1c, the

calibration curve increased within the same concentration range.[54] This suggested that affinity/avidity between anti-AFP antibody towards AFP was stronger than anti-HbA1c antibody towards HbA1c. Although further investigations are necessary, our results demonstrate a wide applicability of the present method in case of several other proteins simply by changing the ALP-labeled antibody. Additionally, each dynamic range covered a clinically important reference concentration range for HbA1c and AFP.

CRP antigen measurement in serum

The utility of the present method was examined for the measurement of CRP antigen in serum samples. For this application, an increase in background fluorescence owing to ALP, intrinsically present in human serum, was of concern. Therefore, we measured the response of hydrophobic coating in capillary towards a dilution series of control serum. The influence was relatively small when serum was diluted more than 32 times (Figure S1†). Thus, we introduced a standard CRP sample solution and spiked serum (diluted 1:100) into each capillary, which immobilized ALP-labeled anti-CRP antibody in the soluble coating (Figure 9). As antigen concentration increased, fluorescence intensity in each sample decreased similar to previous experiments. Furthermore, the fluorescence intensity for standard and diluted serum sample was almost overlapping. This indicated that non-specific reaction or non-specific adsorption due to serum impurities was minimal when serum was used in a 100-fold dilution. Thus, the

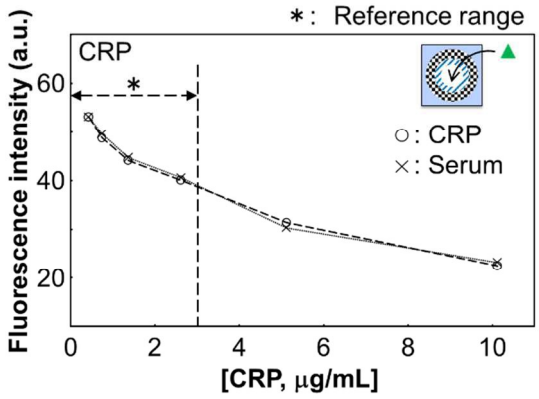

Figure 9 Response curve of single-step capillary sensors for CRP. (○): CRP standard solution, (×): spiked serum

EIA capillary sensor is applicable for measurement of various serum proteins.

Conclusions

We developed a single-step, easy-to-use EIA capillary sensor consisting of a three-layered structure composed of substrate-immobilized hydrophobic coating, hydrogel coating, and a soluble coating containing ALP-labeled antibody. First, a lipophilic fluorescent substrate was synthesized and a simple enzyme reaction at the substrate-immobilized hydrophobic coating-aqueous solution interface was examined; the effect of lipophilic base in this reaction was investigated. Further, the molecular sieving effect of hydrogel and immunoreaction-dependent molecular sieving was investigated. Lastly, the utility of this method in measuring clinically-important proteins was investigated. An antigen concentration-dependent response was successfully obtained by a simple introduction of sample solution via capillary action. As compared to the previous microfluidic studies, comparable sensitivity and analysis time was realized with easy and single-step procedure as immunochromatography and paper-based fluidic devices. This one-step EIA capillary sensor is expected to contribute towards the development of bioassay tools for simultaneous medical diagnosis of several analytes by simply arraying different single-step capillary sensors.

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Notes and references

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† Electronic supplementary information (ESI) available. See DOI:

‡ These authors contributed equally to this work.

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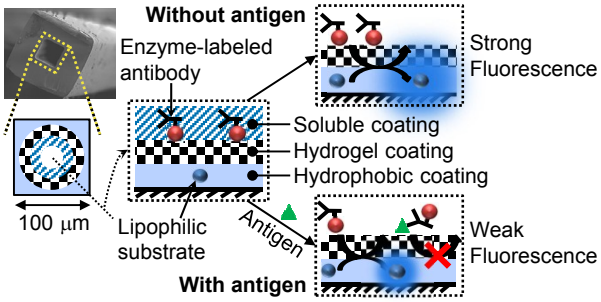

A single-step, easy-to-use enzyme immunoassay capillary sensor, composed of substrate-immobilized hydrophobic coating, hydrogel coating, and soluble coating containing enzyme-labeled antibody was developed.