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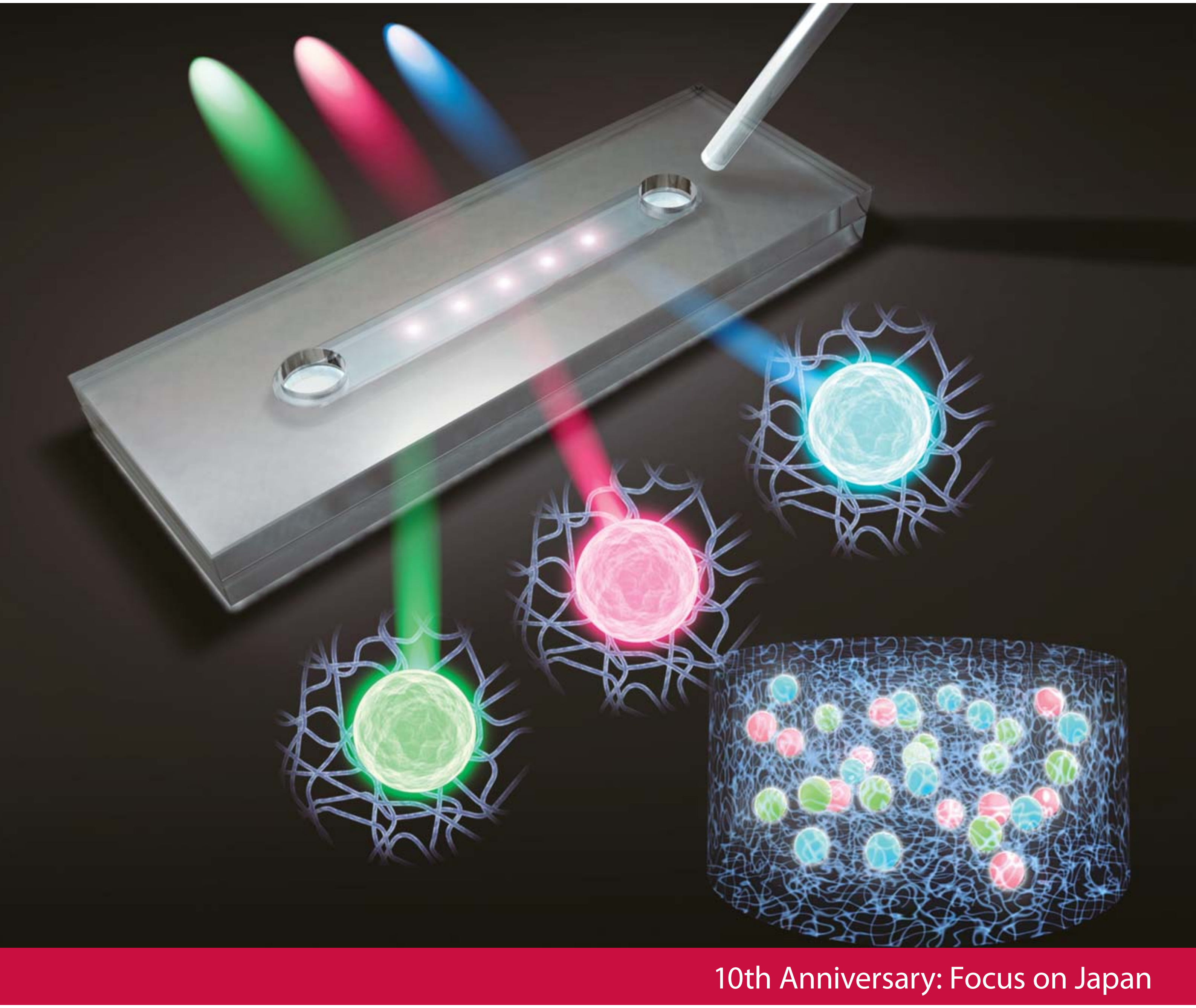

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Immuno-pillar chip: a new platform for rapid and easy-to-use immunoassay

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Immuno-pillar chip: a new platform for rapid and easy-to-use immunoassay†‡

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We present a new rapid and easy-to-use immunoassay chip which we have named the immuno-pillar chip. It has hydrogel pillars, fabricated inside a microchannel, with many antibody molecules immobilized onto 1 μm diameter polystyrene beads. To evaluate the chip performance, we applied it to the sandwich assay of C-reactive protein (CRP), α-fetoprotein (AFP) and prostate-specific antigen (PSA), a cardiac and inflammation marker, tumors and prostate cancer markers, respectively. For detection of disease markers, we confirmed the chip provides rapid analysis (total assay time of about 4 min) with high sensitivity, it is easy-to-use (no special skills are needed), and it uses small volumes of the sample and reagent (0.25 μL each). Moreover, multiplex assay for three biomarkers was also possible. Additionally, the immuno-pillar chip has a big advantage of having hardly any influence on the assay results even if the introduction quantities of the sample or reagents are different.

Introduction

Immunoassays are widely used for medical diagnostics, drug discovery, biological studies, *etc.*[1] and they represent some of the most vigorous activities in the field of μ-TAS (micro-total analysis systems) and Lab-on-a-Chip systems.[2–5] The reduced consumption of sample and reagents and provision of rapid analyses are inherently possible by miniaturizing immunoassay systems.

Since the first chip-based immunoassay experiment,[6] detection sensitivity and assay times have been further improved by immobilizing antibody molecules onto beads as a solid support.[7–11] This is called the bead-bed format. We have published a number of studies that successfully demonstrated the power of this format in improving detection sensitivity and reducing assay times to detect human secretory immunoglobulin A,[7] carcinoembryonic antigen,[12] interferon-gamma,[13,14] and brain natriuretic peptide.[15] More recently, a compact automated apparatus using this format was developed, and we have made rapid and sensitive assays of total and Japanese cedar pollen specific immunoglobulin E in human sera,[16] methamphetamine in hair[17] and osteocalcin in human sera.[18] However, liquid handling for this format needs to use relatively high pressure due to packing of beads inside the microchannel, and it is difficult to remove bubbles in the liquids, though highly sensitive detection and rapid assay are realized. Therefore, the development of simpler formats is desired for clinical applications such as point-of-care (POC) diagnostics.[19–22]

On the other hand, immunoassay chips with integrated three-dimensional (3-D) hydrogel structures using immobilized antibodies have been reported.[23,24] Immobilization of antibodies within 3-D hydrogel structures offers several advantages over 2-D surface immobilization used by most immunoassay chips, including immobilization capacity and antibody activity. Moreover, in this format, the high pressure which is required for the bead-bed format is unnecessary and there is no problem with bubbles because the hydrogel structures inside the microchannel are arranged sparsely. However, though the detection sensitivity is high, the assay time is not very fast. This probably results from the size and shape of the 3-D hydrogel structures. The chip fabrication method is also somewhat complicated.

Here we report a new on-chip platform called the immuno-pillar chip. It provides rapid analysis and high sensitivity, requires just small volumes of sample and reagents, is easy-to-use, and is low cost. By using a combination of immobilization of antibody molecules onto 1 μm diameter polystyrene beads and 3-D hydrogel pillars inside a microchannel, the immuno-pillar chip overcomes the disadvantages of the two above-mentioned formats.

Materials and methods

Materials and reagents

Phosphate-buffered saline (PBS, pH 7.4) solution was prepared with phosphate-buffered saline powder (Wako Pure Chemical Industries, Osaka, Japan) and Millipore water (Millipore Co., France). Carbonate buffer (CB, pH 9.7) was prepared with sodium carbonate and sodium hydrogen carbonate (Wako Pure Chemical Industries). Polystyrene bead suspension (1 μm in diameter with 1% CV) was purchased from Polysciences, Inc. (Warrington, PA); the beads were washed with CB prior to use.

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Human C-reactive protein (CRP) was obtained from Scipac Ltd. (Kent, UK). Affinity chromatographically purified goat anti-human CRP polyclonal antibody and FITC-conjugated goat anti-human CRP polyclonal antibody were acquired from Bethyl Laboratories, Inc. (Montgomery, TX). Human α-fetoprotein (AFP), affinity chromatography purified rabbit anti-human AFP polyclonal antibody and HRP-conjugated mouse anti-human AFP monoclonal antibody were purchased from Dako Japan (Kyoto, Japan). HRP-conjugated mouse anti-human AFP monoclonal antibody was labeled with DyLight 649 using a labeling kit (Pierce, Rockford, IL). Human prostate-specific antigen (PSA) and mouse anti-human PSA monoclonal antibody were obtained from Funakoshi Co. (Tokyo, Japan). Anti-mouse IgG Alexa Fluor 555 was purchased from Invitrogen Co. (Carlsbad, CA). Human serum was purchased from Gemini Bio-Products Inc. (West Sacramento, CA). Bovine serum albumin (BSA) was obtained from Pierce (Blocker™ BSA). A polyethylene glycol (PEG)-based photocrosslinkable prepolymer solution (MI-1) and a photoinitiator solution (PIR-1) were purchased from Kansai Paint Co., Ltd. (Osaka, Japan).

Fabrication of immuno-pillar chip

Fig. 1 shows a photograph and a schematic design of the microchip. It was fabricated using a conventional injection molding technique by Sumitomo Bakelite Co., Ltd. (Tokyo, Japan). The microchip substrate (70 mm × 30 mm × 0.75 mm) was a cyclic olefin copolymer (COC). The COC substrate is hydrophobic with a surface contact angle of 95°. Forty straight rectangular microchannels were fabricated on a COC substrate. The width, depth and length of each rectangular microchannel were 1000, 50, and 6500 μm, respectively. The inlet and outlet diameters were each 0.9 mm. An injection molding part with inlet and outlet through-holes as well as microchannels were bonded to another COC substrate by thermal bonding technique.

Anti-human CRP coated beads were prepared in a 1.5 mL microtube. The anti-CRP solution (200 μL, 1 μg mL^{-1}) was added to the polystyrene beads (100 μL), and the suspension was stirred gently at room temperature, followed by overnight incubation at 4 °C. After incubation, the anti-CRP coated beads were washed with PBS and blocked with 1% BSA for 45 min at room temperature. Typically, 400 μL of the anti-CRP coated beads in PBS solution (A solution) were obtained. A mixed solution (P solution) of the photocrosslinkable prepolymer (PP) and photoinitiator (PI) was prepared in another 1.5 mL microtube. The PI solution (10 μL) and Millipore water (100 μL) were added to the PP solution (200 μL), and the mixture was stirred using a vortex. 100 μL of A solution were added to 100 μL of P solution in a 1.5 mL microtube. Then, the mixture was stirred gently at room temperature, and was used as stock solution (AP solution) to fabricate the immuno-pillars.

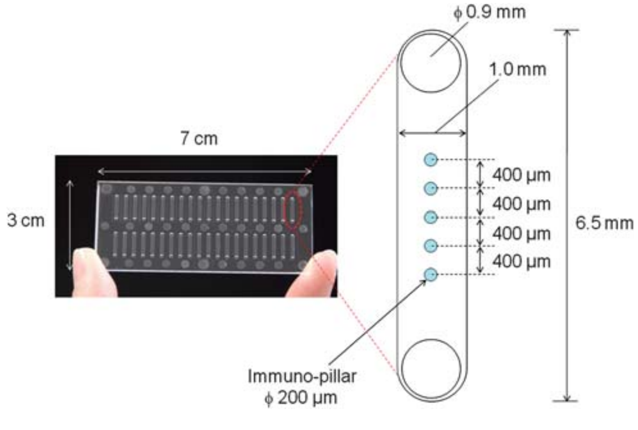

Fig. 1 Photograph and schematic of the microchip.

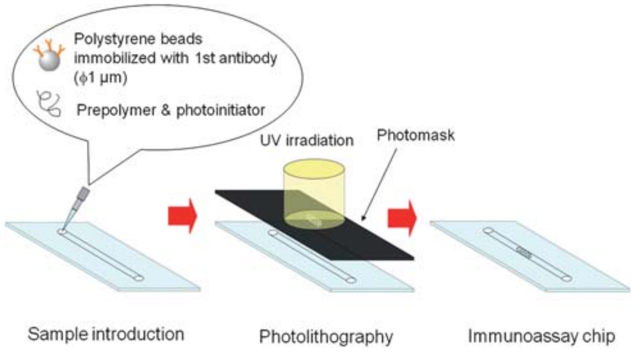

Fig. 2 Schematic illustration of fabrication procedure for the immuno-pillar chip.

A schematic illustration of the fabrication procedure for the immuno-pillar chip is shown in Fig. 2. First, 0.25 μL of the AP solution was introduced into the microchannel by using a pipette (PIPETMAN®, Gilson S.A.S., Villiers le Bel, France) and then the microchannel was filled with the AP solution. Second, UV light (365 nm, 20 mW) from a mercury lamp was irradiated for *ca.* 10 s through a photomask covering the microchannel. The photocrosslinkable prepolymer was polymerized in the microchannel according to the mask pattern. Then, the exposed areas became hydrogel pillars which included many anti-CRP antibody immobilized beads. Third, the non-polymerized solution was removed from the outlet by suction using a vacuum pump, and the microchannel with hydrogel pillars was washed with PBS. Finally, to prevent non-specific binding to the surface of the microchannel and hydrogel pillars, 1% BSA in PBS was introduced and then kept in the microchannel for 1 h at room temperature before washing it out with PBS. We called this microchip the immuno-pillar chip. In this work, the diameter and height of each immuno-pillars were 200 and 50 μm, respectively. In all, five immuno-pillars were fabricated inside the microchannel and their arrangement is shown in Fig. 1. The immuno-pillars were relatively isolated from each other. Although the pillars were physically fixed inside the microchannel without chemical bonding to the surface, they did not move or break during the high-speed flushing. This arrangement leads to their easy fabrication and also to easy handling of liquids for assay.

The fabrication protocol of the immuno-pillar chips for AFP and PSA was the same as the above-mentioned protocol for CRP.

Assay procedures

Fig. 3 shows a schematic illustration of the assay procedure with the immuno-pillar chip. First, 0.25 μL of the sample solution (1%

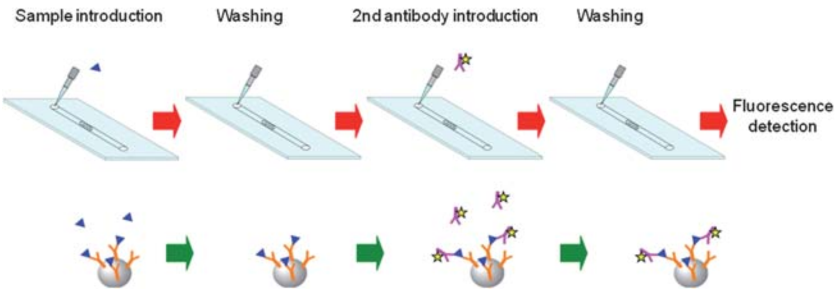

Fig. 3 Schematic illustration of assay procedure with the immuno-pillar chip.

BSA–PBS) was manually introduced into the microchannel from the inlet with a pipette. After incubation, the unreacted sample solution was sucked from the outlet with the pipette, and then the microchannel was washed three times with PBS. Second, 0.25 μL (1 μg mL^{-1}) of the fluorescent-labeled secondary antibody solution (PBS) was introduced into the microchannel. After incubation, the microchannel was washed three times with PBS to remove the unreacted fluorescent-labeled secondary antibody solution. All of these operations were done using a pipette for liquid transfers. Finally, the fluorescence signal from an immuno-pillar was detected by using an inverted fluorescence microscope (IX-71, Olympus, Tokyo, Japan) equipped with a CCD camera (EB-CCD, Hamamatsu Photonics, Hamamatsu, Japan) and three lasers (air-cooled Ar ion laser, λ = 488 nm, 35LAL415, Melles Griot, Carlsbad, CA; diode-pumped solid state laser, λ = 532 nm, CDPS532S, JDS Uniphase Co., San Jose, CA; He–Ne laser, λ = 632.8 nm, 05LHP991, Melles Griot). The fluorescence intensity was obtained by averaging the fluorescence signal of 3–5 immuno-pillars. The fluorescence signal was calculated by dividing fluorescence intensity of the entire pillar by the area of the pillar using commercial software (Aqua-Cosmos, Hamamatsu Photonics, Hamamatsu, Japan).

In the case of CRP assay, an Ar ion laser was used to detect FITC. In the case of AFP and PSA assay, a diode-pumped solid state laser and a He–Ne laser were used to detect Alexa Fluor 555 and DyLight 649, respectively. In the case of multiplex assay, the Ar ion laser for CRP, the diode-pumped solid state laser for PSA and the He–Ne laser for AFP were used to detect FITC, Alexa Fluor 555 and DyLight 649, respectively. Each fluorescence was measured by using the appropriate combination of filters (Olympus) built into the microscope.

Results and discussion

Immunoassay

In order to evaluate the immuno-assay chip, we carried out a sandwich immunoassay for CRP as a model sample. CRP is a known cardiac and inflammation marker. In our assay procedures, total assay time was obtained by adding all the times for the first and second incubations, washings and detection. In the present study, the time for the first incubation was equal to that of the second one. The total time for washings and detection was below 2 min. Thus, the total assay time for our assay was calculated by multiplying the incubation time by two plus 2 min.

First, we evaluated the performance of the immuno-pillar chip for standard CRP solutions (1% BSA–PBS). The CRP calibration curves are shown in Fig. 4(A–C). The error bars reflect ±1 standard deviation based on fluorescence measurements of 3–5 immuno-pillars. Incubation times of 1 min, 3 min and 5 min corresponded to *ca.* 4 min, 8 min and 12 min of the total assay time, respectively. In spite of the very short assay times, all three calibration curves showed that the fluorescence signal increased with increasing CRP concentration. The calibration curve for the 3 min incubation was very similar to that of the 5 min one. The limit of detection (LOD) for 1 min, 3 min and 5 min incubations was ~100 pg mL^{-1} which gave a signal at 3 SDs (standard deviations) above the background; the slope of the calibration curve for the 1 min incubation was gentle. From

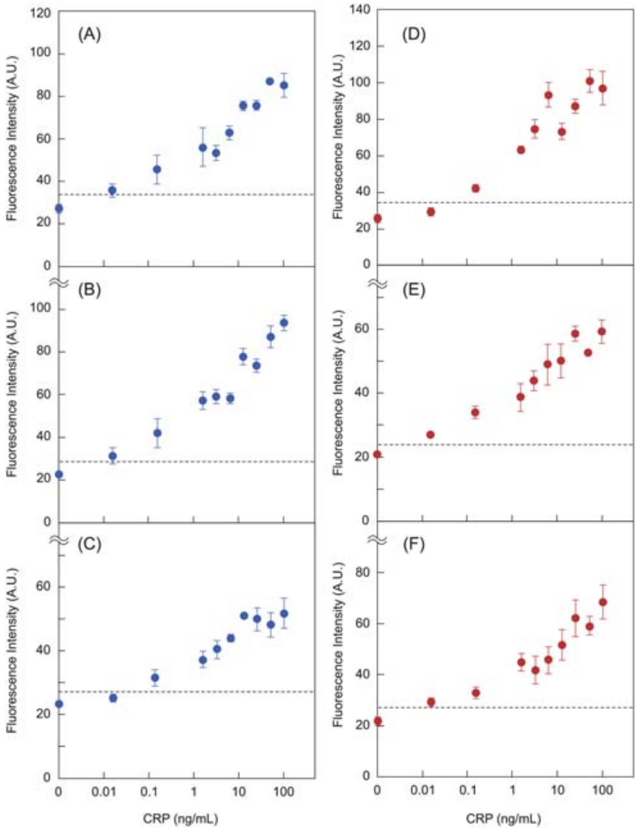

Fig. 4 Calibration curves of CRP. (A) 5 min incubation (standard samples). (B) 3 min incubation (standard samples). (C) 1 min incubation (standard samples). (D) 5 min incubation (serum samples). (E) 3 min incubation (serum samples). (F) 1 min incubation (serum samples). The dashed line represents the signal level at 3 SDs above the background.

these results, we confirmed that the immuno-pillar chip had a high sensitivity in spite of the short assay time and easy assay procedure. For actual CRP diagnoses, the cut-off values for several diseases are at higher concentrations.[25] The detection range of the immuno-pillar chip could be easily changed by using a lower concentration of fluorescence labeled secondary antibody or a lower power of the excitation laser beam (data not shown). In particular, shifting the detection range for a high sample concentration is not difficult, although it is very difficult to shift it for a low concentration.

The features of the immuno-pillar chip of rapid assay and high sensitivity were derived from the immuno-pillar itself and the 1 μm diameter polystyrene beads for the immobilization of capture antibodies. Although we had no detailed information on the pore size of the immuno-pillar, it was likely 100 nm or more because the fluorescence particles leaked from the pillars when we made them containing 100 nm diameter fluorescence particles instead of antibody-immobilized polystyrene beads (ESI†). The diffusion of the antigen and antibody in the pillar was not slow (Fig. S1 and S2, ESI†). Therefore, the antigen and antibody could smoothly enter the pores of the pillar and could diffuse within the pillar. Moreover, a pillar contained about 32 700 beads according to a calculation for our experimental conditions. We thought the numbers of reaction sites for an antigen–antibody reaction were dramatically increased by using these beads inside the pillar. In our previous studies, we demonstrated that the packed bead-bed immunoassay showed good performance regarding assay speed and detection sensitivity.[12–18] It was obvious that the smaller the diameter of the beads was, the greater the number of reaction sites (surface area of the beads) per unit volume (Fig. S3, ESI†). However, smaller beads often lead to serious problems in the assay, including generation of a back-pressure during the solution introduction procedure and the need to remove air bubbles. We previously found that the use of beads with a diameter below 25 μm was very difficult.[14] In the immuno-pillar chip, however, it was possible to use 1 μm diameter beads because the beads were fixed into hydrogel pillars rather than in a bead-bed format.

Next, we evaluated the performance of the immuno-pillar chip for serum samples which were spiked with the desired concentrations of CRP. The CRP calibration curves are shown in Fig. 4(D–F). Like the results of standard samples, the results showed good performance for serum samples. The scattering of the signal intensity in the high concentration region might be due to influence of proteins in the serum. The LOD for 1 min, 3 min and 5 min incubations was ~100 pg mL^{-1}, which had a signal at 3 SDs above the background.

Table 1 The LODs for CRP, AFP and PSA in 1% BSA–PBS and serum samples were obtained by using the immuno-pillar chips

Sample		Incubation time		
		1 min	3 min	5 min
CRP	In 1% BSA–PBS	~100 pg mL^{-1}	~100 pg mL^{-1}	~100 pg mL^{-1}
	In serum	~100 pg mL^{-1}	~100 pg mL^{-1}	~100 pg mL^{-1}
AFP[a]	In 1% BSA–PBS	~100 pg mL^{-1}	~100 pg mL^{-1}	~100 pg mL^{-1}
	In serum	~1 ng mL^{-1}	~1 ng mL^{-1}	~100 pg mL^{-1}
PSA[a]	In 1% BSA–PBS	~5 ng mL^{-1}	~1 ng mL^{-1}	~100 pg mL^{-1}
	In serum	~5 ng mL^{-1}	~5 ng mL^{-1}	~100 pg mL^{-1}
Triplex	In serum	—	—	~100 pg mL^{-1}

[a] In the assay of AFP and PSA, the concentration of the fluorescent-labeled secondary antibody solution was 50 μg mL^{-1} and 50 μg mL^{-1}, respectively.

Moreover, we also assayed the standard and serum samples of AFP and PSA. All results are summarized in Table 1. From these results, we judged the immuno-pillar chip had great potential for analysis of serum samples and would be suitable as an immunoassay chip for POC diagnostics because it was quick, had high sensitivity, was easy-to-use, and needed only small sample and reagent volumes.

Multiplex immunoassay

The immuno-pillar chip developed here can be easily applied to multiplex assay. For instance, if the pillars are made with three kinds of beads where three different antibodies are immobilized respectively, a triplex assay becomes possible (Fig. 5(A)). Since the fabrication protocol of these pillars is the same as the above-mentioned CRP assay, the number of each kind of beads in the pillar is one third, *ca.* 10 000. We carried out a triplex assay for CRP, AFP and PSA by fabricating a suitable immuno-pillar chip. AFP and PSA are well known markers for tumors and prostate cancer, respectively. 0.25 μL of serum solution which was spiked with CRP, AFP and PSA was used as the sample.

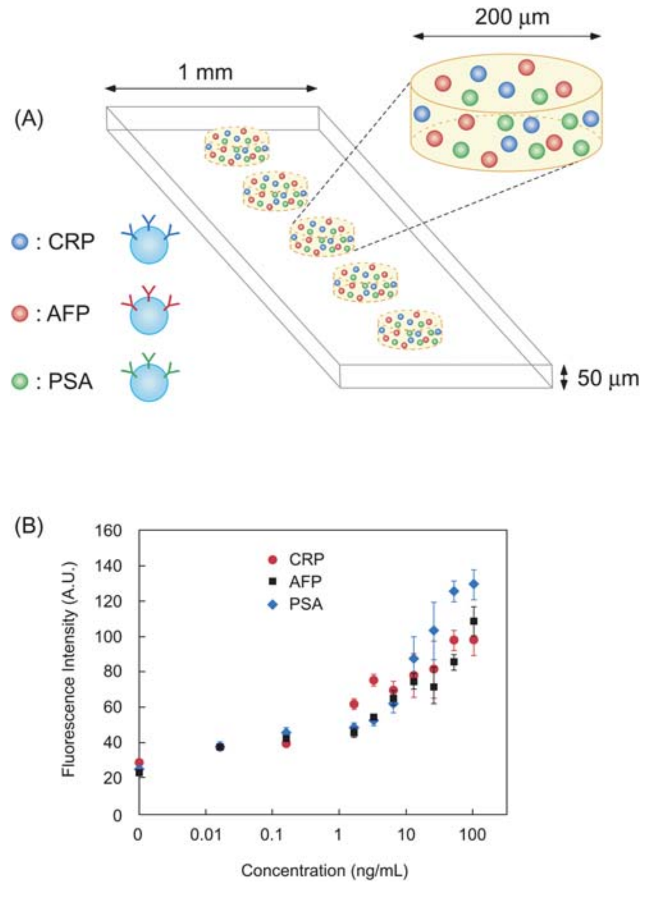

Fig. 5 (A) Schematic illustration of the immuno-pillar chip for the triplex assay. (B) Calibration curves of CRP, AFP and PSA.

0.25μL (200 μg mL^{-1} for CRP, 50 μg mL^{-1} for AFP and 100 μg mL^{-1} for PSA) of the mixture solution (PBS) of fluorescent-labeled secondary antibodies was used as the secondary antibody solution. The incubation times for the sample and secondary antibody solutions were constant at 5 min for each. The total assay time was about 15 min for one sample. The assay results are shown in Fig. 5(B). All three calibration curves showed that the fluorescence signal increased with increasing sample concentration, and good performance was obtained for the multiplex assay in serum. The LOD for CRP, AFP and PSA was ~100 pg mL^{-1} which gave a signal at 3 SDs above the background (Table 1). We noted that the LODs for three markers in this assay were almost the same as that of the single assay. Although the cut-off values for actual diagnoses of these three markers are at higher concentration,[26] the detection range of the immuno-pillar chip can be changed by the experimental conditions as shown before. From these results, we judged the immuno-pillar chip had great potential for multiplex assay of serum samples. Moreover, shortening of the assay time should be achieved by optimizing the number of beads in the pillar and the concentrations of secondary antibodies.

Quantitative assay of small volume sample

Even if the introduction quantities of the sample or reagent differ, the immuno-pillar chip has hardly any influence on the assay results. From the viewpoint of simplicity of assay, this feature is a big advantage of the immuno-pillar chip which does not have other microfluidic devices. We investigated the dependence of fluorescence intensity in CRP assay on the introduction quantities of the sample and FITC-labeled secondary antibody solution (Fig. 6). In the experiment looking at the dependence for the sample and reagent quantities, the introduction quantities of FITC-labeled secondary antibody solution and sample solution were 0.5 μL (200 μg mL^{-1}) and 0.5 μL (100 ng mL^{-1}), respectively. The incubation times for the sample and secondary antibody solution were constant at 5 min for each. The assay results were the same when the introduction quantities of the sample or FITC-labeled secondary antibody solution were changed. Their CV values were 1.6% and 2.3%, respectively. This was attributed to the numbers of the antigen and the secondary antibody that take part in the antigen–antibody reaction being limited around the pillar. In the assay using the immuno-pillar chip, since the sample and secondary antibody solutions introduced into the microchannel did not flow, the sample and the antibody that could take part in the antigen–antibody reaction were limited to only the amounts which reached the pillar by diffusion within the incubation time. From a calculation using the diffusion constant D ($=6 \times 10^{-7}$ cm^2 s^{-1})[27] of IgG (M_w: 140 000), the diffusion distance during 5 min (longest incubation time) was estimated to be about 190 μm. The diffusion distance for the FITC-labeled secondary antibody might be shorter than this value. Since the molecular weight of CRP (M_w: 11 500–135 000)[28] is only a little bit smaller than that of IgG, the distance through which it can move may be almost the same. Thus, only CRP and secondary antibody, which were present within a distance of about 190 μm from the pillar, could participate in the antigen–antibody reaction inside the pillar. This did not depend on the introduction quantities of the sample and FITC-labeled secondary antibody solutions.

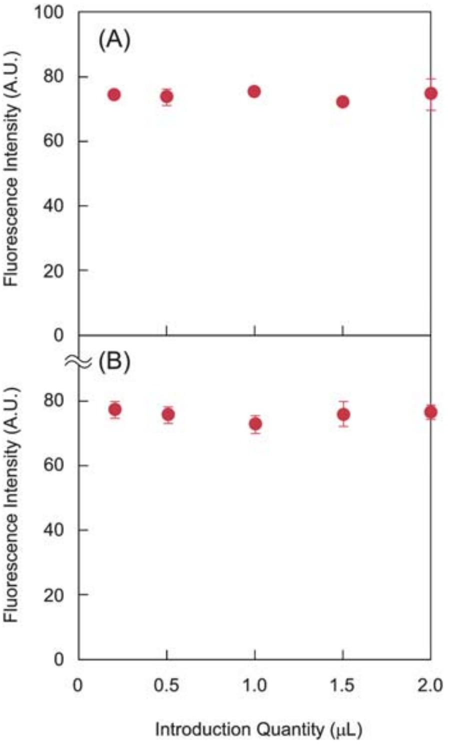

Fig. 6 Dependences of fluorescence intensity for the introduction quantities of the sample and FITC-labeled secondary antibody solution. (A) The fluorescence intensities were measured by changing the introduction quantity of the sample solution (from 0.2 μL to 2 μL). The introduction quantity of the secondary antibody solution was fixed at 0.5 μL. (B) The fluorescence intensities were measured by changing the introduction quantity of the secondary antibody solution (from 0.2 μL to 2 μL). The introduction quantity of the sample solution was fixed at 0.5 μL.

In general, when the sample is 1 μL or less, a small difference in the amount of the sample being assayed deteriorates the measurement accuracy. So, a number of microdevices that can dispense minute samples accurately have been pursued.[29–31] However, complex structures or extra apparatuses such as high voltage power supplies and pumps are necessary for these devices. The immuno-pillar chip can quantitatively assay minute samples without these added components. This feature is extremely valuable for clinical diagnosis applications.

Conclusions

In this work, we designed and fabricated a new platform, the immuno-pillar chip, for rapid and easy-to-use immunoassay and we demonstrated its application. The immuno-pillar chip had hydrogel pillars which included many antibody-immobilized 1 μm diameter polystyrene beads. The chip had ideal features for a practical immunoassay chip: assay was rapid (taking less than 4 min) and sensitive; it was easy-to-use (no pump was required for liquid handling); only small volumes of sample and reagents (0.25 μL) were needed; and professional knowledge or special skills for the assay were not necessary. Moreover, multiplex assay was also possible. The immuno-pillar chip developed here has great potential for practical immunoassay and POC diagnostics. If a small and low cost detection system can be developed such as with an LED and an avalanche photodiode or a photomultiplier tube, a combination of the immuno-pillar chip and such a detection system would lead to a mobile POC diagnostics apparatus. Such an apparatus would be very useful for emergency medicine situations and in home care. Indeed, we are in the progress of developing a compact detection system now. Furthermore, we are examining the liquid handling system regarding automation of the introduction and removal of washing buffer and reagents.

Acknowledgements

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