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PAPER

Highly sensitive detection of platelet-derived growth factor on a functionalized diamond surface using aptamer sandwich design†

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Aptamer-based fluorescence detection of platelet-derived growth factor (PDGF) on a functionalized diamond surface was demonstrated. In this work, a sandwich design based on the ability of PDGF to bind with aptamers at its two available binding sites was employed. It was found that this sandwich design approach significantly increases the fluorescence signal intensity, and thereby a very low detection limit of 4 pM was achieved. The effect of the ionic strength of $MgCl_2$ buffer solution was also investigated, and the most favourable binding for PDGF-BB occurred at a Mg^{2+} concentration of 5.5 mM. Since the aptamers bind to the target PDGF with high affinity, fluorescence detection exhibited high selectivity towards different biomolecules. The high reproducibility of detection was confirmed by performing three cycles of measurements over a period of three days.

Introduction

The fabrication of simple biosensing platforms for the detection of specific proteins is of great interest owing to their significance in clinical diagnostics and therapeutics. Aptamers are single-strand DNA or RNA oligonucleotides that have the ability to bind to proteins[1–5] and cells[6,7] with high selectivity.[8] The high affinity of aptamers can be attributed to their ability to fold upon binding with the target molecule. Moreover, aptamers have high stability and are easy to synthesize owing to their target diversity and tight-binding capability. Thus, aptamer-based biosensors with their unprecedented advantages over antibodies and enzymes are attracting tremendous attention.

In recent years, the rapid detection of platelet-derived growth factor (PDGF) has increased in importance owing to its role in the regulation of cell growth and division. Chemically, PDGF is a dimeric glycoprotein composed of two A (-AA) and two B (-BB) chains, or a combination of both chains (-AB). PDGF-BB is known to be directly involved in cell transformation processes such as tumor growth and progression.[9] It is often overexpressed in human malignant tumors and is known as a potential protein marker for cancer diagnosis.[10] PDGF protein sensing has been previously demonstrated using nanoparticles,[11] electrochemistry[12] and fluorescence.[13,14] The reported methods involved labeling the aptamer or using an aptamer–primer complex as part of the sensing element. Detection methods involving labeled aptamers are advantageous because they can provide faster real-time information on cancer-related proteins than conventional techniques such as electrophoresis, autoradiography and enzyme-linked immunosorbent assay (ELISA).[15,16]

With the extensive development of diamond surface chemistry, considerable effort has been devoted to the development of different types of biosensor using diamond substrates. Their key advantages over other conventional materials such as glass,[17,18] gold[19] and silicon[20] are their high surface stability and the absence of significant biological interface degradation over time.[21] Concurrently, the amide bonds formed by the interaction of carboxyl-terminated biomolecules with an amine-terminated diamond surface are more stable than the commonly used Au–thiol surface bonds.[22] The binding stability can be attributed to the fact that the carboxylic group is an oxidized aldehyde group, whereas a Au–thiol surface exhibits rapid degradation owing to the easy hydrolysis of the thiol group under basic conditions.[21,23] Previously, our research group reported an extensive study on the effect of different passivation layers on diamond surfaces using oxygen, fluorine and hydrogen terminations. In particular, fluorine treatment resulted in a significant increase in hydrophobicity, and the electronegativity of fluorine contributes to the suppression of nonspecific binding to a great extent, thereby significantly increasing the signal to noise ratio of the resultant fluorescence signal. Finally, the high reusability and reproducibility of chemical and biochemical modification of the sp^3-carbon-based biointerface make diamond an attractive material for biosensor applications.[21,22,24]

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In this work, we have designed a fluorescence-labeled aptamer-based sensing platform for sensitive PDGF detection. A diamond surface functionalized by a direct amination technique was used as the substrate for the PDGF-binding aptamer, which was subsequently bound to the target PDGF-BB protein. Finally, the binding of a labeled aptamer to PDGF-BB was used to indicate the presence of the target aptamer. A fluorine passivation layer formed on the diamond surface outside the immobilized area suppressed the physical adsorption and significantly improved the signal to noise ratio. We were able to achieve detection limits in the sub-nanomolar range. This simple labeled PDGF detection technique offers high sensitivity and selectivity. Finally, we demonstrated the reusability of the sensor through a series of hybridization and denaturing cycles. The detection strategy reported in this work has the potential to be extended to the detection of other proteins.

Materials and methods

Protein and oligonucleotides

The PDGF-binding aptamer, PDGF-BB, PDGF-AB, PDGF-AA, bovine serum albumin (BSA), adenosine triphosphate (ATP), glucose oxidase (GO_x), calmodulin and urease were purchased from Sigma Genosys Company (Hokkaido, Japan). The 5′ end of all aptamers was terminated with a carboxyl group (COOH). Without using linker molecules, this carboxyl group can covalently bind to DNA and amine-terminated diamond. The method used has been described in detail elsewhere.[25] The end of the PDGF-binding aptamer used for optical measurement by fluorescence microscopy was labeled with Cy5. In the fluorescence observations, two different types of probe DNA were immobilized on the diamond substrate, the PDGF-binding aptamer (5′-CAGGCTACGGCACGTAGAGCATCACCATGATCCTG-3′) and a control aptamer (5′-CAGCGTACGGCACGTACCGATTCACCATGAAGCTG-3′), as well as labeled PDGF aptamer (Cy5-5′CAGGCTACGGCACGTAGAGCATCACCATGATCCTG-3′) and labeled control aptamer (Cy5-5′CAGCGTACGGCACGTACCGATTCACCATGAAGCTG-3′).

Synthesis of partially functionalized diamond surface

The polycrystalline diamonds used in this study were obtained by microwave-plasma-assisted chemical vapor deposition (MPCVD). The substrates were synthesized by the approach used in our previous studies.[23,24] The substrates were H-terminated by hydrogen plasma treatment for 30 min. Partial surface amination of the H-terminated diamond was performed by irradiation with UV light for 4 h with a continuous flow of 100 sccm of ammonia gas. The wavelength of the UV light used was 257.3 nm. Prior to UV light irradiation, nitrogen gas was introduced into the UV chamber for 5 min to remove other gases. This functionalization procedure was performed at room temperature and atmospheric pressure, which allowed the modification to be performed in a short time. In this experiment, amination of 18% of the diamond surface was achieved, as determined by X-ray photoelectron spectroscopy (XPS). Fluorination was performed by inductively coupled plasma (ICP) with a C_3F_8 gas source at a pressure of 3 Pa and a plasma power of 500 W. The coverage of fluorine on the diamond surface was 40% after 50 s of fluorine treatment.

Covalent immobilization of aptamer

The PDGF-binding aptamer was immobilized as the probe aptamer on the partially aminated diamond surface *via* covalent bonding. The concentration of the immobilized probe aptamer used was 20 μM. The probe aptamer with 3× sodium saline citrate (SSC) buffer solution, 0.1 M *N*-hydroxysuccinimide (NHS) and 0.4 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) were mixed in a 2 : 1 : 1 ratio. The sample was incubated for 2 h at 38 °C in a humidified chamber. After immobilization, the sample was washed once in phosphate-buffered solution with Tween-20 (PBS: 1 mM, NaCl: 2 mM, NaH_2PO_4: 8 mM, Na_2HPO_4; 0.1% Tween-20) for 5 min and washed three times for 3 min each with deionized water to remove the physisorbed PDGF-binding aptamer. PDGF-BB was then bound to the immobilized aptamer at room temperature for 1 h, followed by treatment with PBSM (PBS, 0–10.0 mM $MgCl_2$, pH 7.2) for 7 min. After that, labeled PDGF aptamer (1 μM) was bound to PDGF-BB for 1 h at 25 °C, and rinsed again with PBSM (PBS, 0–10.0 mM $MgCl_2$, pH 7.2) for about 5 min. The regeneration was performed by rinsing in 10% sodium dodecyl sulfate (SDS) solution for 30 min.

Fluorescence measurement

A circular micropattern array (20 μm diameter dots) was fabricated on a functionalized diamond surface by photolithography. Inside each micropattern was a partially amine-terminated diamond surface used to immobilize the probe aptamer, and outside of the micropattern was a fluorine-modified diamond surface, which acts as the background surface. Because the fluorine-modified diamond surface is superhydrophobic, the nonspecific adsorption of the probe aptamer and PDGF-BB was minimal. Fluorescence observation was performed using an Olympus IX71 epifluorescence microscope.

Results and discussion

Aptamers with high affinity to the PDGF-BB chain have been developed to suppress PDGF-BB-induced cell proliferation by inhibiting the binding of PDGF-BB to its receptor.[26,27] These target specific aptamers were used to design a sandwich-type aptamer detection mechanism[21] to detect PDGF-BB. In this

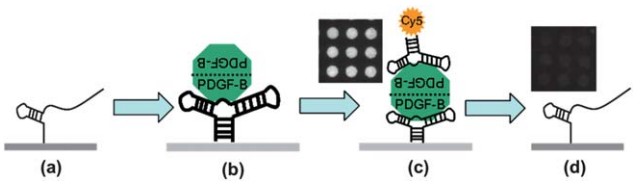

Fig. 1 Procedure for preparing aptamer sandwich design for fluorescence observation: (a) the PDGF-binding aptamer (probe aptamer) was immobilized on a partially aminated diamond surface without a linker molecule; (b) PDGF-BB was bound to the probe aptamer; (c) the labeled PDGF aptamer was bound to the other site of PDGF-BB; and (d) the PDGF sensor was initialized using SDS to release PDGF-BB from the probe aptamer.

work, two types of aptamers are used: one is the PDGF-binding aptamer (probe aptamer) and the other is a labeled PDGF aptamer that binds to the top of the target PDGF-BB. The schematic shown in Fig. 1 illustrates the detection methodology. Firstly, the PDGF-binding aptamer is covalently immobilized on the diamond surface without a linker molecule through amide binding (Fig. 1(a)). Secondly, the target PDGF-BB is bound to the immobilized aptamer, forming a stable three-way helix junction with a single-strand loop at the branch point (Fig. 1(b)). Finally, the labeled PDGF aptamer is bound to the top-binding site of PDGF-BB (Fig. 1(c)) and the fluorescence is measured. The reusability of the sensor was investigated by removing PDGF-BB from the PDGF binding aptamer using SDS solution (Fig. 1(d)). The binding of the synthesized probe aptamer and fluorescent aptamer to PDGF-BB was confirmed by fluorescence observation. A strong fluorescence signal was detected when PDGF-BB binds with two aptamers on the diamond surface (Fig. 1(c)).

In this study, to confirm the selectivity of the sandwich design mechanism, four different types of sandwich aptamer assay were examined. The fluorescence signal was observed in the conventional sandwich design (PDGF binding aptamer/PDGF-BB/labeled PDGF aptamer) as shown in Fig. 2(a). However, in the case of the PDGF binding aptamer/PDGF-BB/labeled control aptamer structure (Fig. 2(b)) no fluorescence signal was observed because the labeled control aptamer does not bind with PDGF-BB. In Fig. 2(c), the fluorescence signal intensity in the sandwich design (control aptamer/PDGF-BB/labeled PDGF aptamer) was low because the control aptamer, which acts as a probe aptamer, does not bind to PDGF-BB owing to the sequence mismatch. Similarly, in the case shown in Fig. 2(d), no fluorescence signal was observed because there is no PDGF-BB acting as a bridge between the probe aptamer and labeled PDGF aptamer. We suggest that using the sandwich design approach, the probability of aptamer misbinding is less than that of one aptamer binding detection. These results demonstrate the feasibility of using sandwich design aptamers to detect the presence of PDGF-BB.

The dye/DNA and protein/DNA interactions are affected by various key factors such as the pH and ionic strength of the buffer solution.[28] At pH 7.2, the effect of the buffer solution was investigated by varying the concentration of $MgCl_2$. Note that divalent ions such as Mg^{2+} and Ca^{2+} are usually more effective than monovalent ions such as Na^+ for binding assay stabilization.[29,30] It has been reported that Mg^{2+} stabilizes nucleic acids and facilitates their folding into biologically active secondary and tertiary structures.[29] The effect of metal ion concentration on fluorescence intensity for all the sandwich aptamer designs is shown in Fig. 3. We found that the optimum concentration of Mg^{2+} for PDGF-BB detection on the sandwich aptamers is 5.5 mM. At this concentration, the ratio between the fluorescence signal intensities of the PDGF binding aptamer/PDGF-BB/labeled PDGF aptamer and PDGF binding aptamer/PDGF-BB/control labeled aptamer structures is highest. However, the fluorescence signal degraded gradually above concentrations of 7.0 mM Mg^{2+}. On the other hand, in the case of the PDGF binding aptamer/PDGF-BB/control labeled aptamer structure (Fig. 2(b)), the background fluorescence signal was still prominent at a concentration of 2.5 mM $MgCl_2$. However, when the concentration of $MgCl_2$ was increased to 5.5 mM, the intensity of the background signal was considerably reduced. In other sandwich designs such as the control aptamer/PDGF-BB/labeled PDGF aptamer structure (Fig. 2(c)) and PDGF binding aptamer/labeled PDGF aptamer structure (Fig. 2(d)) the fluorescence signal was negligible. Our results are in agreement with those of Fang *et al.* and Huang *et al.*, who observed that the affinity of DNA decreases with increasing buffer salt

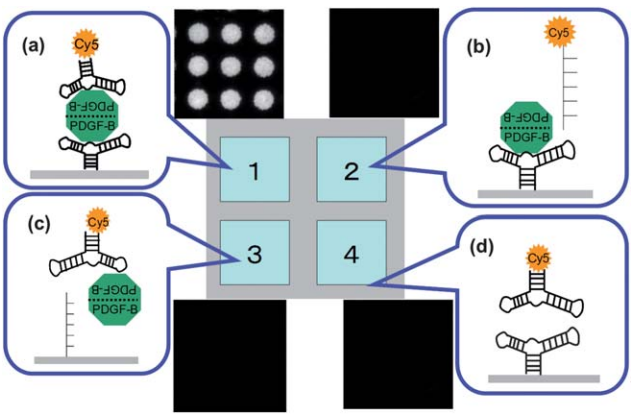

Fig. 2 Schematic of four different types of sandwich design approach based on the aptamer. (a) The sandwich design (PDGF binding aptamer/PDGF-BB/labeled PDGF aptamer) structure was successful for PDGF-BB detection. The fluorescence can be most clearly seen. In contrast, for (b) the PDGF binding aptamer/PDGF-BB/labeled control aptamer structure and (c) the control aptamer/PDGF-BB/labeled PDGF aptamer structure, the sandwich design failed because the aptamer was partially unfolded, causing only one of the three helix junctions to be effective; therefore, no fluorescence signal was detected. Similarly in (d), without the presence of PDGF-BB (PDGF binding aptamer/labeled PDGF aptamer) no fluorescence signal was observed.

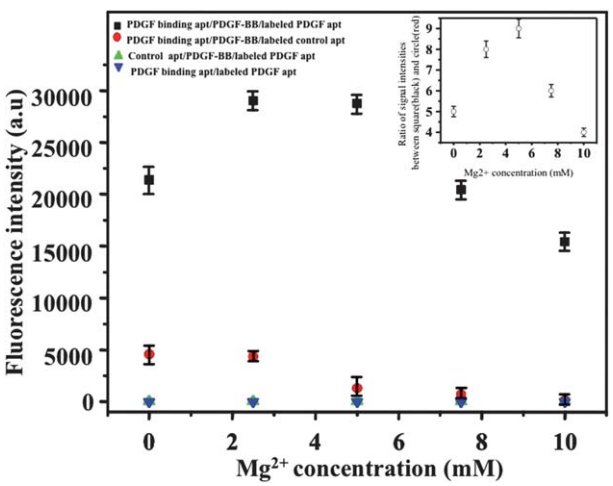

Fig. 3 Effect of Mg^{2+} cation concentration in PBS on fluorescence intensity for Mg^{2+} concentrations from 0 to 10 mM after adding the labeled PDGF aptamer for different sandwich designs. The inset shows the ratio between the signal intensities (R_{SI}) of the PDGF binding aptamer/PDGF-BB/labeled PDGF aptamer and PDGF binding aptamer/PDGF-BB/labeled control aptamer structure for each Mg^{2+} concentration. The concentrations of the aptamer, PDGF and labeled PDGF aptamer are 20 μM, 100 nM and 1.0 μM, respectively.

concentration. It is clear that the ionic strength of the buffer solution is important in this labeled sandwich detection design.

Similarly, the binding activity of PDGF-BB detection in various physiological salt concentrations of NaCl in PBS buffer solution was also carried out on the sandwich aptamer (the PDGF binding aptamer/PDGF-BB/labeled PDGF aptamer). The result is presented in Fig. 4. The fluorescence signal intensity shows a small initial decrease in fluorescence signal intensity when the NaCl concentration was increased from 1 mM to 150 mM. But when the NaCl concentration was >200 mM, the signal intensity was decreased drastically. Nevertheless, this sandwich system has the potential to be used in bioassay for protein detection in biological specimens and also applicable to clinical samples since it works at physiological salt concentration (~150 mM).

The sensitivity of each sandwich design was evaluated by varying the concentration of PDGF-BB and measuring the resulting fluorescence signal intensity. A wide range of concentrations of PDGF-BB from 0.001 nM to 100 nM was used for detection. As shown in Fig. 5, the fluorescence signal intensity clearly increases with increasing PDGF-BB concentration. The inset in Fig. 5 shows that the sensor is highly sensitive and that the detection limit of PDGF-BB is as low as 4 pM. In clinical samples, the PDGF concentrations in the blood serum and plasma of normal individuals and cancer patients have been found to be in the sub-nanomolar range: 0.4–0.7 nM in human serum and 0.008–0.04 nM in human plasma.[26,27] The local PDGF concentration in a tumor area is expected to be higher than that in blood samples as a result of its dilution in blood and short lifetime in circulation.[31] We expect that the range of concentration of PDGF-BB detection achieved in this study will be sufficiently wide for potential application in the preliminary diagnosis of cancer.

The selectivity of the sensor was investigated by evaluating the difference in fluorescence signal intensity for different biomolecules bound to the probe aptamer as shown in Fig. 6. Since the PDGF binding aptamer has very high specificity toward PDGF-BB, the fluorescence signal intensity of the aptamer sandwich design is much higher than that for other biomolecules. A PDGF dimer composed of two different types of monomer (A and B chains) occurs in three variants: PDGF-BB, PDGF-AB and PDGF-AA. It is shown in Fig. 6 that the aptamer used here binds to these variants with different affinities. The increase in fluorescence intensity caused by PDGF-AA (an isoform of PDGF-BB) is about 6-fold lower than that caused by PDGF-BB. Therefore, this sensor can distinguish isoforms with high selectivity. On the other hand, in the case of PDGF-AB, the fluorescence signal intensity was between those of the target PDGF-BB and PDGF-AA. PDGF-AB consists of A and B chains and it has one site that binds to the aptamer. Since the structures of A and B chains are similar, the fluorescence signal is higher than that in the case of PDGF-AA but is approximately half that of PDGF-BB. This is because the PDGF A chain and B chain have 60% similarity in their amino acid sequences. Furthermore, the A chain is more acidic than the B chain, which may cause the lower affinity of PDGF-AA.[27] For biomolecules such as BSA, ATP, calmodulin, urease and glucose oxidase, the test failed to cause any significant change in fluorescence signal intensity. Even though BSA usually contains a quite high

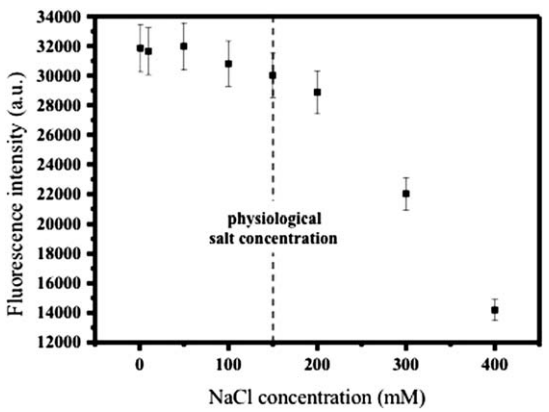

Fig. 4 The effect of NaCl salt concentration in PBS buffer solution on fluorescence signal intensity for the binding activity of PDGF-BB sandwich aptamer design (PDGF binding aptamer/PDGF-BB/labeled PDGF aptamer). The salt concentration was tested from 1 mM to 400 mM.

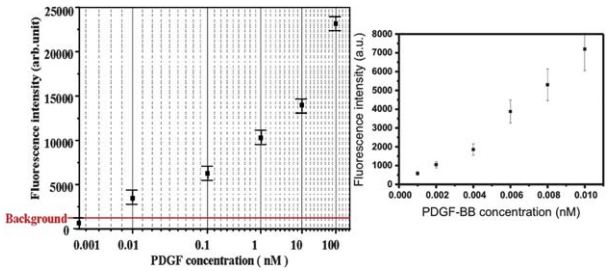

Fig. 5 Response of the sensor for a wide range of PDGF-BB concentrations from 0.001 nM to 100 nM. Figure on the right side shows that the detection limit of PDGF-BB was as low as 4 pM. The concentrations of the PDGF-binding aptamer and labeled PDGF aptamer were 20 μM and 1 μM, respectively.

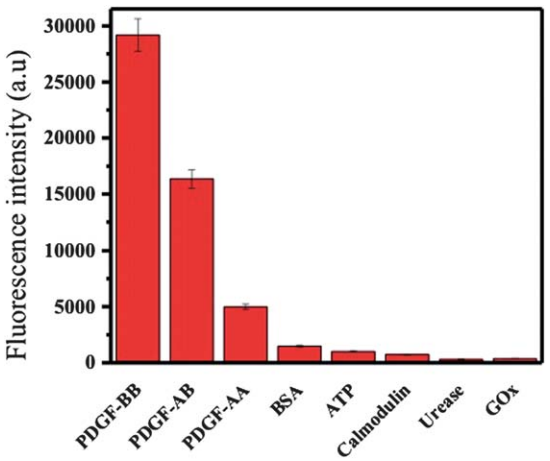

Fig. 6 Fluorescence signal intensity detected using different types of protein showing a comparison of the binding capability on the PDGF-B chain (PDGF-BB) with those of other growth factors and biomolecules, such as the PDGF isomers PDGF-AA and PDGF-AB, serum albumin, ATP, calmodulin, urease and glucose oxidase. The concentrations of the three isoforms and biomolecules (BSA, ATP, calmodulin, urease and glucose oxidase) were all 100 nM. This experiment was conducted in PBS with 5.5 mM $MgCl_2$ buffer solution.

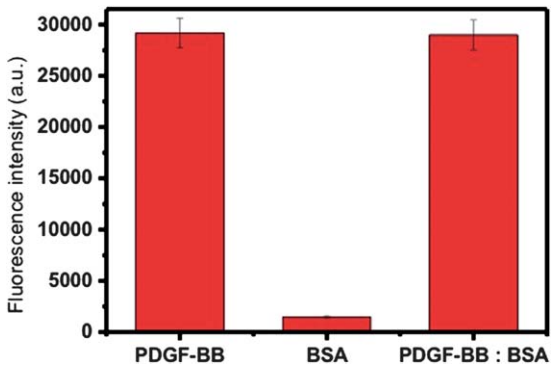

Fig. 7 The fluorescence signal intensity was compared with PDGF-BB, BSA and PDGF-BB containing a biologically relevant concentration of BSA at 1 : 1 ratio in PDGF detecting sandwich design, respectively. The concentrations of biomolecules were 100 nM.

concentration of proteins, it does not affect the selectivity of aptamers towards intensity PDGF-BB. Also we observed a low fluorescence signal intensity when the concentrations of BSA, ATP, calmodulin, urease and glucose oxidase were increased to 1 μM owing to the fluorine treatment, which significantly suppresses the background fluorescence and subsequently increases the signal to noise ratio (see ESI, S1†). Therefore, this sensor can distinguish isoforms with high selectivity. However, when the sample contains biologically relevant concentration such as BSA at 1 : 1 ratio as in Fig. 7, scarce reduction signals are observed. The reduction signal is caused by the interference of PDGF binding aptamer and PDGF-BB interactions.

A diamond surface has the ability to achieve very high stability. To investigate the stability of the PDGF-binding aptamer on directly aminated polycrystalline diamond, we conducted repetitive cycles involving the binding and denaturing of aptamers in 10% SDS solution for 30 min. As a result of this treatment, PDGF-BB and the labeled PDGF aptamer were released from the PDGF-binding aptamer, and the detection was again carried out by subsequently binding PDGF-BB and the labeled PDGF aptamer. Fig. 8 indicates that the fluorescence signal intensity was maintained when the denaturing-binding cycle was performed three times over a period of three days. The

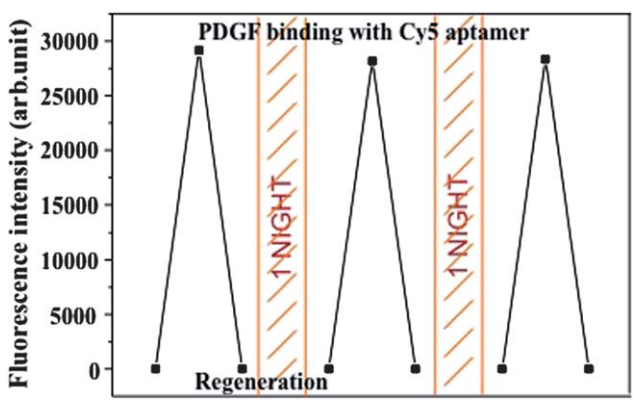

Fig. 8 Reproducible detection of PDGF-BB using aptamer sandwich design observed for three cycles over three days. The sensor was stored in a refrigerator after each cycle. The concentrations of PDGF-BB and labeled PDGF aptamer were 100 nM and 1 μM, respectively.

reproducibility is due to the fact that the aptamers, which are covalently immobilized on the diamond surface, are stable and maintain their ability to bind selectively to PDGF-BB even after repeated cycles. We consider that this aptamer-based sensor has greater potential to be used as a long-term reusable sensor than protein-based sensors.

Conclusions

We have developed a highly sensitive aptamer sandwich design for PDGF-BB detection on a functionalized diamond surface, and demonstrated the reusability of the aptamer as a sensing probe for an optical-based sensor. The aptamer sandwich design successfully detected PDGF-BB at a concentration of as low as 4 pM. We also demonstrated that the sandwich design is highly selective to PDGF-BB even in the presence of different biomolecules. The simplicity and specificity of this approach hold great potential for protein analysis and cancer diagnosis. The same assay concept can also be used in biosensors for *in vivo* or *in vitro* protein monitoring. The increasing interest in aptamer research will expand their future applications to the clinical, biological and immunological fields.

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