Fast and Single-step Fluorescence-based Competitive Bioassay Microdevice Combined PDMS Microchannel Arrays Separately Immobilizing Graphene Oxide-Analyte Conjugates and Fluorescently-labelled Receptor Proteins

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In this study, a fast and easy-to-use capillary-type microdevice for competitive bioassay is proposed. The device is composed of polydimethylsiloxane (PDMS) microchannel arrays that separately immobilize polyethylene glycol (PEG) coating, which contained graphene oxide (GO)-analyte conjugates and fluorescently-labelled receptor proteins. The working principle of the device involved the spontaneous dissolution of the PEG coating, subsequent mixing and reaction with analyte to give fluorescence response, triggered by the capillary action-mediated introduction of a sample solution. For principle verification, a competitive biotin assay was successfully demonstrated within 20 s in a single-step operation by detecting the change in fluorescence via microscopy.

Keywords Graphene oxide, single-step bioassay, fluorescence resonance energy transfer, biotin, microchannel array

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Introduction

Microplates are a widely accepted experimental tool in the field of bioassay. However, long analysis time, high sample volume, and complicated operation are still recognized as the problems to be solved by many researchers in this field.1–4 Recently, a large number of studies on technological developments of microsystems, microchemistry, and fully automated analytical systems have been reported in attempts to solve these problems.5–8 However, simultaneously solving these problems is still challenging. Recently, we proposed designing “single-step” microdevices based on square glass capillaries9–13 or polymer capillaries.14–16 For these approaches, the inner capillary wall is functionalized by physical adsorption or covalent bonding of analytical reagents or by coating the wall with reagent-containing films. By integrating all reagents involved in the analysis onto the capillary wall, a rapid response could be achieved based on small reaction scale, low amount of sample, and simple analytical procedure based on the capillary action-mediated introduction of the sample solution.

Graphene oxide (GO), which is a two-dimensional carbon nanomaterial, has recently gained attention because of its exceptionally high quenching ability based on fluorescence resonance energy transfer (FRET),17 and this property has been applied in DNA sensors,18,19 immunosensors,20–22 and aptasensors.23–27 Furthermore, recently, we reported a capillary-type homogeneous immunoassay microdevice based on the fluorescence quenching function of the GO immobilizing antibody13 and a heterogeneous immunoassay microdevice based on the size separation and fluorescence quenching functions of GO-containing hydrogel.16 These microdevices successfully eliminated the complicated operations for the detection of proteins; however, the microdevices still presented with the underlying limitation of detectable molecular size, i.e., small molecules could not be detected. In order to detect small molecules by using GO with a single-step operation, the immobilization of the GO-small molecule conjugate and a fluorescently labelled receptor for the small molecule within the same capillary is indispensable. However, a conventional capillary cannot be applied because these reagents react with each other during the immobilization procedure. Thus, in order to demonstrate a single-step detection of small molecule by using the fluorescence quenching function of GO, we selected biotin as the model small molecule sample. Here, a capillary-type competitive biotin assay microdevice was prepared by combining PDMS microchannel array with a coating that contained the GO-biotin conjugate and with a coating that contained fluorescently-labelled streptavidin (Fig. 1) for demonstration of a fast and single-step analysis of small molecules with low amounts of the sample solution.

Experimental

Materials

The PDMS prepolymer (SILPOT 184) and curing agent (SILPOT184 CAT) were purchased from Dow Corning Toray (Tokyo, Japan). Biotin and N-succinimidyl d-biotinate (biotin-NHS) were purchased from Tokyo Chemical Industry (Tokyo, Japan). Alexa Fluor® 488 streptavidin conjugate (F-SA) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Branched polyethylenimine (PEI), poly(dimethyl siloxane-co-methyl-(3-hydroxypropyl)-siloxane)-graft-PEG methyl ether (PDMS-PEG), and bovine serum albumin (BSA) were purchased...
from Sigma-Aldrich (St. Louis, MO, USA). Single-layer GO dispersed in water was purchased from ACS Material (Medford, MA, USA). 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethane sulfonic acid (HEPES) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were purchased from DOJINDO (Kumamoto, Japan). Acetonitrile, N-hydroxysuccinimide (NHS), sodium hydroxide, polyethylene glycol 20000 (PEG 20000), ascorbic acid, glucose, and urea were purchased from Wako Pure Chemical Industries (Osaka, Japan). The deionized water for dilution had resistivity values of more than $1.8 \times 10^7 \, \Omega \cdot \text{cm}$ at 25°C.

Preparation of the GO–biotin conjugate

For the preparation of the GO–biotin conjugate, biotin-NHS (20 mg) was dissolved in acetonitrile (2.6 mL), 1 vol% of PEI in acetonitrile was added, and the mixture was stirred for 2 h at room temperature. Then, the acetonitrile was evaporated, and a crude mixture of biotin-immobilized PEI was obtained. For covalently binding the biotin-immobilized PEI onto GO, the carboxylic group on GO (200 μg mL$^{-1}$) was activated by EDC (500 μg mL$^{-1}$) and NHS (600 μg mL$^{-1}$), then biotin-immobilized PEI (5 μg mL$^{-1}$) was added to the activated GO aqueous solution, and the mixture was gently shaken for 3 h at room temperature. The solution was centrifuged and rinsed with 100 mM HEPES buffer (pH 7.4) several times, and the remaining activated sites in GO were blocked by overnight incubation with BSA (1 mg mL$^{-1}$). After centrifuging and rinsing several times, the GO-biotin conjugate was obtained.

Preparation of PDMS microchannel array for detection of biotin

To prepare a PDMS microchannel array for the detection of biotin, a PDMS substrate possessing microchannel arrays (channel width: 520 μm and channel depth: 200 μm) was fabricated using a previously reported method.$^{14}$ Then, a solution containing PEG 20000 (1 mg mL$^{-1}$), PDMS–PEG (1 vol%), and the GO–biotin conjugate (360 μg mL$^{-1}$) or F-SA (4 μg mL$^{-1}$) was introduced into an air plasma-treated microchannel array, and PEG coatings containing the reagents were formed by vacuum drying for 2 h. By combining these two PDMS microchannel arrays, the present microdevice was developed (Fig. 1).

Single-step biotin assay using the microdevice

A single-step biotin assay was conducted by introducing the sample solution into the present microdevice by capillary action. Fluorescence images were captured for measuring the fluorescence intensity. For the preparation of the standard curve, various concentrations (0, 0.5, 1, 2.5, 5, 10, and 25 μg mL$^{-1}$)
of biotin solutions (100 mM HEPES buffer, pH 7.4) were used. Selectivity of the present microdevice was investigated by introducing 100 μg mL⁻¹ solutions of potential interferents such as urea, ascorbic acid, and glucose. Fluorescence images were obtained with a fluorescence microscope (VB-S20, Keyence, Osaka, Japan) and converted into a numerical response using the ImageJ software (NIH, Bethesda, MD, USA).

Results and Discussion

For the preparation of the GO–biotin conjugate, PEI was chosen as a linker between GO and biotin. Biotin-NHS easily reacted with PEI and formed an amide bond; the resulting product (biotin-immobilized PEI) was immobilized onto GO by covalent bonding. The obtained GO–biotin conjugate was dispersible in water. Thus, by introducing the solution containing the GO–biotin conjugate, PEG, and PDMS–PEG into a PDMS microchannel array, followed by vacuum drying, the soluble PEG coating that contained the GO-biotin conjugate was homogeneously immobilized at the inner surface of a PDMS microchannel array. PEG coating that contained F-SA was also homogeneously immobilized. In our previous studies, PEG was used as a soluble coating reagent for glass capillary; however, when using a PDMS microchannel array, the PEG coating was robustly bound onto the PDMS microchannel surface. Therefore, when the sample solution was introduced, the dissolution of the PEG coating was relatively slow and non-uniform along the lengthwise direction of the microchannel (data not shown). On the other hand, fast and homogeneous dissolution was observed when PDMS–PEG, which possessed high affinity for the surface of PDMS substrate as well as PEG, was chosen as an additional reagent. By combining the two PDMS substrates that immobilized the GO-biotin conjugate and F-SA, a capillary-type PDMS microchannel array for biotin assay was successfully fabricated (Fig. 1).

As shown in Fig. 2, in the presence of biotin, F-SA competitively reacted with the free biotin in the sample solution, and the GO-biotin conjugate dissolved from PEG coating located at the opposite PDMS surface. Therefore, the fluorescence intensity of the microchannel filled with the sample solution containing biotin (5 μg mL⁻¹) was higher than that of the microchannel filled with the blank solution (Fig. 2A). The competitive reaction and quenching based on FRET occurred subsequently and reached a plateau within 20 s (Fig. 2B). These results suggested that the free biotin in the sample solution rapidly reacted with F-SA and inhibited F-SA from binding to the GO-biotin conjugate. The most important observations of the present microdevice were the short analysis time, low amount of sample, and easy-to-use detection system for small molecules. The normalized fluorescence intensity, calculated by dividing the obtained fluorescence intensity at the particular biotin concentration by the fluorescence intensity for blank buffer solution, increased with an increase in biotin concentration in the sample solution (Fig. 3). The errors indicating standard deviation were calculated from the results of three replicate experiments. The coefficient of variation (CV) value for each biotin concentration (0, 0.5, 1.0, 2.5, 5, 10, and 25 μg mL⁻¹) was 3.6, 5.6, 3.4, 1.8, 2.1, 1.0, and 2.3%, respectively. For low biotin concentrations, the fluorescence intensity exhibited a linear relationship with the biotin concentration. The calculated limit of detection was approximately 0.40 μg mL⁻¹ based on the 3σ value of blank measurements (N = 3). Finally, we investigated the selectivity of the present microdevice by using various sample solutions containing urea, ascorbic acid, or glucose (concentration: 100 μg mL⁻¹ each), and the results are shown in Fig. 4. In comparison to the fluorescence intensity of the sample solution containing biotin (A, 10 μg mL⁻¹), the

![Figure 3](image3.png)

**Fig. 3** Standard curve of normalized fluorescence intensity versus biotin concentration. The linear range was approximately 0 - 2 μg mL⁻¹.

![Figure 4](image4.png)

**Fig. 4** (A) Selectivity investigation of the present microdevice using (A) biotin (10 μg mL⁻¹), (B) urea (100 μg mL⁻¹), (C) ascorbic acid (100 μg mL⁻¹), (D) glucose (100 μg mL⁻¹), and (E) buffer. The normalized fluorescence intensity of A was significantly higher than that of B, C, D, and E. The normalized fluorescence intensities of B, C, and D exhibited non-significant differences compared with that of E by Tukey’s test at 1% confidence level.
fluorescence intensities of all the interferents were sufficiently weak and almost equal to the case of the absence of biotin. The errors indicating standard deviation were calculated from the results of four replicate experiments. The significant difference of A against all the other samples and the non-significant differences between B, C, D, and E were confirmed by Tukey’s test at 1% confidence level. Therefore, we concluded that the present microdevice exhibited selective response to biotin.

Conclusions

In this study, a fast and single-step capillary-type PDMS microchannel array for biotin assay that uses low amounts of the sample solution was developed. By surface modification and combining the microchannel array of two independent PDMS substrates, the GO-biotin conjugate and fluorescently-labelled streptavidin, which are reactive reagents, were successfully immobilized on the same inner capillary surface. The reaction time was approximately 20 s with a simple operation involving sample introduction by capillary action. The increase in the fluorescence intensity with increasing biotin concentration and selectivity to biotin were confirmed. Currently, the error bars for the measurements are still large; however, by optimizing the coating method and the material, the present microdevice is expected to be useful for analyzing biotin in a vitamin containing two kinds of reagents, is generally applicable for fast and single-step detection of various small molecules and requires low volume of the sample by replacing the analyte immobilized on GO and the fluorescent receptor.

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