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Rapid and simple immunosensing system for simultaneous detection of tumor markers based on negative-dielectrophoretic manipulation of microparticles

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ABSTRACT

We report here a rapid, simple, and simultaneous immunosensing method for two tumor markers, alpha-fetoprotein (AFP) and carcinoembryonic antigen (CEA), by applying the negative-dielectrophoretic (n-DEP) manipulation of microparticles. Microparticles modified with different antibodies rapidly accumulated to designated areas of poly(dimethylsiloxane) (PDMS) fluidic channels modified with different antibodies within 1 min by n-DEP upon the application of AC voltage. The presence of specific antigens, AFP or CEA, permitted the irreversible capture of microparticles via the formation of immuno-complexes between the PDMS surface and the microparticles. Uncaptured microparticles redispersed after switching off the AC voltage. The fluorescent intensity from the irreversibly captured microparticles allowed us to determine the concentration of AFP and CEA in the sample. Neither the unreacted analytes nor the microparticles required separation steps, since we detected the fluorescent signals only from the microparticles captured on the PDMS surface. The detectable concentration range shifted to lower values when the amount of the antibody on the PDMS surface increased. The range for both AFP and CEA assays was 0.1–100 ng/mL, which was sufficient to cover the concentration required for the medical diagnoses. We simultaneously detected the concentrations of AFP and CEA by using a device, with two channels modified for different antibodies. Since n-DEP was used for the rapid manipulation of the microparticles toward the PDMS surface, the time required for the assay was substantially short; 1 min for forcing and 5 min for redispersion of the microparticles and sensing.

1. Introduction

In recent years, immunoassays have attracted significant interest as a bioanalytical method to determine biomarkers for high-throughput screening of diseases in their early stages and for monitoring the condition and recurrence of these diseases following medical treatments. The use of a single marker determination, however, is insufficient for an exact diagnosis of a disease due to individual variation. Therefore, researchers have focused on and developed immunodevices for multianalyte sensing to detect biomarkers simultaneously [1–3].

Both multilabeling and multispacing systems have been used to accomplish multisensing. Multilabeling has been accomplished by using different labels, including enzymes [4–6], radioisotopes [7–9], lanthanide chelates [10,11], fluorescent dyes [12,13], and metal compounds [14]. The distinguishable signals allow us to determine targets simultaneously. However, combining multiple labels in a single assay often leads to overlapping signals [13,14] and a decrease in the analytical efficiency due to different optimal conditions for each assay [2]. Spatially separated microarrays of antigen or antibody have also been applied for simultaneous immunosensing using optical [15,16], fluorescent [17,18], electrochemical [19,20], and piezoelectric [21] signals. For detecting signals from different regions, specific apparatus with significant spatial resolution are required, such as charge-coupled device (CCD) cameras or multi-channeled electrochemical workstations. An affinity microcolumn and capillary have also developed to apply toward the multisensing of targets [22,23].

We reported the development of the microfluidic device for the immunosensing based on the microparticle trapping by the negative-dielectrophoresis (n-DEP) [24,25]. However, a period as

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long as 40 min was required for the single assay. Moreover, subsequent introduction of the solutions containing antigen and antibody was also required. We also reported the rapid patterning with particles and cells and immobilization on the substrate based on the combination of the n-DEP and cross-linking chemistry [26–28]. In a previous contribution, we applied the rapid patterning method for particles to the sandwich-type immunosensing systems using accumulation and redispersion of microparticles based on n-DEP [29]. The use of n-DEP manipulation of microparticles restricted the time taken for assays to within 3 min and accomplished separation-free sensing of unreacted target molecules and microparticles. Furthermore, the detection range was tunable as a function of the time during the accumulation of the microparticles via n-DEP. In this study, we applied the n-DEP device to the simultaneous sandwich-type immunosensing of two tumor markers.

As significant tumor markers, we used alpha-fetoprotein (AFP) and carcinoembryonic antigen (CEA) in this research. AFP, a glycoprotein of approximately 70 kDa was synthesized from the fetal yolk sac, gastrointestinal tract, and liver. AFP levels increase in the cases of patients with conditions related to hepatocellular cancer, yolk sac cancer, liver metastasis from gastric cancer, and nasopharyngeal cancer [30,31]. To detect AFP levels, various immunoassay methods have been exploited, such as fluorescence spectrometry [32], electrochemical spectrometry [33], inductively coupled plasma mass spectrometry (ICPMS) [11,34], and atomic absorption spectrometry [35]. Carcinoembryonic antigen (CEA) has been also used as a tumor marker to indicate lung, liver, pancreatic, breast, ovarian, and prostate cancers [31]. For detecting CEA, researchers have developed sensing systems based on chemiluminescent [36], time resolved fluorescent [37], electrochemical [38,39], and thermal lens microscopic methods [35]. AFP and CEA levels in the normal human serums have been used for cancer diagnosis, and the cut-off values for positive tests are <10.0 and <2.5 ng/mL, respectively [40,41].

Sandwich-type multisensing systems for tumor markers such as CEA, AFP, CA125, and CA15-3 have been reported using electrochemical reactions [1,39] and chemiluminescent immunoassays [42]. However, these methods require step-wise operations. Therefore, simplicity (one-step), rapidity, and miniaturization are also desired in newly developed multisensing systems. In this paper, we demonstrate rapid, simple, and simultaneous immunosensing by applying the n-DEP manipulation of microparticles recently reported. We placed poly(dimethylsiloxane) (PDMS) fluidic channels modified with different antibodies above an interdigitated array (IDA) electrode and introduced microparticles modified with different antibodies into the channels. Subsequently, an appropriate AC voltage was applied to the IDA template to drive the formation of particle patterns on the ceiling of the PDMS channels. When the specific antigens, AFP, and CEA, were present in the microparticles suspensions, the accumulated microparticles were irreversibly trapped in the designated areas of the PDMS channels via the formation of sandwich-type immuno-complexes between the PDMS surface and the microparticles. The fluorescent intensity from the trapped microparticles allowed us to determine the concentration of the analytes in the samples since the irreversibly captured microparticles depended on the concentration of the analytes. Neither the unreacted analytes nor the microparticles were required since only the fluorescent signals from the microparticles irreversibly captured on the PDMS surface were detectable. The channels modified with different antibodies for AFP and CEA could define new multisensing systems based on the spatial separation of fluorescent signals. Since the n-DEP phenomena allowed for the rapid manipulation of the microparticles toward the PDMS surface, the time required to complete an assay was substantially shorter compared to those of conventional assays.

2. Materials and methods

2.1. Materials

All chemicals of reagent grade were obtained from Wako Chemicals (Osaka, Japan) and used as received. We purchased mouse monoclonal anti-AFP antibody (AFP antibody-1), rabbit polyclonal anti-AFP antibody (AFP antibody-2), and AFP protein from Lab Vision Co., Inc. (Fremont, CA, USA) [43]. Two different mouse monoclonal anti-CEA antibodies (CEA antibody-1 and CEA antibody-2) and CEA protein were purchased from Abcam (Cambridge Science Park, UK) as liquids. The stock solution of each antibody and antigen was prepared by dilution with phosphate-buffered saline (PBS), which consisted of 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, and 137 mM NaCl (pH 7.6; conductivity 1.54 S/m). We prepared the aqueous solutions using distilled, deionized water from a Milli-Q filtration unit (Nihon Millipore Ltd., Tokyo, Japan) and an Aquarius GS-200 system (Advantec, Tokyo, Japan). PDMS elastomer and a curing agent (Silpot 184) were obtained from Dow Corning Toray Silicone Co., Ltd. (Tokyo, Japan). We purchased polystyrene latex microparticles with incorporated yellow-green (YG) fluorescent dye (Fluoresbrite Plain Microspheres, 2 μm diameter) from Polysciences Inc. (Warrington, PA, USA) and indium tin oxide (ITO) glass from Sanyo Vacuum Industries Co., Ltd. (Tokyo, Japan).

2.2. Fabrication of ITO-IDA electrode and PDMS microfluidic channels

The DEP immunosensing device consisted of an ITO-IDA electrode and a PDMS microfluidic channel substrate. We fabricated the ITO-IDA electrode with two comb-type band arrays with 10 microband electrode elements by photolithography and a wet-etching process. Each microband electrode was 1.0 mm long and 20 μm wide, separated by 50 μm from adjacent microbands. The IDA electrode exposed to solution was by 750 μm × 750 μm, defined by using an insulating layer of SU-8 with 1.4 μm thickness. We characterized the IDA electrode with cyclic voltammetry and found that each microband of the IDA electrode independently demonstrated the same electrochemical behavior.

The PDMS microfluidic channel (150 μm channel width, 50 μm gap, and 14 μm height) was fabricated by curing the pre-polymer on a silicon mold. The SU-8 masters of four microfluidic channels were patterned by photolithography [44], and the surface of the mold was treated with 3,3,4,4,5,5,6,6,6-nonafluorohexyl trichlorosilane (ShinEtsu Corp., Tokyo, Japan) to prevent adherence of the channel materials to the mold. We degassed the elastomer with a PDMS pre-polymer and curing agent in a 10:1 (v:v) ratio for 1 h and poured the mixture over the silicon mold. After curing for 1 h at 80 °C in an oven, we peeled the PDMS microfluidic channel form from the mold.

2.3. Immobilization of antibodies on the PDMS microchannels and microparticles

The AFP antibody-1 and CEA antibody-1 were immobilized on each PDMS microfluidic channel by physical adsorption. The hydrophobic PDMS channel was placed on a flat PDMS sheet, which was pretreated with O_2 plasma for 30 s to make the surface hydrophilic. The hydrophilic pretreatment of PDMS sheet ensured suction of the antibody solution into the PDMS channel to immobilize the antibody on the hydrophobic PDMS channel. We injected 3 μL aliquots of 1.0 or 10 ng/mL AFP antibody-1 and 10 ng/mL or 10 μg/mL CEA antibody-1 into every other line of the microfluidic channels. After incubation overnight at 4 °C, the antibody-immobilized PDMS microfluidic channel was washed three times with PBS. We modified the yellow-green fluorescent

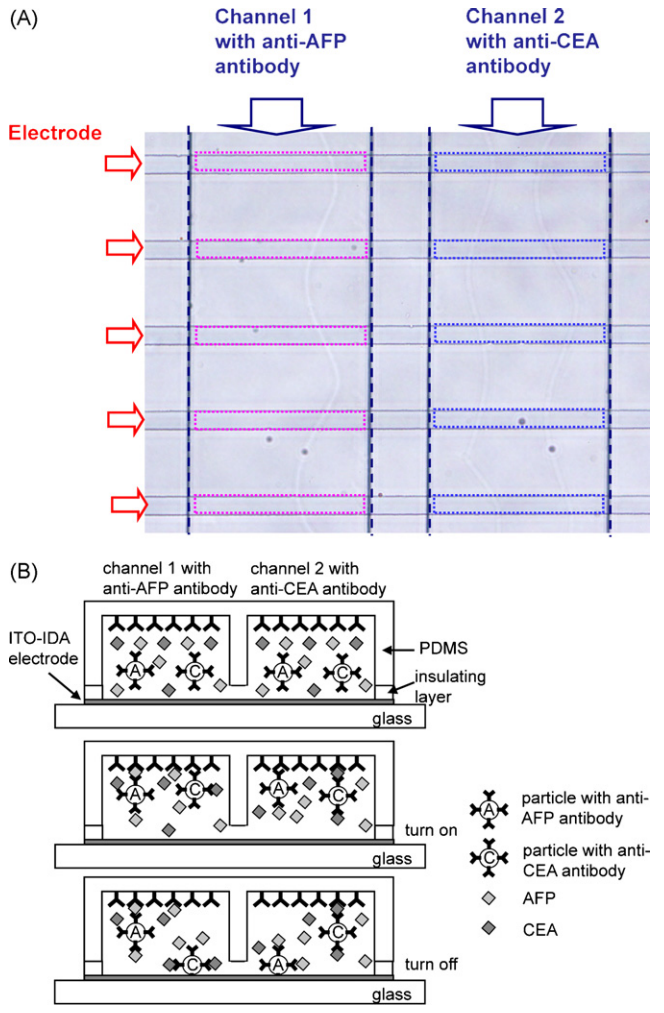

Fig. 1. n-DEP device with two channels modified with different antibodies. (A) Top view picture of the n-DEP device. (B) Schematic representation (side view) of the accelerated immunosensing based on the n-DEP particle manipulation.

microparticles with the antibody as follows. Microparticles were incubated with a 10 μL aliquot of 10,000-fold diluted AFP antibody-2 or 10 ng/mL CEA antibody-2 at 4 °C overnight. The resulting antibody-coated microparticles were also washed three times with PBS.

2.4. Manipulation of probe microparticles and immunosensing via the irreversibility of n-DEP behavior

We prepared standard solutions (0.1 ng/mL–10 μg/mL) of AFP and CEA by serial dilution in PBS to calibrate the immunodevice. The sample suspension was prepared by mixing the antibody-coated fluorescent microparticles with various concentrations of analyte solutions. The electrical conductivity of all the final sample mixtures containing 1.2×10^9 particles/mL microparticles was 0.36 S/m, which was suitable to manipulate microparticles using n-DEP in the device [24,25,29]. We dropped a 20 μL sample suspension onto the ITO-IDA electrode, and then perpendicularly covered the antibody-immobilized PDMS microfluidic channel to the IDA electrodes to assemble the immunosensing device. Fig. 1A shows the optical microscope image of the IDA electrode and the two perpendicularly overlapping PDMS microfluidic channels modified with different antibodies. Since some fluorescent microparticles remained on the IDA electrode, the overall channel height of the immunosensing device was approximately 18 μm (PDMS height 14 μm, insulating layer 2 μm, particle diameter 2 μm). We introduced the sample suspension, containing microparticles and analytes into the DEP device and applied an AC voltage of 10 V_{pp} (peak-to-peak voltage) with a 2 MHz frequency to the IDA template to accumulate microparticles on the PDMS surface via the n-DEP force as shown in Fig. 1B. The fluorescence from the accumulated microparticles was measured in the designated areas (see the dashed-square zones in Fig. 1A) under a fluorescent microscope with a CCD camera. The voltage was applied for 1 min to capture the microparticles on the PDMS surface via irreversible immunoreactions. For multisensing using two microparticles with different antibodies, we twice repeated the particle alignments over 40 s with a 1 min interval to increase the opportunity for specific antibody to contacting the microparticles on the PDMS surface. After turning off the voltage, the microparticles that were not captured redispersed. The fluorescence could be intensely obtained from the microparticles attached to the surface by focusing the microscope on particles trapped. Therefore, no separation step for unreacted molecules was required. The difference in the fluorescence intensities immediately before and 5 min after turning the DEP voltage off was calculated and used as the degree of the immunoreaction at the PDMS surfaces. Each data point of fluorescent intensity was evaluated as the average of four data points which were obtained from four different experiments.

3. Results and discussion

3.1. Rapid immunoassays of AFP and CEA

We performed the AFP sensing by using the microparticle manipulation with the n-DEP. Fig. 2A shows fluorescent images of the microparticles before and 5 min after the AC voltage was turned off. We treated the channels 1 and 2 with PBS containing 1.0 and 10 ng/mL AFP antibody-1, respectively. After applying the AC voltage, the microparticles immediately moved and integrated into the designated areas for fluorescence measurements indicated in the dot squares in the microfluidic channels (shown as broken lines, see Fig. 2A(a)) within a few seconds (see Fig. 2A left). The microparticles were irreversibly captured at the ceiling of the PDMS channel via the sandwich formation of an antibody–antigen–antibody immuno-complex in the presence of 1.0 ng/mL AFP while unreacted microparticles redispersed into the bulk solution by Brownian motion and sedimented as a result of gravity after switching the AC voltage off (see Fig. 2A(a) right). As a result, fluorescence intensities in channels 1 and 2, 5 min after the voltage was turned off, were approximately 23% and 56% of the initial intensities, respectively. On the other hand, almost all the microparticles redispersed in the absence of AFP antigen (see Fig. 2A(b) right). Therefore, we evaluated the immunosensing performance from the redispersion behavior of the microparticles after the voltage was turned off. Slight signal intensities were observed from the channels without AFP due to the non-specific adsorption of fluorescent microparticles.

Fig. 2B shows the calibration plots for AFP obtained using the channels treated with 1.0 and 10 ng/mL AFP antibodies-1. The intensity from the captured microparticles in the PDMS channel treated with 1.0 ng/mL AFP antibody increased with increasing AFP concentration up to 1000 ng/mL and saturated at higher concentrations (see Fig. 2B(a)). When the PDMS channel was treated with 10 ng/mL antibody, the intensities from the captured microparticles were larger than those obtained in the channel treated with 1.0 ng/mL, especially in the lower concentration region. The increase of the antibody concentration used for the treatment of PDMS channel may result in the increase of the amount of the immobilized antibody. These results indicated that we could con-

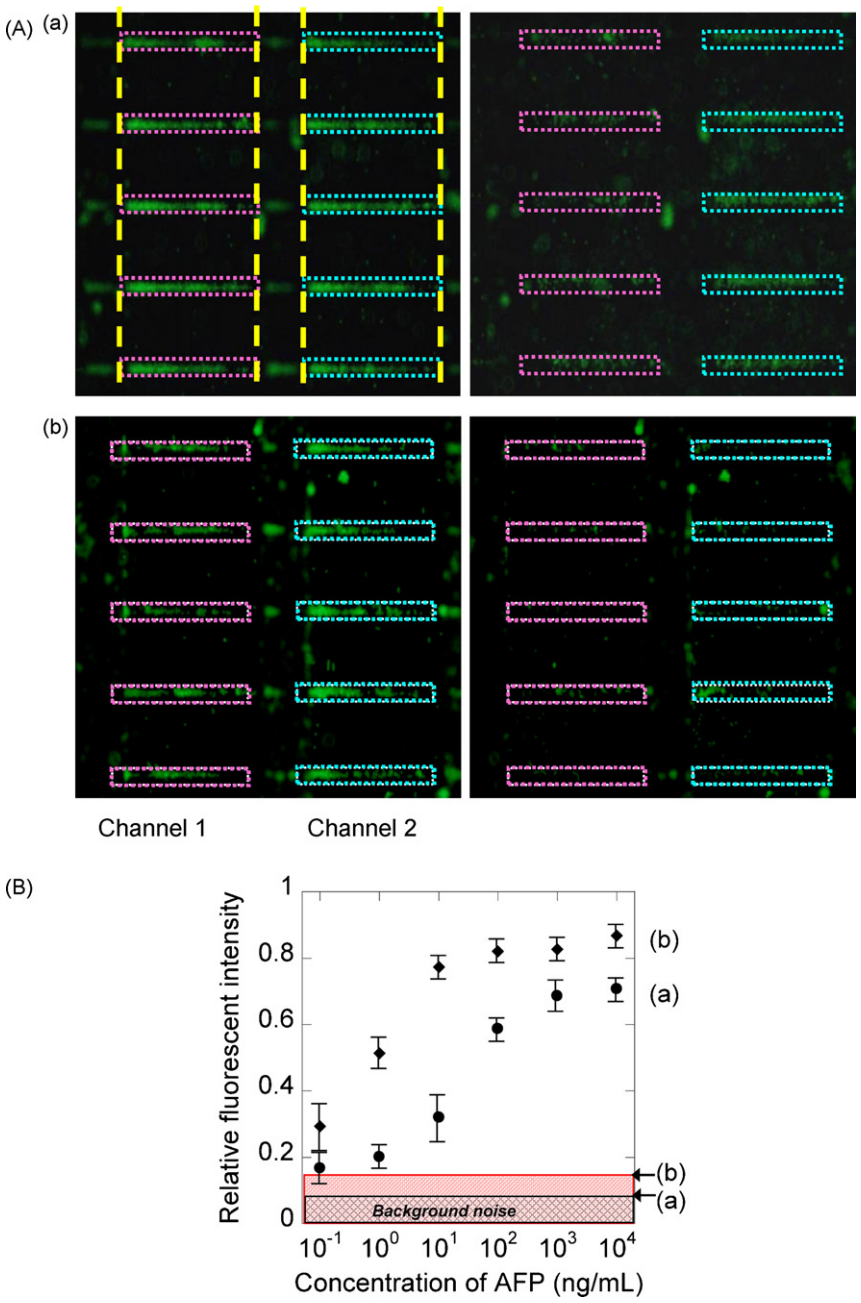

Fig. 2. AFP assay using the n-DEP device. (A) Fluorescent images of the microparticles before (left picture) and 5 min after (right picture) the AC voltage was turned off (a) in the presence of 1.0 ng/mL AFP and (b) in the absence of AFP. Channels 1 and 2 were treated with 1.0 and 10 ng/mL AFP antibody-1, respectively. (B) Calibration plots for AFP obtained using the channels treated with (a) 1.0 and (b) 10 ng/mL AFP antibodies-1.

trol the detectable concentration range by regulating the antibody concentration for the treatment of the PDMS channels. Shadowed boxes in Fig. 2B depict the background noise levels due to the non-specific adsorption of microparticles for the channels treated with 1.0 and 10 ng/mL mouse AFP antibody. The signal intensities obtained in the concentration range of interest were higher than those for the background noise levels.

The CEA assay was also conducted by using a similar method for AFP. We prepared the probe microparticles by incubating microparticles in the PBS containing 10 ng/mL CEA antibody-2 and introducing the resulting microparticles into the channels 1 and 2 treated with 10 ng/mL and 10 μg/mL CEA antibody-1, respectively. Fig. 3A shows the redispersion tendency of the CEA assay. Microparticles captured at the designated areas in the PDMS channels in the presence of 1.0 ng/mL CEA were significantly larger than those captured in the absence of CEA antigen (see Fig. 3A(a) right and (b) right). Fluorescence intensities in channels 1 and 2, 5 min after the voltage was turned off, were approximately 37% and 74% of the initial intensities, respectively. Fig. 3B shows the calibration plots for CEA obtained using the channels treated with 10 ng/mL and 10 μg/mL CEA antibodies. The intensities depended on the CEA concentration, and the detectable range shifted to the lower concentration region when we treated the PDMS channel with a higher CEA antibody concentration. No clearly irreversible trapping occurred without n-DEP accumulation of the microparticles in this experimental period, indicating that n-DEP accumulation facilitated the rapid immunoreactions between the PDMS surface and the microparticles. A concentration as low as 0.1 ng/mL for both AFP and CEA could be detected within 6 min by using the n-DEP accelerated system for the immunoreactions. Therefore, the

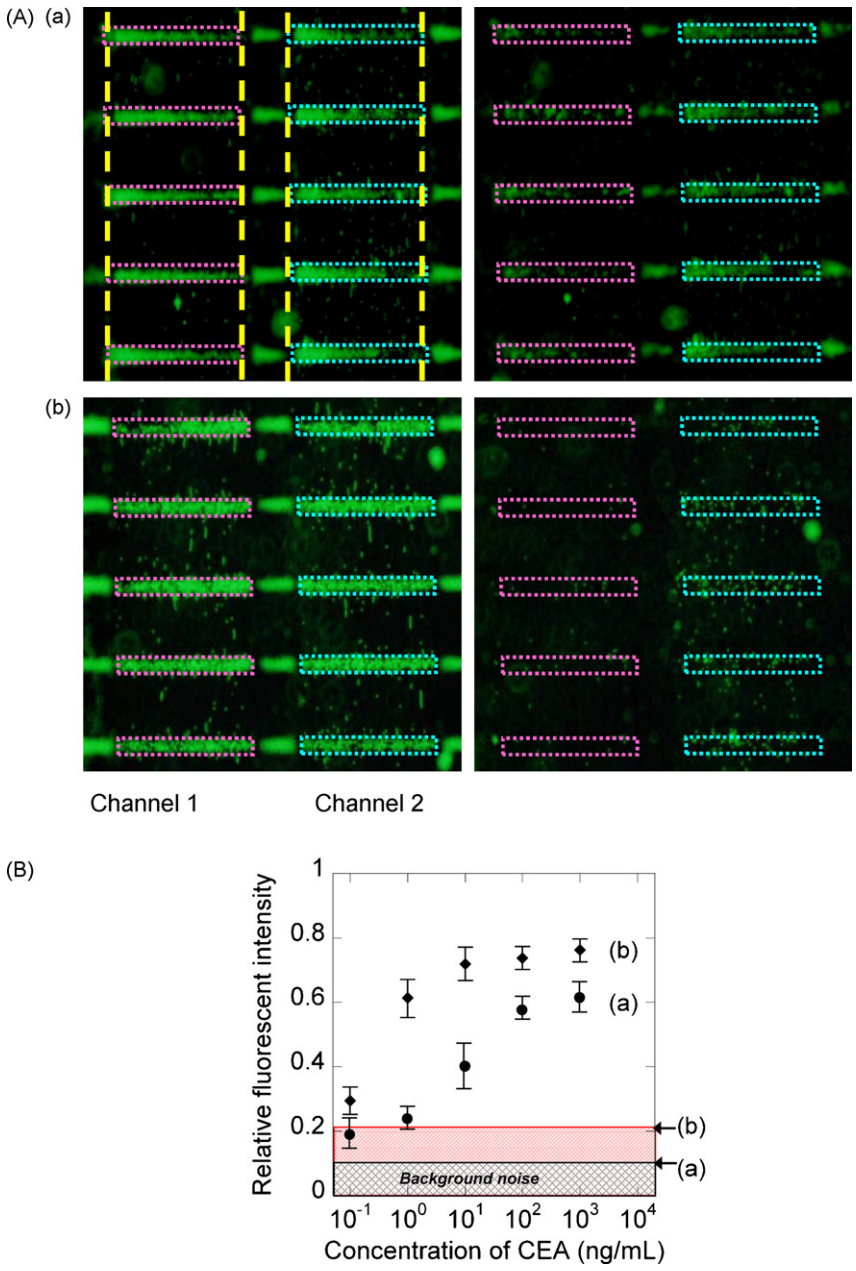

Fig. 3. CEA assay using the n-DEP device. (A) Fluorescent images of the microparticles before (left picture) and 5 min after (right picture) the AC voltage was turned off (a) in the presence of 1.0 ng/mL CEA and (b) in the absence of CEA. Channels 1 and 2 were treated with 10 ng/mL and 10 μg/mL CEA antibody-1, respectively. (B) Calibration plots for CEA obtained using the channels treated with (a) 10 ng/mL and (b) 10 μg/mL AFP antibodies-1.

detection range in this work fully covered the concentration levels required to measure serum samples. Moreover, using the present multi-channel based immunosensing device, the detection range could be controllable as a function of the analyte concentration.

3.2. Simultaneous multianalyte immunosensing performance for the tumor markers CEA and AFP

To develop the multi-sensing device, the PDMS channels 1 and 2 were treated with 1.0 ng/mL AFP antibody-1 and 10 ng/mL CEA antibody-1, respectively. Two different microparticles modified with AFP antibody-2 and CEA antibody-2 were added to the sample solution containing various concentrations of AFP and 20 ng/mL CEA. We immediately introduced the suspension solution containing two different microparticles and both analytes to the DEP device to measure the fluorescent intensity. Fig. 4A shows the relative fluorescent intensity as a function of the AFP concentration obtained from channel 1 for AFP (see Fig. 4A(a)) and channel 2 for CEA (see Fig. 4A(b)). CEA concentrations remained constant at 20 ng/mL in this multi-sensing experiment. The fluorescent intensity obtained from channel 1 for the AFP assay increased with increasing AFP concentration while that obtained from channel 2 for the CEA assay remained practically constant. The calibration curve for the AFP assay in the sample coexisting with CEA corresponded with the curve in the sample containing only AFP (see Fig. 4A(a) and (c)). The results indicated that we could accurately determine the AFP concentration in the presence of other target molecules and the probe microparticles for other targets.

The calibration curve for CEA also characterized the samples with and without 20 ng/mL AFP (see Fig. 4B(a) and (c)). Although the intensities for CEA obtained from the sample without AFP were slightly larger than those from the sample obtained with 20 ng/mL

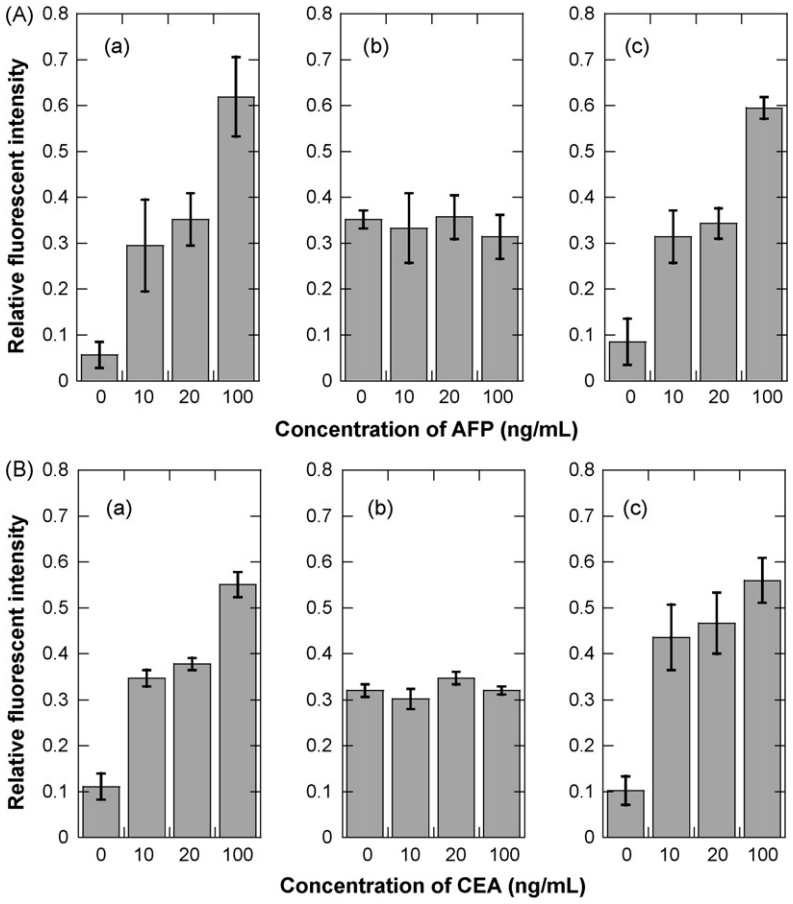

Fig. 4. Relative fluorescent intensities as a function of the (A) AFP and (B) CEA concentrations, respectively. (A) Fluorescent intensities were simultaneously obtained from (a) channel 1 for AFP and (b) channel 2 for CEA of a multi-sensing device injected with sample solutions containing various concentrations of AFP and 20 ng/mL CEA. (c) Obtained from the sample containing only AFP. (B) Fluorescent intensities were simultaneously obtained from (a) channel 1 for AFP and (b) channel 2 for CEA of a multi-sensing device injected with sample solutions containing 20 ng/mL AFP and various concentrations of CEA. (c) From the sample containing only CEA.

AFP, we observed a similar curve for CEA. Signals for AFP obtained from channel 1 were independent of the concentration of CEA (see Fig. 4B(b)). The signal intensities obtained from the multi-sensing system using the mixture of two different microparticles were smaller than those obtained for the single analysis. This fact might be due to a hindrance of the spatial contact of the microparticles with the antibody on the PDMS. The accumulation of microparticles with non-specific antibody would block the objective microparticles from interacting with the designated area of the PDMS surface. We should again note that the time required for the immunoreaction at the surface could be significantly shortened to less than 60 s by the n-DEP process. Total assay time required for the simultaneous determination of two different analytes was also substantially shortened: as little as 6 min, including 1 min for forcing and 5 min for the redispersion of the microparticles. This method, based on the combination of the microparticle manipulation with n-DEP and the microarray for the different antibodies, should be applicable to many immunoassay protocols.

4. Conclusions

We demonstrated the simultaneous immunosensing of AFP and CEA by using a microparticle manipulation based on n-DEP upon the application of AC voltage. Microparticles modified with antibodies for AFP or CEA quickly accumulated to designated areas on PDMS fluidic channels, which were also modified with antibodies for AFP or CEA. The presence of specific antigens, AFP or CEA, permitted the irreversible capture of microparticles by the formation of immuno-complexes. Since uncaptured microparticles redispersed into the solution after the AC voltage was turned off, the fluorescent intensity emitted from the irreversibly captured microparticles increased with increasing AFP and CEA concentrations. The detectable concentration range shifted to lower concentrations when the amount of the antibody at the PDMS surface increased. The range of the present system was 0.1–100 ng/mL for both the AFP and CEA assays, a value range that sufficiently covered the concentrations required for medical diagnoses. We achieved the simultaneous sensing of AFP and CEA by using a device with two fluidic channels modified with different antibodies. In the presence of various concentrations of AFP and 20 ng/mL CEA, the signals of the AFP detection channel increased with increasing AFP concentration while the signals for the CEA detection channel remained constant. Similar results were also obtained for the CEA sensing using the sample containing a constant concentration of AFP (20 ng/mL). The time required for the assay was substantially short, 1 min for forcing and 5 min for the redispersion of the microparticles and sensing. The present multianalyte immunosensing based on n-DEP should be widely recognized as a rapid and simple (one-step) assay system without complicated stepwise processes such as separation and washing.

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