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Electrochemical aptamer-based sensor prepared by utilizing strong interaction between DNA aptamer and diamond

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Stable and continuous biosensing of electroactive species in vivo have been achieved by boron-doped diamond (BDD) electrodes owing to the outstanding electrochemical properties. However, the present problem of the biosensing by BDD electrodes is how to specifically measure/detect the target molecules, including electrochemically inactive species. A possible solution is to fabricate an electrochemical aptamer-based (E-AB) sensor using a BDD electrode. In a preliminary investigation, we found that the DNA aptamers strongly adsorb on BDD surface and the aptamer-adsorbed BDD apparently worked as the E-AB sensor. The present study reports the performance of the aptamer-adsorbed BDD electrode as an E-AB sensor. Doxorubicin (DOX), a widely used chemotherapeutic, was chosen as a target molecule. The sensor could be prepared by just dipping BDD in aptamer solution for only 30 min, and the electrochemical signals were dependent on DOX concentration. The adsorption of DNA was strong enough for continuous measurements and even a sonication treatment. Such behaviors were not observed when using gold and glassy carbon electrodes. In a kinetic measurement, distortion by a sluggish response was observed for both association and dissociation phase, indicating that the interaction between DOX and the aptamer involves several kinetic processes. By fitting to Langmuir isotherm, limit of detection of 49 nM and maximum detectable concentration of 2.3 μM were obtained. Although the sensitivity was lower than well-established E-AB sensors of gold, the values are within a drug's therapeutic range. Overall, the present work demonstrates that a DNA aptamer and a BDD electrode is an effective combination for an E-AB sensor with stable sensitivity and a wide variety of DNA aptamers can be applied without any special treatment.

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Introduction

Among a variety of electrode materials, a boron-doped diamond (BDD) electrode shows outstanding electrochemical properties such as wide potential window in water, low background current, high chemical/physical durability, and biocompatibility.[1–5] Such properties enable highly sensitive and stable measurements of electroactive molecules in situ in the living body.[6–10] However, the present problem in the biosensing by BDD electrodes is how to specifically detect targets, including electrochemically inactive species. One of the most promising methods is the use of DNA aptamers that can specifically binds to a target molecule. When an aptamer combined with a redox probe molecule is immobilized on an electrode surface, target-specific measurement can be achieved by monitoring the change in the redox signal of the probe in response to the aptamer-target binding, without adding reagents or electrochemical mediators.[11] Such a system is called an electrochemical aptamer-based (E-AB) sensor and enables a target specific, reversible, real-time, and continuous biosensing in situ.[12,13]

Immobilization of DNA onto BDD surface has been achieved by electrografting of aryldiazonium salts, followed by amide condensation[14] and azide/alkyne click reaction.[15] Although these surface-modified BDD electrodes are fascinating platforms for electroanalysis, complicated multistep procedures would be undesirable in preparation process. On the other hand, it has been previously reported that DNA strongly adsorbs on BDD surface during the direct electrochemical measurement of DNA.[16] The adsorption of DNA was supposed to be caused by electrostatic interaction between negative charges of the DNA backbone and positively polarized surface of BDD. Although the origin of the strong adsorption is still unclear, the adsorption is supposed to be specific to BDD. In our preliminary experiments, we checked an affinity of the DNA aptamer for BDD. It was found that the DNA aptamers adsorbed on BDD surface strongly and the aptamer-adsorbed BDD apparently worked as an E-AB sensor.

We herein report the performance of BDD E-AB sensors fabricated by utilizing the adsorption of the DNA aptamer on BDD surface. Doxorubicin (DOX), a widely used chemotherapeutic, was chosen as a target molecule because the performance of a DOX-specific aptamer has been proved well.[17–19] A specific interaction was found between the aptamer-adsorbed BDD and DOX, resulted in the modulation of electrochemical signals of the probe molecule conjugated with the DNA aptamer. The adsorption was strong enough to endure several measurement cycles and even a sonication treatment. Such target-specific signals could not be observed when using gold and glassy carbon electrodes, which supports the strong and stable adsorption of the aptamer is intrinsic to the BDD electrodes. Considering the signals were dependent on a measurement speed, the mechanism of the sensor was proposed. Although the sensitivity was less than well-established gold E-AB sensors,[18] both the limit of detection and maximum detectable concentration of DOX are within the drug's therapeutic range in the human blood. Overall, the present work demonstrates that a DNA aptamer and a BDD electrode is an effective combination for an E-AB sensor with stable sensitivity and a wide variety of DNA aptamers can be applied without any special treatment.

Experimental

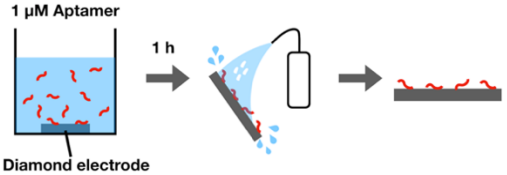
Preparation of BDD electrode

BDD electrodes were prepared on p-type silicon wafers using a microwave plasma-assisted chemical vapor deposition system (MPCVD, model AX2520M, ASTeX, Inc., USA). Before deposition, the silicon wafers were scratched with a diamond powder (0–1 µm, Lot# 603064, Kemet, UK) on a polishing pad (H1000, Nitta Haas Inc., Japan), followed by sonication in ethanol for 10 min. Methane and trimethylborane were used as a carbon and a boron source, respectively. A boron-to-carbon atomic ratio was fixed to 1% in gas phase and methane-to-hydrogen ratio was *ca.* 2.8%. Deposition was carried out for 2 h under a plasma power of 5.0 kW and a chamber pressure of 105 Torr. Substrate temperature was typically around 950–1100 °C during deposition. Referring to our previous report,[20] the actual boron concentration in BDD was estimated to be *ca.* 2.6×10^{21} cm^{-3}.

Aptamer design and immobilization

The aptamer probe was synthesized by Biosearch Technologies (USA) with the sequence of 5'-H_2N-$(CH_2)_6$-ACCATCTGTGTAAGGGGTAAGGGGTGGT-MB-3' (28 mer). This sequence is suspected to support a hairpin structure with DOX interaction.[17] The 3' end was conjugated with methylene blue (MB) to modulate a target binding-induced charge transfer.[18] MB is suitable for a redox probe having a redox potential of *ca.* –250 mV (*vs.* Ag/AgCl) where little interfering redox signal appears in biological environment.

The aptamer solution (4 µM in Tris-EDTA buffer, pH 8.0) was stored at –20 °C in 100 µL aliquot. An aliquot was melted at room temperature before each experiment. After BDD electrodes were sonicated for 5 min in pure water, BDD electrodes were attached to the electrochemical cell for the aptamer immobilization. Surface of BDD electrodes were contacted with 1 µM aptamer solution diluted with phosphate buffer saline (PBS, pH 7.4) for more than 30 min, then rinsing with pure water for 1 min. The procedure is illustrated in **Scheme 1**.



Scheme 1 Schematic illustration of the fabrication of BDD E-AB sensor.

Electrochemical measurement

Electrochemical measurements were conducted using a three-electrode system in a 1 mL single compartment Teflon cell: a BDD, a platinum wire, and an Ag/AgCl (saturated KCl) as a working, a counter, and a reference electrode, respectively. A BDD electrode was attached to the cell by using an O-ring (JIS P-7, the geometric surface area of BDD: 0.363 cm^2). Squarewave voltammetry (SWV) was recorded by EmStat^{3+} (PalmSens, Netherlands) or ALS852cs (CH Instruments, Inc., USA). Typically, SWV was taken with 70 Hz frequency, 25 mV amplitude, and 1 mV step. A response of aptamer sensor was evaluated by SWV measured after reaching the adsorption/desorption equilibrium state between the aptamer and DOX (approximately 10 min). Here, reduction currents are shown in positive values for easy understanding of MB reduction signals. Moreover, in order to normalize the MB signals of different electrodes, a signal gain was defined as follows:

$$\text{Gain}\ (\%) = \frac{I_{\mathrm{p}} - I_{\mathrm{p},0}}{I_{\mathrm{p},0}} \times 100$$

where I_p is a MB reduction peak current and $I_{p,0}$ is I_p in background solution (PBS). Details of calculations and flow measurements are described in **ESI**.

Gold (Au) and glassy carbon (GC) electrodes were polished firstly with 1 µm diamond suspension (40-6540, Buehler, USA), and then 0.05 µm alumina suspension (40-6301-00, Buehler) on a microcloth (40-7212, Buehler). After polishing, the electrodes were cleaned by sonication in ethanol and pure water. GC electrode was further polished on a microcloth to remove remained alumina on the surface.[21] Soon after polishing, the Au electrode was electrochemically treated as described in a literature.[22]

Chemicals

Doxorubicin (DOX), ifoxphamide (Ifex), and cisplatin (CDDP) were purchased from FUJIFILM Wako Chemicals (Japan). Dacarbazine (DTIC) and mitomycin C (MTC) were purchased from Tokyo Chemical Industry Co., Ltd. (Japan). All reagents were used without any further purification. Pure water was prepared by DIRECT-Q 3 UV (Merck).

Results and discussion

Adsorption of aptamer on BDD surface

First, we examined time dependence and stability of adsorption of the DNA aptamer on BDD surface. Adsorbed aptamers were quantified by the reduction signal of MB conjugated at the 3' end of the aptamer. Although the electrodes were rinsed thoroughly with pure water for 1 min, a well-defined reduction signal of MB was observed at around –250 mV in SWV measurement. The MB signals were compared among BDDs prepared by changing dipping time from 30 min to 360 min. (**Fig. 1a**). The signals did not show any correlation to dipping time with deviation less than 10% from the average. The difference in signals might be caused partly by the different surface morphology of BDD electrodes due to the polycrystalline nature. This result shows that 30 minutes is enough for aptamer adsorption on BDD surface. The following experiments were conducted using BDD dipped in aptamer solution for more than 30 min.

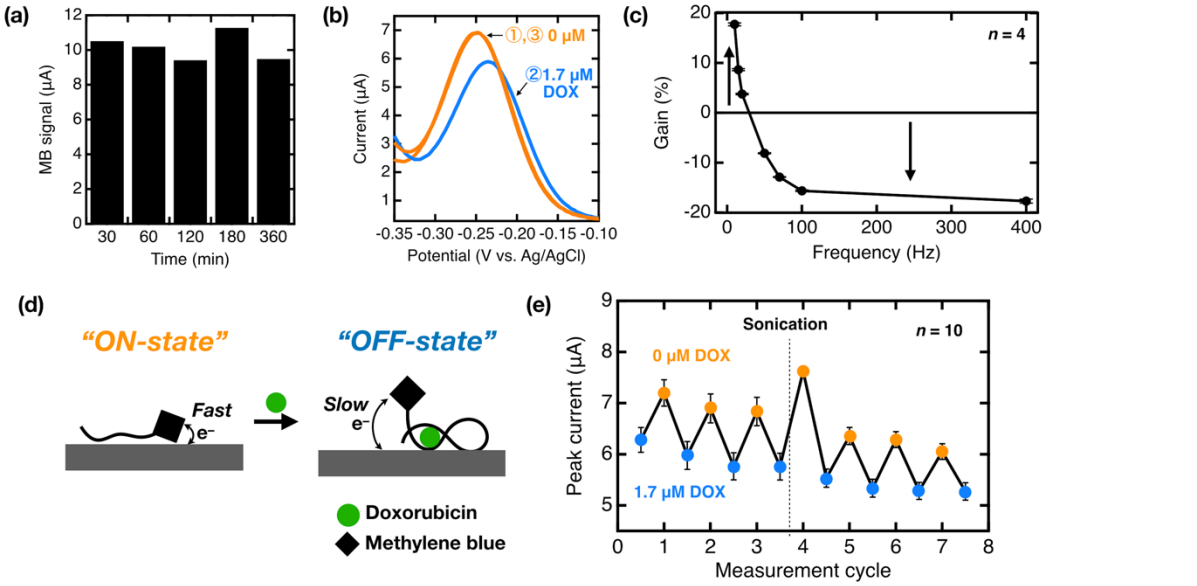


Fig. 1 SWV measurements on BDD electrodes treated by dipping into aptamer solution. (a) Dependence of a reduction signal of MB on dipping time (30–360 min). (b) A reduction signal of MB for 1.7 µM DOX solution. (c) Signal gains in SWV for 1.7 µM DOX measurement. A negative gain was obtained in high frequency measurements. The gain increased to positive values with decreasing the frequency. (d) A schematic illustration of how the BDD E-AB sensor works. (e) Repeatability of the sensor response were examined with the repetitive measurements of 1.7 µM DOX (odd numbers) and 0 µM DOX (even numbers). Sonication treatment of 3 min in PBS was performed at the dotted line.

The MB signal was measured with and without target molecule (**Fig. 1b**). The signal decreased in 1.7 µM DOX solution. After changing to background solution, the signal recovered to the initial level. These results indicated that the distance of MB and BDD electrodes increased after DOX interaction.

The above SWV measurements were taken with a pulse frequency of 70 Hz. It is known that the behavior of the SWV signal of a redox probe depends on an SWV pulse frequency. Generally, an SWV signal of a redox molecule attached to an electrode surface decreases with decreasing pulse frequency. Each pulse in SWV can be considered as chronoamperometry. SWV with a low frequency corresponds to low current recorded at long time after potential step in chronoamperometry. Therefore, the signal of the adsorbed redox probe decreases in low frequency SWV. On the other hand, the rate of such signal changes is affected by the distance between a redox probe and a sensor surface. The closer a redox probe is attached to an electrode surface, the faster the SWV signal decreases for low frequency measurements. This causes "signal-on" (signal increase) and "signal-off" (signal decrease) behaviors that depend on a pulse frequency.[23] **Fig. 1c** shows the plot of a signal gain versus a pulse frequency for 1.7 µM DOX measurements on the BDD E-AB sensor. The gain decreased in high frequency measurements (signal-off) and crossover to signal-on state was occurred in low frequencies. The result indicates that the aptamer-DOX interaction causes increase in the distance between the MB and BDD surface. When interacting with DOX, the conformation of aptamer changes so as to increase an average distance between MB and the sensor surface.

Considering above results, the plausible mechanism of the sensor behavior was presented in **Fig. 1d**. In this work, BDDs were prepared by the CVD process. The as-prepared BDD is known to have hydrogen terminated surface.[24,25] Such surface would be positively polarized due to the difference in electronegativity between carbon and hydrogen atoms. The hydrogen-terminated BDD can adsorb the aptamer through the electrostatic interaction with negative charges on the DNA backbone.[16] Actually, oxygenation of BDD surface suppressed the aptamer adsorption (**Fig. S2)**. The aptamer-attached BDD electrodes works as an E-AB sensor by the modulation of MB signal conjugated on the aptamer. In target free solution, the sensor generates large signal of MB probe, having a "lying-down" form of the aptamer caused by a multiple point electrostatic interaction with the BDD surface ("ON-state" in **Fig. 1d**). After the aptamer binds to the target molecule, the average distance between MB and the BDD surface increases, leading to slow electron transfer ("OFF-state" in **Fig. 1d**). Note that, considering the mechanism of DNA attachment on BDD surface through electrostatic interaction, we believe the amine modification at 5' end is not required for function. Influence of the terminal structure on the sensor performance is now under investigation.

Fig. 1e shows the sensor performance for repetitive measurements of DOX. SWV was taken in 1.7 µM DOX and background solution for 3 cycles. After each measurement, the solution was replaced to the fresh solution after a gentle rinse. The signal decreased in DOX solution and recovered in background solution. Even after a sonication treatment in PBS for 3 min, the behavior was preserved without a notable decrease in signals. The result shows that the aptamer adsorption is strong enough for repeated measurements and even a sonication treatment. Overall drift of the signal might be caused by DOX adsorption on the BDD surface.

Gold (Au) and glassy carbon (GC) electrodes were used for comparison. Although the clear reduction signal of MB was observed on an Au electrode, the signal intensities were not correlated to DOX concentration and decreased during repetitive measurements (**Fig. 2a**). The signal decreased especially after DOX measurements. The interaction between the aptamer and the gold surface might be weaker than a DOX-aptamer interaction. This result indicates that the aptamer adsorption on Au electrodes is not stable enough to obtain a sensor behavior.

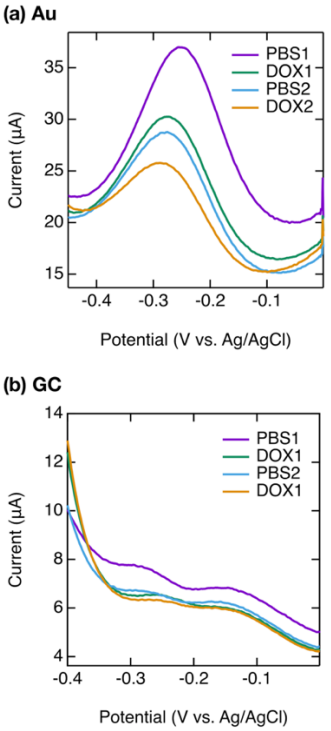


Fig. 2 SWV on (a) Au and (b) GC electrodes treated by dipping into 1 µM aptamer solution for 1 h. PBS and DOX represent measurements in 0 µM and 1.7 µM DOX solution. Measurements were performed in the order in annotations (from top to bottom).

In the case of a GC electrode, a reduction signal of MB was quite small regardless of DOX concentration (**Fig. 2b**). It is well-known that GC surface is terminated with various functional groups such as –OH, –C=O, –COOH, and others.[21] Such oxygen containing groups, especially –OH and –COOH, suppresses adsorption of DNA.[26,27] In contrast, BDD electrodes have little oxygen groups and the surface is terminated by hydrogen as mentioned before. This uniformity of the BDD surface generates quite strong and reproducible interaction between aptamer and the electrode. It is concluded that the sensor behavior is specific to hydrogen terminated BDD electrodes.

Sensor performance

The signal gains of the sensor were collected with varying DOX concentration (**Fig. 3a**). Note that a positive gain corresponds to decrease in a reduction signal of MB. The gains showed a nearly first order response at the low DOX concentration and approached to the saturation value. The behavior is fitted to a Langmuir isotherm (the dotted line in **Fig. 3a**), leading to the apparent equilibrium dissociation constant (K_d) of 486 ± 49 nM. This value is smaller than E-AB sensor of an Au electrode using the same DNA sequence (824 nM).[18] Limit of detection and maximum detectable concentration of DOX were 49 nM and 2.3 μM, respectively. These values are not greater than those in the Au E-AB sensor (10 nM and 10 μM), but coincides with a drug's therapeutic range in the human blood.[28–30]

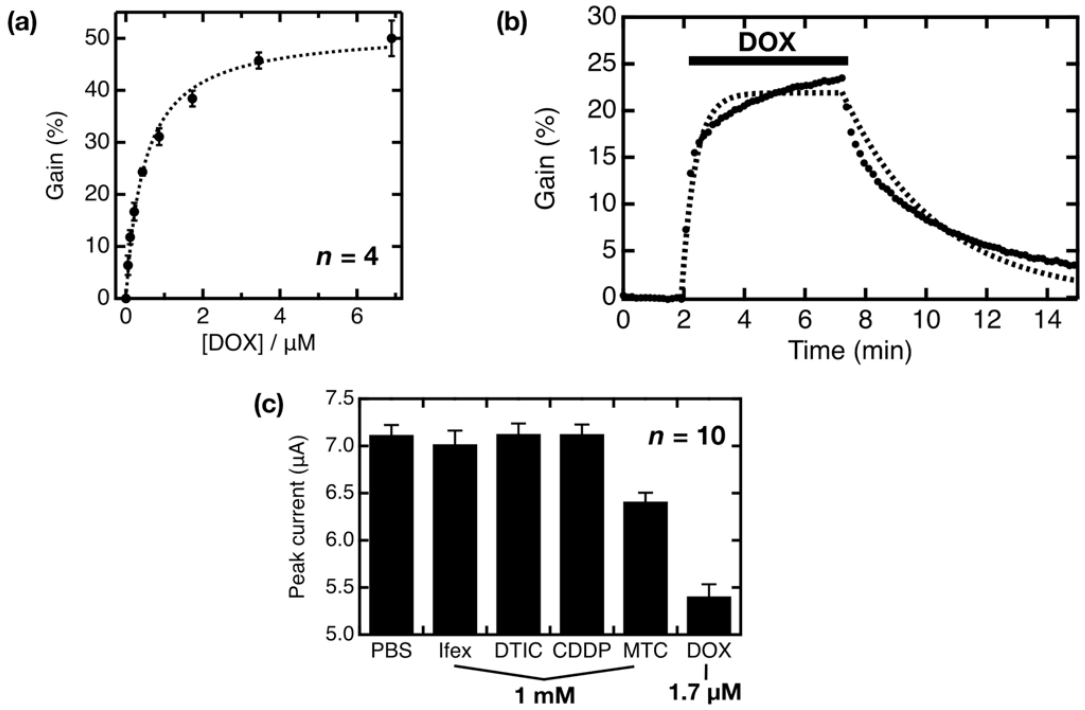


Fig. 3 (a) Signal gains of the BDD aptamer sensor for DOX concentration. (b) A time profile of the sensor response to DOX injection; 0.86 μM DOX was flowed for 5 min (indicated as bar) between PBS flows. (c) A specific response of the sensor to DOX was demonstrated by comparing with signals of other chemicals.

A drugs' concentration in our bodies changes with a certain time scale depending on drugs and individuals. Therefore, the sensor should be compatible with sensitivity and time resolution to monitor the concentration change. Flow measurements were conducted to estimate a kinetic constant for the adsorption and desorption of DOX on the sensor surface (**Fig. 3b**). DOX solution (0.87 μM) were injected for 5 min and the response was fitted to a one-phase association and dissociation model. A clear difference could be seen between experimental data plots and fitted results. A sluggish response was observed in both association and dissociation phase. These behaviors indicate that the interaction between DOX and the sensor surface involves several kinetic processes. This is probably because the binding of a target molecule to the aptamer proceeds with a variety of folding patterns and kinetics on the sensor surface. Additionally, the complicated behavior is inevitable because the aptamer immobilization on the BDD is not be achieved by a specific covalent bonding but by a nonspecific multipoint interaction. Nonetheless, the general sensor kinetics would be evaluated using the fitting results. The fitted results of k_{on} = 2.25 μM min^{-1} and k_{off} = 0.32 min^{-1} are much smaller than those in the same aptamer sensor on an Au electrode (k_{on} = 3.00 μM min^{-1} and k_{off} = 1.35 min^{-1}),[5] showing slow kinetics of the BDD E-AB sensor. Especially desorption was slow, having 4 times smaller value of k_{off} compared to the Au sensor. Using the BDD sensor, it takes more than 2 min to obtain 90% of the saturated level of signal in the association phase, which is comparable to α-phase plasma clearance time of DOX spanning from 6 to 26 min.[43] On the other hand, the gain did not recover to the initial level even after 7 min in the dissociation phase. These results show that slow but strong interaction of DOX with the BDD surface besides the aptamer-DOX binding degraded the sensor performance. Overall signal drift in **Fig. 1e** would also be caused by the same manner.

Lastly, the sensor specificity was investigated in **Fig. 3c**. SWV was taken using drugs commonly used with DOX, such as ifosphamide (Ifex), dacarbazine (DTIC), cisplatin (CDDP), or mitomycin C (MTC), with more than 500-fold higher

concentration than DOX. Reduction signals of MB for Ifex, DTIC, and CDDP were almost consistent with that of PBS. On the other hand, the signals for MTC and DOX decreased to 10% and 25% respectively, compared to that for PBS. The signal decrease for MTC must be caused by nonspecific adsorption of MTC molecules on the BDD electrode. A quite strong adsorption of MTC on the BDD surface was confirmed by the other experiment (**Fig. S3**). The adsorption of MTC on the surface would interfere the aptamer-BDD interaction. The same effect can occur for any other molecules which adsorbs on BDD surface. It is very probable that the overall signal drifts for DOX measurement (**Fig. 1e** and **3b**) originate the same mechanism. However, the signal modulation is specific to DOX considering the high signal change for low concentration. It can be concluded that the aptamer-attached sensor works as an E-AB sensor.

Conclusions

We fabricated the electrochemical aptamer-based (E-AB) sensor using a boron-doped diamond (BDD) electrode, in which electrostatic interaction between the DNA aptamer and BDD surface was a key process. The adsorption of DNA was strong enough for the repetitive measurements and even a sonication treatment. The sensor response was fitted to a Langmuir isotherm: limit of detection of 49 nM and maximum detectable concentration of 2.3 µM. Although the sensor did not work better than gold E-AB sensors, the values coincides with a drug's therapeutic range in the human blood. Overall, the present work demonstrates that a DNA aptamer and a BDD electrode is an effective combination for an E-AB sensor with stable sensitivity and a wide variety of DNA aptamers can be applied without any special treatment. In order to achieve fast response with high sensitivity on an E-AB sensor of BDD, the followings are required in an aptamer immobilization process: (1) specific and covalent bonding of an aptamer to BDD surface and (2) introducing a bio-resistant surface on BDD for "stand-up form" of an aptamer.[11,26,31] Such a BDD E-AB sensor is now under investigation.

Conflicts of interest

There are no conflicts to declare.

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