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On-chip graphene oxide aptasensor for multiple protein detection

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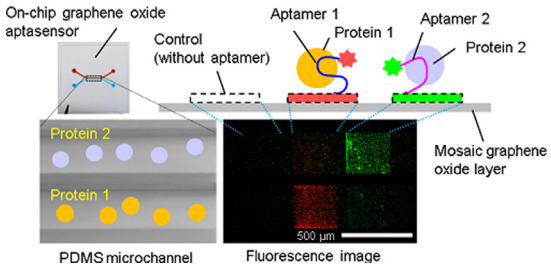
HIGHLIGHTS

- On-chip aptasensor built on graphene oxide surface fixed on solid support.
- Multichannel configuration realizes simultaneous detection of 3–5 samples.
- Versatility of graphene oxide aptasensor formed on solid surface was studied.
- DNA and RNA aptamers immobilized on graphene oxide surface maintain their activity.
- Simultaneous multiple protein detection was demonstrated.

GRAPHICAL ABSTRACT



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ABSTRACT

The versatility of an on-chip graphene oxide (GO) aptasensor was successfully confirmed by the detection of three different proteins, namely, thrombin (TB), prostate specific antigen (PSA), and hemagglutinin (HA), simply by changing the aptamers but with the sensor composition remaining the same. The results indicate that both DNA and RNA aptamers immobilized on the GO surface are sufficiently active to realize an on-chip aptasensor. Molecular selectivity and concentration dependence were investigated in relation to TB and PSA detection by using a dual, triple, and quintuple microchannel configuration. The multiple target detection of TB and PSA on a single chip was also demonstrated by using a 2×3 linear-array GO aptasensor. This work enables us to apply this sensor to the development of a multicomponent analysis system for a wide variety of targets by choosing appropriate aptamers.

Abbreviations: GO, graphene oxide; TB, thrombin; PSA, prostate specific antigen; HA, hemagglutinin; TBA, thrombin binding aptamer; PSAA, prostate specific antigen binding aptamer; HAA, hemagglutinin binding aptamer.

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1. Introduction

Graphene is a two-dimensional sp^2 carbon sheet with atomic layer thickness [1,2]. First, graphene was isolated, and it attracted attention owing to its unique structure and properties. Now graphene oxide (GO) has started to generate considerable interest. GO is an oxidized form of graphene and it also has an atomically thin sheet-like structure, which contains nanometer-sized graphene-like sp^2 domains [3–5]. Of several useful properties of GO, an important one for the present study is that it is an excellent acceptor for fluorescence resonance energy transfer (FRET) in the entire visible wavelength region. This makes GO a promising material for a FRET-based biosensor. In addition, unlike graphene, GO is water dispersible and readily synthesized on a large scale [6,7]. Another useful property is its strong molecular adsorption via a π–π interaction. Thus, GO exhibits a high affinity to aptamers, which are selected single-strand oligonucleotides that bind to specific targets [8,9]. Aptamers offer many advantages as a molecular recognition probe. They have wide variety of targets, can be flexibly designed without loss of activity, and they are easy and cheap to produce. There are DNA and RNA-type aptamers, which we can use to suit our purpose. DNA is more stable and easier to mass-produce than RNA. On the other hand, RNA is flexible in structure and thus an RNA aptamer can have wider variety of targets than a DNA aptamer [10]. All the above features make GO suitable for biological applications.

By using a GO and dye-conjugated aptamer, we can realize a unique type of FRET biosensor. By using GO, the selective biological response of the aptamer can be converted to a fluorescence signal, which is a measurable physical quantity. In detail, the GO aptasensor detection process is as follows. At the initial stage, when the dye-conjugated aptamer is adsorbed on the GO surface via π–π interactions, the dye is located close to the GO surface and its fluorescence is well quenched by GO and is barely observable. If a target is present in the sample, the aptamer forms a complex with the target and leaves the GO surface. At the same time, the dye molecule also leaves the GO surface and the fluorescence of the dye recovers. By employing this concept, early studies on this type of sensor have demonstrated DNA sensors (i.e., sensors for complementary DNA) [11–16] and aptasensors in an aqueous dispersion of GO [17–21].

In contrast, we have proposed and developed a protein detection system that works on a GO layer supported on a solid surface [22]. In our system, the aptamer terminus opposite the dye-labeled end is firmly fixed to the GO surface by a pyrene linker molecule that shows a strong affinity to the sp^2 domains in the GO [5,23,24]. Thus, the aptamer stays close to the GO surface after forming an aptamer/protein complex. The system allows us to undertake molecular detection on a solid surface, unlike with conventional GO aptasensors which operate in a solution. This is a useful point for realizing an on-chip sensor.

Based on this system, we have recently demonstrated the first example of a linear-array GO aptasensor by forming a linear array of several different aptamers on the surface of a GO layer and implementing a microfluidic device across the array [25]. A linear-array GO aptasensor is a smart approach for comparing the responses of different aptamers on a single chip simultaneously. It is also advantageous that the on-chip sensor requires 1 μL or less of solution, and no out-of-chip sample pretreatment, such as labeling or the mixing of liquids. The recognition process is complete within 2 min of adding the sample solution.

In general, aptasensors are versatile because the sensors can be extended to the detection of many different targets by replacing the aptamers. Although we have already demonstrated that this approach works for thrombin detection, we have not yet confirmed whether the versatility is valid for an aptamer immobilized on a solid surface. Thus, in this work, we investigated the extension of our GO FRET aptasensor to other types of DNA and RNA aptamers. We also increased the number of microchannels, which allowed us to obtain a consistent reference-to-sample comparison, by implementing a dual, triple, and quintuple channel microfluidic device on a single chip. If we use one channel as a reference, it can be employed as an internal standard for eliminating the effect of fluorescence degradation caused by laser exposure and other noises. The precise reference comparison allows us to confirm molecular selectivity and the concentration dependence of the fluorescence intensity in relation to thrombin (TB) and prostate specific antigen (PSA) detection by using TB- and PSA-binding DNA aptamers (TBA and PSAA), respectively. We also fabricated the GO aptasensor by using an RNA aptamer, namely hemagglutinin (HA)-binding RNA aptamer (HAA). We found that both DNA and RNA aptamers immobilized on a GO surface maintained the biological activity needed to realize an on-chip aptasensor. Based on these findings, we demonstrated the multiple target detection of TB and PSA on a single chip by using a 2×3 linear-array GO aptasensor.

2. Experimental

2.1. Materials

The DNA and RNA aptamers were purchased from Sigma Genosys. The 5′- and 3′-termini of the aptamer sequences were modified with green fluorescence 6-carboxyfluorescein (FAM) and an amine group. The aptamer sequences were as follows: TBA (5′-GGTTGGTGTGGTTGG-3′) [26], PSAA (5′-TTTAATTAAAGCTCTC-CATCAAATAGCTTTTTTTTTT-3′) [27], and HAA (5′-GUCGGCA-UGCGGUA-3′) [28]. Another TB-binding DNA aptamer, TBA29 (5′-AGTCCGTGGTAGGGCAGGTTGGGGTGACT-3′) [29] was modified with red fluorescence TAMRA instead of FAM. The DNA and RNA aptamers were dissolved in 100 mM phosphate buffer solution and DNase/RNase-free DI water, respectively, and prepared as 100 μM solutions. Phosphate buffer (pH 7.4) and RNase-free DI water (UltraPure™ DNase/RNase-Free Distilled Water) were purchased from Nacalai Tesque Inc. and Life Technologies, respectively. 1-Pyrenebutanoic acid–succinimidyl ester was purchased from Invitrogen. *N*,*N*-Dimethylformamide (DMF) was obtained from Kanto Chemical Co., Inc. Alpha-human thrombin (ICN Biochemicals), alpha-human albumin (ICN Biochemicals), and PSA from human semen (Sigma–Aldrich), were dissolved in DI water. Influenza A H3N2 (A/Aichi/2/1968) Hemagglutinin protein (Sino Biological Inc.) was dissolved in DNase/RNase-free DI water. DI water (Millipore, >18 MΩ cm) and DNase/RNase-free DI water, respectively, were used in all the aqueous solution preparation and washing procedures in the DNA and RNA aptamer experiments.

2.2. Apparatus and measurement conditions

An Olympus BX51-FV300 confocal laser scanning microscope (LSM) was used to obtain fluorescence images. We used a 505–525 nm band-pass filter and a 565 nm high-pass filter for the fluorescence observations of FAM (λ_{max}(abs)/λ_{max}(em) = 494 nm/518 nm) and TAMRA (λ_{max}(abs)/λ_{max}(em) = 555 nm/580 nm), respectively, with a 488 nm laser light source. The fluorescence images were obtained through a glass plate with an objective lens (UPlan Apo 10× LSM or a water-immersion objective lens Plan Apo 40× WLSM (Olympus)) within about 2 min of injecting a certain concentration of protein solution and reference water into the sample and the reference channels, respectively. The reaction time was sufficient for the aptamer to complete protein recognition [22]. The required sample volumes for each channel were smaller than 1 μL. Atomic force microscope (AFM) images were recorded in the AC (tapping) mode under atmospheric conditions using a

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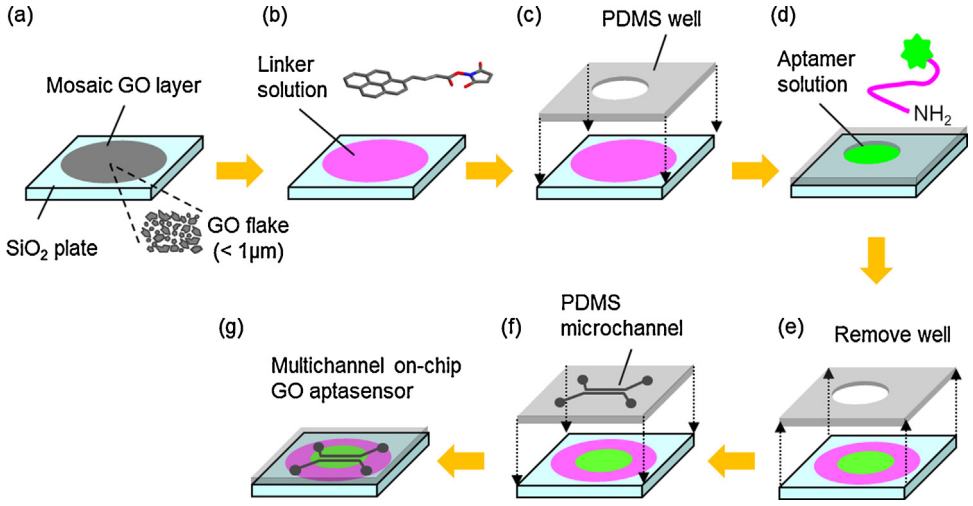

Scheme 1. Multichannel on-chip GO aptasensor preparation procedure.

Dimension FastScan and Dimension 3100 Atomic Force Microscope (Bruker).

2.3. Fabrication of multichannel on-chip GO aptasensor

Scheme 1 illustrates the multichannel on-chip GO aptasensor preparation procedure [22,25]. The GO dispersion was spin coated on a hydrophilic surface consisting of an 18 mm × 18 mm glass plate that was treated with O_2 plasma prior to the GO coating (Scheme 1(a)). The size of the GO pieces and the concentration of the GO dispersion for this procedure are described in detail in Section 3 together with the sensing performance. The GO pieces were then covered with a drop of a 5 mM DMF solution of the linker (1-pyrenebutanoic acid–succinimidyl ester) for 1 h (Scheme 1(b)), rinsed with DMF, and then dried in a nitrogen stream. A polydimethylsiloxane (PDMS) sheet with a hole in it was placed on the plate to form a well (Scheme 1(c)) and 3.5 μL of aptamer solution was poured into the well to cover the GO surface for 1 h (Scheme 1(d)). The amine group at the 3′-terminus of the aptamer was bonded to the pyrene linker by a dehydration reaction and thus the aptamer was immobilized on the GO surface. The PDMS well was removed from the plate and the plate was rinsed with DI water (RNase-free DI water was used for the RNA aptamer) to remove unreacted aptamer, and then dried in a nitrogen stream (Scheme 1(e)). Another PDMS sheet with multiple microchannels was prepared and treated with O_2 plasma for 5 min and then immediately mounted on the glass plate (Scheme 1(f)). The multichannel on-chip GO aptasensor was then ready for detection (Scheme 1(g)). All the above procedures were performed at room temperature.

3. Results and discussion

3.1. Preparation of mosaic GO layer on solid surface as platform for GO aptasensor

First, we describe the preparation of a solid surface covered with a homogeneous GO layer. It would be preferable to have a

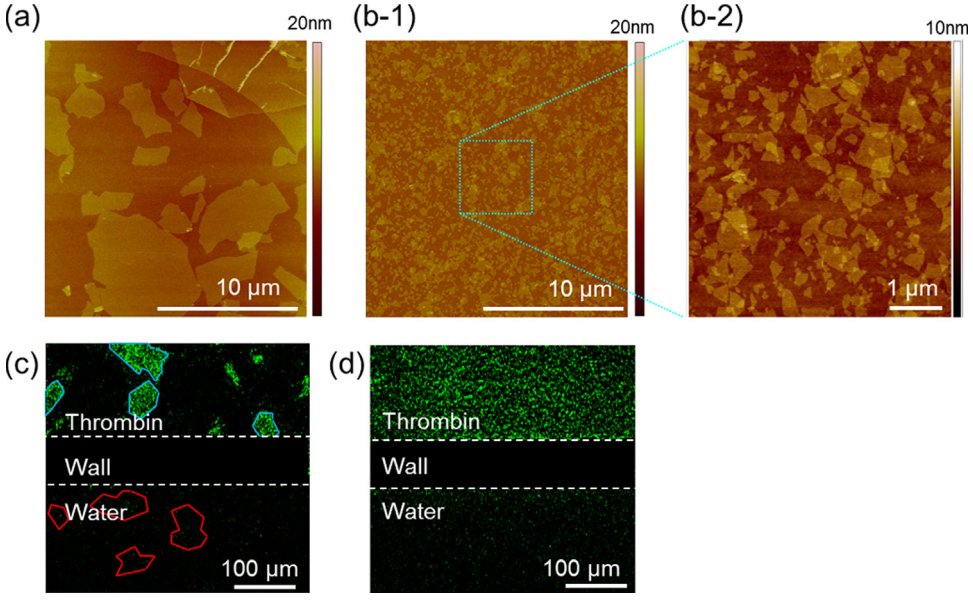

Fig. 1. AFM topographical images of a mosaic GO layer on a SiO_2/Si chip; (a) covered with large GO pieces as synthesized and (b-1, b-2) covered with small GO pieces prepared by ultrasound treatment. (c, d) Fluorescence images of the GO aptasensors taken in the vicinity of the wall between the two microchannels, fabricated on a mosaic GO layer on a SiO_2 surface covered with the same GO dispersion used for (a) and (b-1, b-2), respectively.

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solid surface that is larger than 1 mm × 1 mm in area and covered with a continuous GO monolayer. However, a single GO piece of that size is currently unavailable. Instead, we attempted to cover the surfaces with many small GO pieces with as few overlaps and as high a coverage as possible. We call this a mosaic GO layer, and the surface can be used as a common platform for an on-chip GO aptasensor. We examined the spreading conditions by changing the size of the GO pieces and the concentration of the GO dispersion. An aqueous dispersion of as-synthesized GO contains pieces of various sizes ranging from a few μm to larger than 10 μm. We also prepared a GO aqueous dispersion containing smaller (sub-μm) GO pieces by breaking the large GO pieces into small pieces using ultrasound [30]. The GO dispersion was spin coated on a SiO_2/Si chip and the size of the GO pieces in the dispersion was analyzed with an AFM (Fig. 1(a) and (b)). The coverage of the GO pieces on the SiO_2/Si chip was also estimated from AFM topographical images. We found that when the GO pieces were large, the GO coverage of the substrate was rather poor and not homogeneous with a low GO dispersion concentration, whereas the GO pieces overlapped each other with a high concentration. We determined that the most appropriate size for the GO pieces was a few hundred nanometers since it allowed us to form a homogeneous GO layer with the smallest uncovered area and the least overlapping, (Fig. 1(b)). The coverage exceeded 70% when the absorbance of the GO dispersion was about 0.1 at 400 nm. We used the same conditions determined for the SiO_2/Si chip in a further fabrication process using a glass plate.

We examined the performance of the GO aptasensor in relation to TB detection. TB is a coagulation protein that is present in blood and has a molecular weight about 37 kDa. We used TBA to modify the surface of a mosaic GO layer formed on a glass plate, and then mounted a dual microchannel on it. Fig. 1(c) and (d) compare the fluorescence images of GO aptasensors that were prepared using large and small GO pieces, respectively. The images were obtained after injecting TB solution (100 μg mL^{-1}) into the top channel. The bottom channel was filled with water as a reference. The fluorescence recovery on the GO surface was observed in the sample channel for both chips and there was little fluorescence corresponding to the background signal of the sensor in the reference channel. With the chip prepared using large GO pieces, the coverage of the sample and that of the reference channels were not the same (Fig. 1(c)). In contrast, with the chip prepared using small GO pieces, the fluorescence in the channel was almost homogeneous at least at the resolution of the obtained fluorescence image (Fig. 1(d)). Thus, we can consider the GO coverage of the glass plate to be the same across the same chip. A merit of using a homogeneous mosaic GO layer is that we can easily obtain the detection signal simply by calculating the ratio between the average signal intensity of the sample channel area and that of the reference channel area. As a result, the dual microchannel system allows us to make a precise sample–reference comparison. It also enables us to perform simultaneous observations of the sample and reference signals, which can be used to eliminate the effect of fluorescence degradation caused by laser exposure and other noises. This is an advantage of our on-chip GO aptasensor. Moreover, our on-chip biosensor requires no out-of-chip processes such as labeling or mixing the sample and thus the human errors occurring in the sample preparation procedure can be minimized.

3.2. Sensing performance examined using multichannel configuration

Fig. 2(a) shows the fluorescence image of the GO aptasensor for TB detection, which was larger than 1 mm × 1 mm in area, and on which a dual microchannel was mounted. The fluorescence in the top channel, which was filled with the TB solution, was much brighter than that in the bottom channel, which was filled with water. Fig. 2(b) shows the line profile of the fluorescence intensity, which had an average of about 480 pixel data along the horizontal axis for the detection of 100 μg mL^{-1} TB. The signal, an output of the detector of the LSM (photomultiplier tube), was almost constant in the channel along the vertical axis. The results showed that the mosaic GO layer in the microchannel was formed almost homogeneously on a larger than 1 mm × 1 mm scale. The ratio of the fluorescence intensity of the sample channel to that of the reference channel reached about 4:1. The most important merit of using the multichannel configuration is that we can evaluate the response of the sensor quantitatively by using one of the channels as an internal standard.

We have already shown that the aptamer stays on the GO surface after TB recognition by employing AFM analysis using a

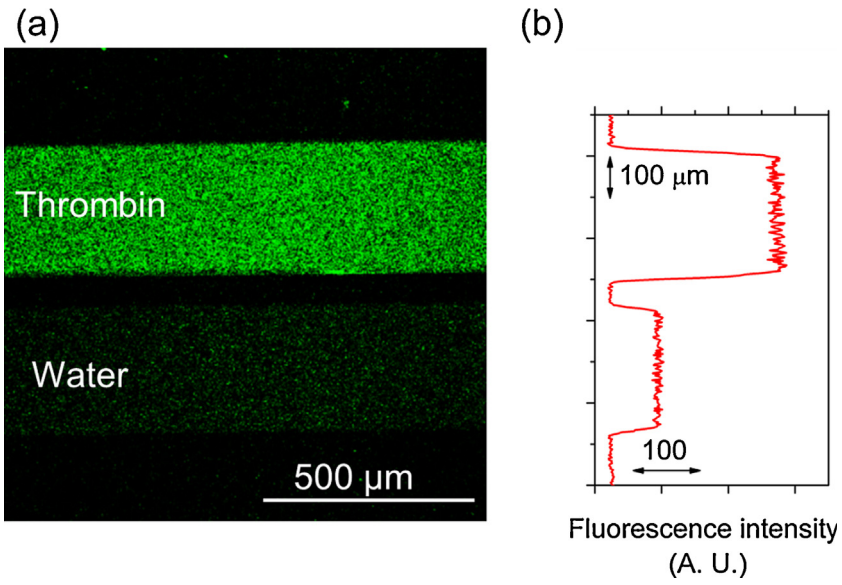

Fig. 2. (a) Fluorescence image of the GO aptasensors after injecting TB solution (100 μg mL^{-1}) and water into the top and bottom channels obtained in an area including two complete microchannels, (b) the line profile of the fluorescence intensity, which was the average of about 480 pixel data along the horizontal axis. The image resolution was 2.8 μm pixel^{-1}.

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single piece of GO [22]. Thus, it is obvious that the fluorescence in the microchannel is derived by the FAM fixed on the GO surface, not by the FAM desorbed from the GO surface, which is floating in the fluid in the microchannel. The results shown in Fig. 1(c) also support this, in which the fluorescence areas after TB addition correspond to GO shapes.

Next we examined the selectivity of this sensor for TB detection. Here we prepared a GO aptasensor with a triple microchannel configuration. We measured the fluorescence images when TB, human albumin solution, and water were injected into the top, middle, and bottom channels, respectively (Fig. 3(a)). Albumin, the most abundant protein in human blood plasma, caused no change in the fluorescence intensity, in the same way as water (Fig. 3(b)). This proved the selectivity of our GO aptasensor for TB detection. We also examined the dependence of the fluorescence intensity on the TB concentration. We used a quintuple microchannel configuration and injected TB solution with four different concentrations and water as a reference into each microchannel (Fig. 3(c) and (d)). The results showed that the fluorescence intensity became weaker as the TB concentration decreased. The above observations could not be achieved without the multichannel on-chip GO aptasensor.

3.3. Application of on-chip GO aptasensor to other proteins

To confirm the versatility of the detection system used in the on-chip GO aptasensor, we studied the performance of the sensor by changing the DNA aptamer from TBA to PSAA. PSA, whose molecular size is similar to that of TB (34 kDa), is the most common serum marker for diagnosing prostate cancer. Fig. 4(a) shows a fluorescence image of the sensor measured after injecting PSA solution, human albumin solution, and water into the top, middle, and bottom microchannels, respectively. A high fluorescence was observed on the GO surface in the top channel

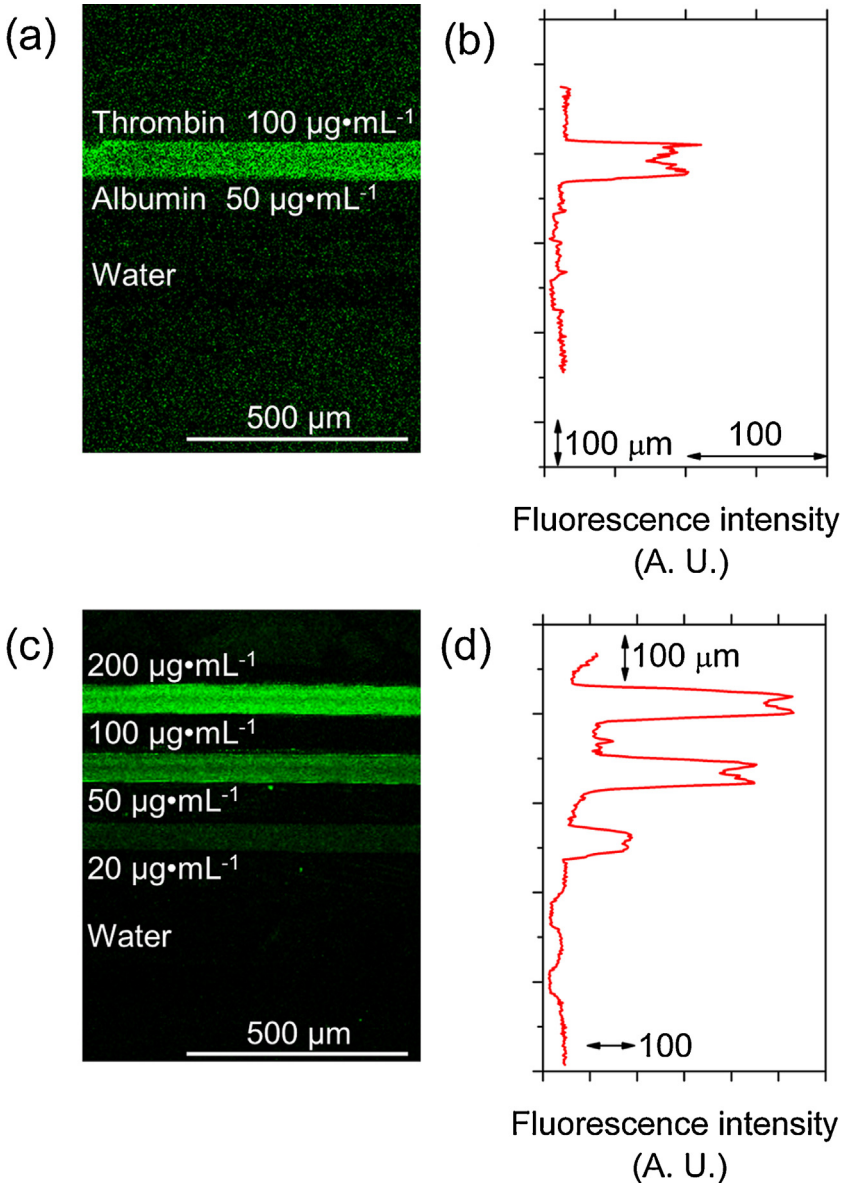

Fig. 3. (a) Fluorescence images of the GO aptasensors after injecting TB solution (100 μg mL^{-1}), albumin solution (50 μg mL^{-1}) and water into the top, middle, and bottom channels obtained in an area including all three microchannels, (c) after injecting different concentrations of TB solution (200, 100, 50, 20, and 0 μg mL^{-1} (water) from top to bottom) into each channels obtained in an area including all five microchannels, and (b, d) the line profiles of their fluorescence intensities, which were averaged along the horizontal axis.

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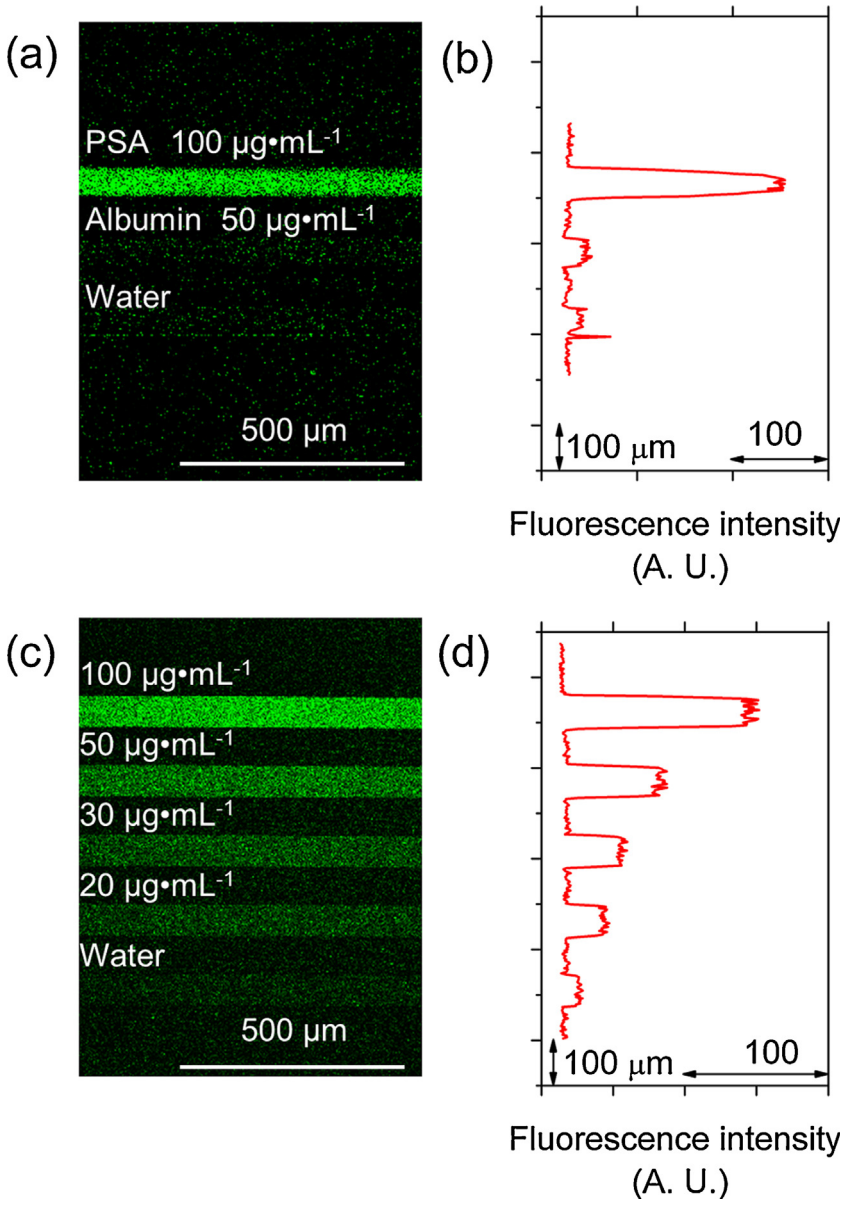

Fig. 4. (a) Fluorescence images of the GO aptasensors after injecting PSA solution (100 μg mL^{-1}), albumin solution (50 μg mL^{-1}) and water into the top, middle, and bottom channels obtained in an area including all three microchannels, (c) after injecting different concentrations of PSA solution (100, 50, 30, 20, and 0 μg mL^{-1} (water) from top to bottom) into each channel obtained in an area including all five microchannels, and (b, d) the line profiles of their fluorescence intensities, which were averaged along the horizontal axis.

and little fluorescence was observed in the other channels (Fig. 4(b)). The result shows that PSA can be detected by using our on-chip GO aptasensor, as well as TB. Therefore, PSAA immobilized on a GO surface maintains the biological activity needed to realize an on-chip aptasensor as well as TBA. Moreover, the selectivity for PSA detection was confirmed simultaneously, because albumin caused no change in the fluorescence intensity. We observed the dependence of the fluorescence intensity on the PSA concentration by using a quintuple microchannel configuration (Fig. 4(c) and (d)). We injected PSA solution with four different concentrations into four microchannels, and water into the fifth channel, which we used as an internal standard. The fluorescence intensity became weaker as the PSA concentration decreased, as found in the TB experiments. The sensing performance of the GO aptasensor was almost unchanged when the DNA aptamers were replaced thus confirming the versatility of the on-chip GO aptasensor.

3.4. Multiple protein detection by using aptamer array configuration

Based on the knowledge we obtained in Sections 3.2 and 3.3, we developed a simultaneous multiple protein detection system on one chip by employing an aptamer array configuration. We fabricated a 2×3 linear-array GO aptasensor by using two different DNA aptamers, TBA29 and PSAA. The TBA29 and PSAA were labeled with red fluorescent TAMRA and green fluorescent FAM, respectively, for ease of distinction (Fig. 5(a)). We used a similar fabrication procedure to one that we reported previously [25]. Fig. 5(b) shows a color-composite fluorescence image of the sensor measured after injecting PSA and TB solution into the top

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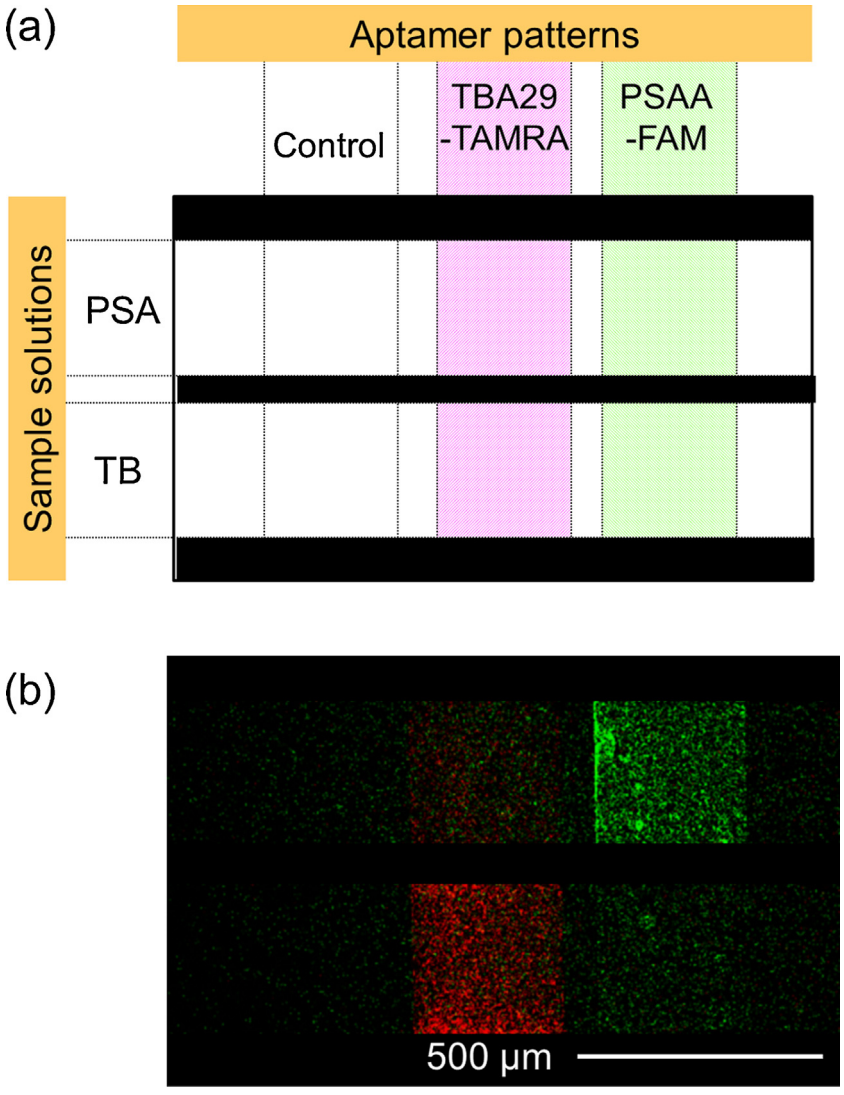

Fig. 5. (a) Layout of a 2 × 3 linear-array GO aptasensor and (b) color-composite fluorescence image of GO aptasensors after injecting PSA solution (100 μg mL^{-1}) and TB solution (100 μg mL^{-1}) into the top and bottom channels. The outside of the channel was blackened to exclude the background fluorescence.

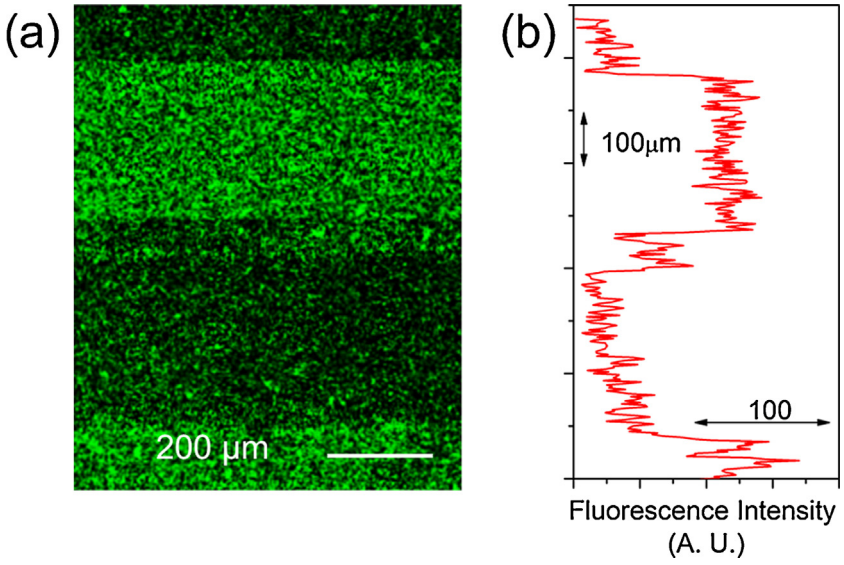

Fig. 6. (a) Fluorescence image of the GO aptasensors after injecting HA solution (1 mg mL^{-1}), and water into the top and bottom channels obtained in an area including whole two microchannels and (b) the line profiles of their fluorescence intensities which were averaged along the horizontal axis.

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and bottom microchannels, respectively (Fig. 5(b)). The red and green fluorescence images were observed simultaneously by using two different filters with the same excitation light source. A large amount of FAM fluorescence was observed at the PSAA position in the upper channel, whereas, little fluorescence was observed in other areas including the control area. In the same manner, a large TAMRA fluorescence was observed at the TBA29 position in the lower channel. Little fluorescence was observed in other areas. The average fluorescence intensities after background subtraction were 60 (TB-PSAA) and 79 (PSA-TBA29) for the cross reactions, 144 (PSA-PSAA) and 140 (TB-TBA29) for the right reactions, respectively. Although cross-reactions were observed, the signal was small enough to distinguish from the right reactions. Thus, a simultaneous multiple protein detection system on a single chip was successfully demonstrated.

A protein detection system built on a homogeneous mosaic GO layer formed on a solid surface is a powerful tool with which to realize a parallel analysis system such as multichannel and/or array sensors.

3.5. Application of on-chip GO aptasensor by using RNA aptamer

With a view to achieving a further increase in the number of detection targets, we extended the GO aptasensor to protein detection by using an RNA aptamer. Since RNA is less stable than DNA, we fabricated the sensor carefully. The detection target we used was HA, whose molecular size is also almost the same as that of TB (37.6 kDa). It is an antigenic glycoprotein found on the surface of influenza viruses. Fig. 6(a) shows a fluorescence image of the sensor measured after injecting HA solution and water into the top and bottom microchannels, respectively. The fluorescence intensity in the top channel was stronger than that in the bottom channel (Fig. 6(b)). The slight fluorescence observed in the bottom channel can be regarded as background fluorescence. Outside the microchannel, a small amount of the dye can be incorporated into PDMS and this may cause fluorescence. The result shows that HA, as well as TB and PSA, can be detected with the on-chip GO aptasensor. However, the fluorescence intensity of the sample channel was not as strong as with TB/PSA detection. Since the molecular size of HA is similar to that of TB/PSA, the steric hindrance between the protein and GO should be almost the same. The difference in the flexibility between DNA and RNA may result in a different sensing performance.

4. Conclusions

The versatility of the on-chip GO aptasensor was confirmed by the detection of three different proteins, namely, TB, PSA, and HA, simply by changing the aptamers but with the sensor composition remaining the same. The results indicated that both DNA and RNA aptamers immobilized on the GO surface maintained the biological activity sufficiently well to realize an on-chip aptasensor. Molecular selectivity and concentration dependence were clearly confirmed in relation to TB and PSA detection by using a dual, triple, and quintuple microchannel configuration. The simultaneous detection of TB and PSA on a single chip was demonstrated by using a 2×3 linear-array GO aptasensor.

This work enables us to extend this system to the development of on-chip biosensors for a wide variety of targets by choosing appropriate aptamers. The sensor is based on the significant change in the FRET efficiency depending on the distance between the dye and GO surface before and after the aptamer recognizes the target protein. The sensing performance thus depends on the size of the target molecule. However, considering the previously reported FRET efficiency [16], detection of ordinary proteins whose size ranges from a few nanometers to several tens nanometers should perform effectively. We can also apply the advantages of on-chip biosensors such as ease of operation, small volume, and high-throughput analysis, to the GO aptasensor. This work will help to provide a potentially useful biosensor chip for medical/pharmaceutical applications.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aca.2014.10.047.

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