Fluorescence-Signaling Aptasensor for ATP and PDGF Detection on Functionalized Diamond Surface

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An aminated diamond-based aptasensor is presented for adenosine triphosphate (ATP) and platelet-derived growth factor (PDGF) detection. In this work, a signal-off detection method was employed to provide a sensitive, selective and reusable platform for the simple detection of PDGF and ATP. The detection limit of 0.2 nM and 0.2 μM of PDGF and ATP were achieved, respectively. It was also demonstrated in this study that the aptasensor is selective for PDGF and ATP over other biomolecules. In addition, the reusability of the aptasensor was confirmed by performing multiple cycles, which shows that diamond is a promising candidate as a material for biosensor applications. Moreover, this detection system does not require a target label and it is unaffected by non-specific binding.
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To utilize an aptamer for biosensor applications, the immobilization of the aptamer to the sensor surface is considered as an important step in sensor design. The design challenges include many factors such as minimizing the non-specific binding, loss of affinity and increasing the accessibility of the aptamer. Aptamers resolve their potential as sensors when they are covalently immobilized on a solid surface. Aptamers are short, single-stranded oligonucleotides that have been selected for high affinity against a target protein, small molecule, or even whole cells. Owing to their high sensitivity, selectivity, temperature stability, low cost, and reusability, aptamers have become the ideal recognition elements for varying biosensors. There are several possible immobilization strategies for DNA based molecules.[1] Antithrombin aptamers were modified with an amino group at the 3′ end, enabling covalent attachment to a glass surface via carbonylimidazole groups[2] or aldehyde groups.[3] Strategies for DNA immobilization in nucleic acid-based sensors with quartz crystal microbalance detection of hybridization were developed several years ago in various formats including biotin attachment and direct adsorption.[4,5] These were adapted to aptamers in biosensors with a biotin labeled anti-IgE aptamer immobilized on a streptavidin modified Au.[6] Biotin or streptavidin immobilization strategies were also used in other aptamer biosensors.[7,8] However, the methods described above take several steps to immobilize the aptamer resulting in a higher risk for chemical contamination and loss of specificity of the sensor.[9] It is therefore vital to develop simple methods for the attachment of aptamers to the surfaces for use in biosensors or diagnostics that involve minimal loss in functionality and non-specific interactions.

Diamond has attracted significant attention because diamond is the best-known electrochemical transducer owing to its chemical stability, low background current, wide potential window and outstanding biocompatibility.[10–12] The diamond surface shows unique properties as it can be modified with hydrogen, oxygen, fluorine, carboxyl group and amino groups, which allows optimizing the properties of the solid or electrolyte interface for biological materials such as enzyme,[13] DNA[14,15] and protein.[16] Furthermore, covalent binding and electrostatic adsorption are the most well known methods that have been used currently in diamond surfaces for DNA immobilization.[17] Of these methods, covalent binding onto several types of termination has been used mainly, because these surfaces provide sites for amide or disulfide bonds.

However, the oxygen terminated surfaces formed by dry oxidation or wet chemical treatment provide several oxygen-related functional groups such as ketone, hydroxyl, and carboxyl groups. The method could subsequently limit the surface coverage of the carboxyl group, which is crucial for the immobilization of biomolecules. However in the case of amination, only NH_2 bonds are expected to form on the diamond surface due to their chemical structure.[14] Furthermore, the aminated diamond commonly keeps the structure for a long time. 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.[18] This is because the Au-thiol surface shows rapid degradation owing to the easy hydrolysis of the thiol group under basic conditions.[10,12] Besides that, fluorine treatment have the hydrophobic behavior and the electronegativity of fluorine contributes to the suppression of non-specific binding to a great extent, which resulted in significantly increasing the signal to noise ratio of the fluorescence signal.

Here, we report a signal-off method designated biosensor on aminated diamond surfaces focusing on fluorescence-signaling aptasensor for adenosine triphosphate (ATP) and platelet-derived growth factor (PDGF) detection. ATP plays an important role as the major carrier of chemical energy in regulation of cellular metabolism, biochemical pathways in cell physiology. It also has been used as an indicator for cell viability and cell injury. Whilst, PDGF is related to tumor growth as a potential cancer marker. It plays an important role in the regulation of cell growth and division. The efficiency of aptasensor for PDGF-BB and ATP was investigated in the view of selectivity, sensitivity, stability and reusability performance on functionalized diamond surface. Combining the bio-recognition elements of aptamers to the solid surface like diamond will represents a significant advancement, especially with respect to biomolecular reaction in vicinity of chemically functionalized carbon-based surface. Therefore, diamond as an active substrate interacting with aptamer immobilized on it has a potential that goes beyond a passive role as a substrate for anchoring biomolecules.

Experimental Methods

Direct amination on diamond surface.— A polycrystalline diamond was deposited by the microwave plasma-assisted chemical vapor deposition method (MPCVD) on a silicon substrate (100) in 1% methane gas diluted with hydrogen gas for 4 h. After deposition, the diamond films were exposed to hydrogen plasma to realize hydrogen termination with a chamber pressure of 50 Torr. For surface functionalization of diamond, partial surface amination of the hydrogen-terminated diamond was performed by low-pressure mercury of ultraviolet light (UV) with the wavelength of 253.7 nm. A Nitrogen gas was introduced into the reaction chamber for 5 min to purge the gases inside the chamber, then ammonia gas (99.9%) was introduced at 100 sccm and the substrate irradiated with UV light for 4 h. The diamond surface was evaluated by X-ray photoelectron

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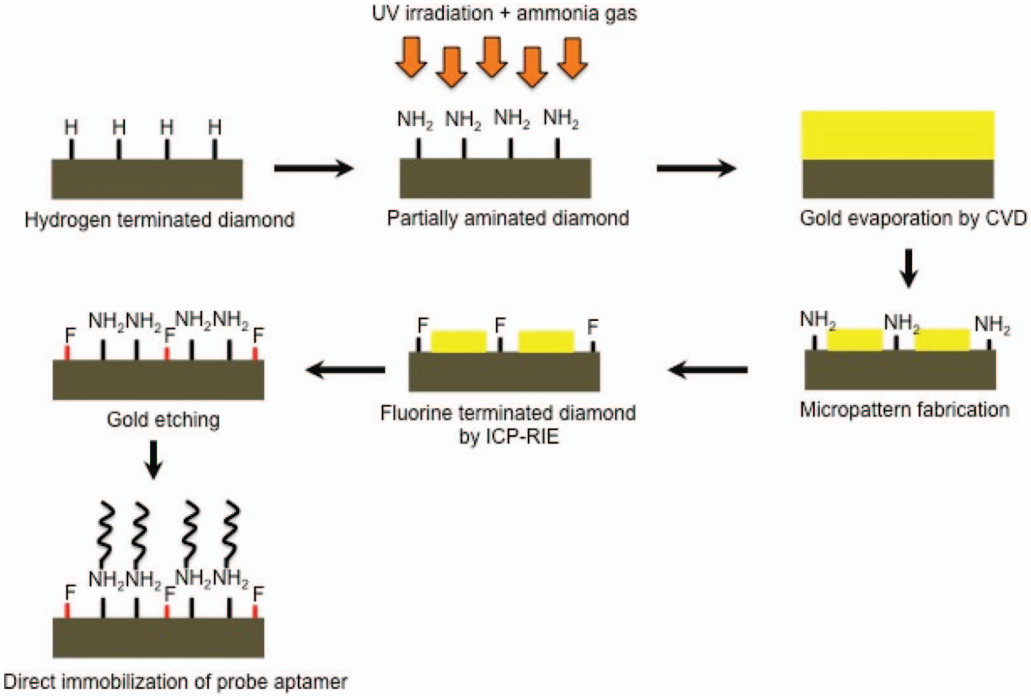

Figure 1. A micropattern fabrication step on diamond surface and probe aptamer immobilization.

spectroscopy (XPS) analysis before and after amination separately using an Ulvac Φ 3300 (Ulvac-Phi, Kanagawa, Japan) with an anode source providing Al Kα radiation.

Fabrication of diamond micropattern.— A micropatterns were fabricated on aminated diamond using Au mask by photolithography. The micropatterns on the partially aminated diamond surface were fabricated using the following steps: (1) deposition of the Au mask by chemical vapor deposition (CVD), (2) prebaking at 80°C for 10 min to remove adsorbed water molecules, (3) coating with photoresist by spin coating, (4) re-baking at 80°C for 20 min, (5) patterning by aligner, (6) immersion in drying solution for 20 s, and (7) etching at the outside of micropatterns by KI solution. For the cleaning of KI solution and Au, the diamond surface of the etched Au was sonicated three times with H_2O for 5 min. The remaining amino group was not destroyed in KI solution after etching the Au mask, because the probe oligonucleotides can be immobilized within the existing amino group. To improve the signal-to-noise ratio, the area outside of the micropattern was fluorinated by C_3F_8 plasma treatment (RIE-101iPH; Samco International, Inc., Kyoto, Japan), because the nonspecific adsorption of probe and target oligonucleotides can be minimized by the negative charge of the fluorine-terminated diamond.[19] A fabrication step of the micropattern on functionalized diamond surface is shown in Figure 1.

Chemicals and reagents.— The PDGF-binding aptamer (i.e., 5′-CAG GCT ACG GCA CGT AGA GCA TCA CCA TGA TCC TG-3′),[20] the complementary DNA of ATP aptamer (5′-TGG ACC CCC TCA TAA CGC CTC CTT CCA-3′)[21] modified with –COOH at the 5′ end and the 27-mer ATP-binding aptamer (i.e., Cy5-5′-TGG AAG GAG GCG TTC TGA GGG GGT CCA-3′)[21] labeled with Cy5 at the 5′ end were synthesized and purified by Sigma Genosis Company (Hokkaido, Japan). The intercalating dye solution (TOTO (1,1-(4,4,8,8-tetramethyl-4,8-diazaundecamethylene)-bis-4-(3-methyl-2,3-dihydro(benzo-1,3 thiazole)-2-methylidene) quinolinium tetraiodide)[22] was purchased from Invitrogen Corporation (Tokyo, Japan). The PDGF isoforms (i.e, PDGF-BB, PDGF-AB, PDGF-AA), adenosine triphosphate (ATP), glucose oxidase (GO_x), calmodulin and urease were purchased from Sigma Genosis Company (Hokkaido, Japan). The bovine serum albumin (BSA) samples were purchased from Sigma Aldrich Company, Tokyo. Two samples were prepared which are of undiluted bovine serum and of a contaminated-ridden sample of 50% BSA. Distilled water (DI) was used to prepare all the solutions.

Covalent immobilization of probe aptamer.— The PDGF-binding aptamer and the complementary DNA of ATP aptamer were used as a probe DNA for PDGF and ATP detection, respectively. These probe DNA were immobilized on the partially aminated diamond surface via covalent binding. The concentration of the immobilized probe DNA used was 20 μM probe DNA + 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 2:1:1 ratio. The sample was incubated for 2 h at 38°C in a humidified chamber. After immobilization, the samples were then 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 physisorbed probe aptamer. In the case of PDGF, the concentration of the intercalating dye solution was 10 μM in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH = 8.0) and the aptamer with intercalating dye sample was incubated at room temperature for 1 hour. Then, the sample was washed again in TE buffer at room temperature for 20 min. The fluorescence signal was observed by epifluorescence microscopy (Olympus IX71; Olympus, Tokyo, Japan). Then, the target protein (PDGF) solution was diluted with 2 × SSC and incubated at room temperature for 1 hour. After that, the sample was rinsed using DI water and again the fluorescence signal was measured. As for ATP, the ATP-binding aptamer was hybridized to the complementary DNA of ATP aptamer (probe DNA) with the concentration of 1.0 μM in 2×SSC for 1 h at 25°C. The sequence is completely complementary to the probe DNA because the ATP-binding aptamer itself bears four pairs of complementary bases at its both ends (i.e., 5′-TGG AAG GAG GCG TTA TGA GGG GGT CCA-3′)[21] and accordingly, its complementary DNA inherently has four pairs of complementary bases (i.e., 5′-TGG ACC CCC TCA TAA CGC CTC CTT CCA-3′) at both of its ends. The affinity of ATP is not affected to the aptamer that even overcomes Watson-Crick base pairs which has been demonstrated by Ying Lu et al. group.[22] Then the fluorescence signal

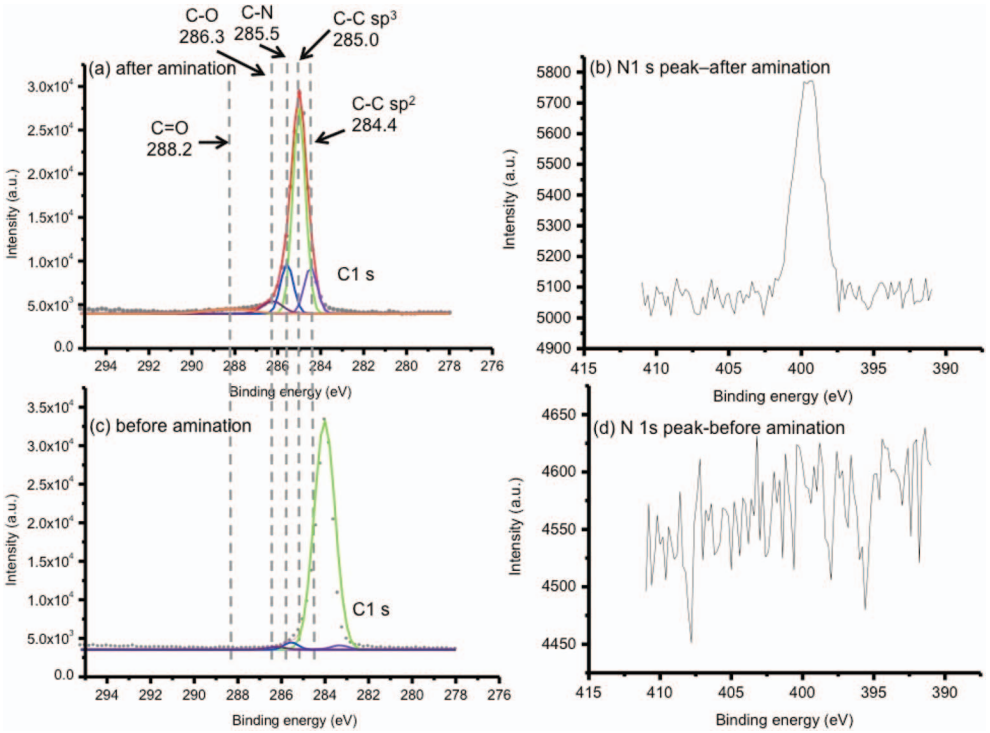

Figure 2. X-ray photoelectron spectra of polycrystalline diamond surface. (a) High resolution scan of the C 1s peak and (b) high-resolution scan of the N 1s peak are compared before and after direct amination.

was measured. Finally, the ATP was introduced and incubated for 1 h at 25°C followed by the fluorescence signal measurement.

Results and Discussion

XPS analysis.— Two different kinds of diamond surface, before and after amination were evaluated by XPS to determine the amine groups generated on the diamond surface by direct amination. Figure 2 shows the XPS spectra for C 1s and N 1s area before and after direct amination. Amino groups are supposed to exist at the high-density regions of the diamond surface by amination processes. The simple reaction of C-H bonds with ammonia gas undergoes a photochemical reaction once exposed to UV irradiation. This resulted in the formation of C-NH_2 on diamond substrate, though steric limitations will limit amine group coverage on the surface to a certain level.[14] In Fig. 2a the main carbon C 1s from diamond was detected at a typical carbon peak position, which includes sp^2 and sp^3, at 284.4 eV and 285.0 eV, respectively. The positions of the peaks at 285.5, 286.2 and 288.2 eV were identified and correspond to C–N, C–O and C=O bonds, respectively. The corresponding ratio of N/C is 0.08. In addition, the existence of the amino group on the diamond was confirmed by the presence of the N 1s peak after amination in Fig. 2b. The density of amino groups is evaluated from the N 1s-to-C 1s peak-area ratio but not from C–N to C–C peak-area ratio due to the very small (0.2 eV) binding energy differences between C–C (sp^3) and C–N.[14] The results show that the amino groups were successfully formed on diamond surface by direct amination.

Fluorescence signal detection.— The illustration diagram of the aptasensing mechanisms of PDGF and ATP are shown in Figure 3.

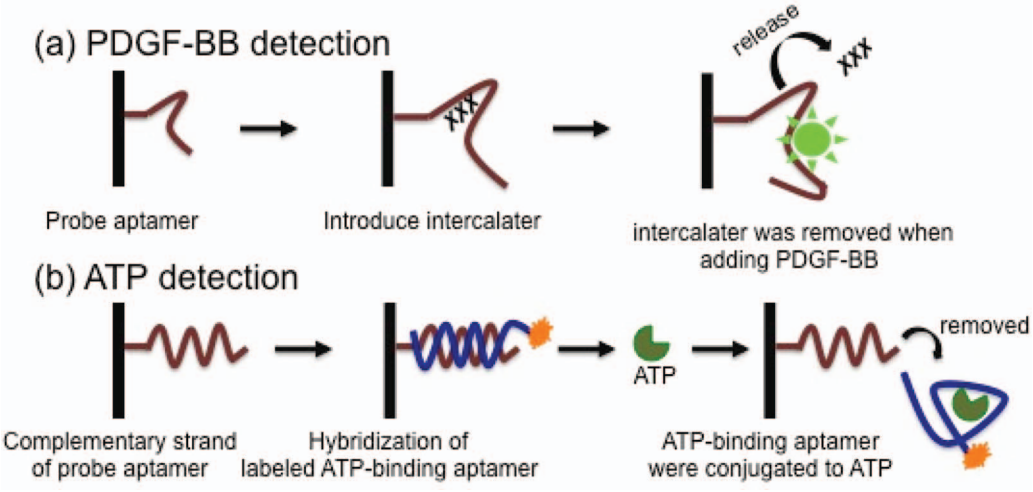

Figure 3. A PDGF-BB and ATP sensing mechanism on aminated-diamond aptasensor.

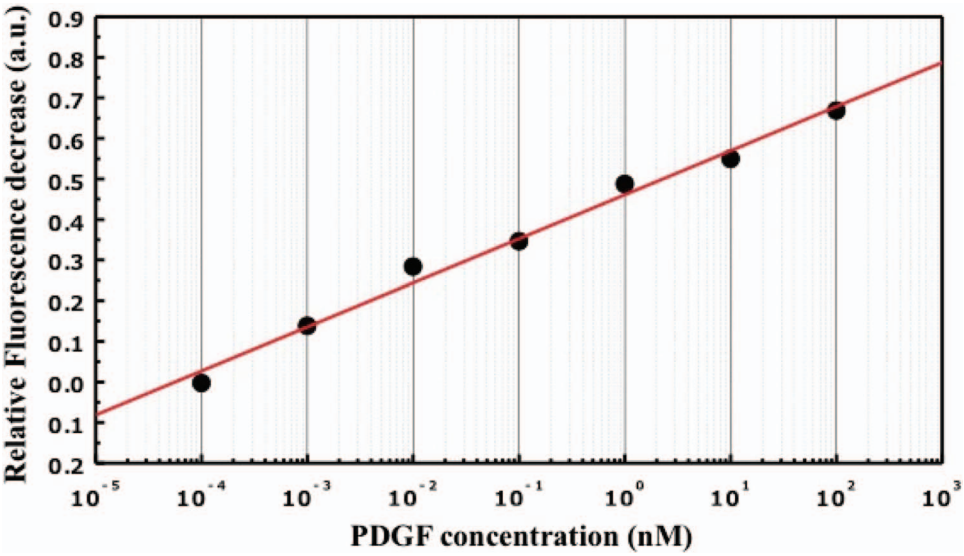

Figure 4. The sensitivity of PDGF-BB aptasensor from range 100 fM to 100 nM. The concentrations of the probe aptamer and intercalater were 20 μM and 10 μM, respectively.

Here, we used the existing DNA aptamer for PDGF and ATP to test the feasibility of signal-off strategy using the molecular light switch, TOTO and labeled ATP-binding aptamer, respectively. A PDGF binding aptamer is reported to form a stem loop structure that can capture the intecalater fluorescent dye, resulting in significant fluorescence signal. As PDGF-BB is added to the PDGF-binding aptamer, the intercalater dye dissociates due to the change of the stem loop structure in three-way helix junctions. This results in a decrease in fluorescence signal intensity indicating the presence of PDGF-BB during the sensing detection method. As for ATP detection, the complementary strand of aptamer was immobilized on aminated diamond surface as probe DNA. The labeled ATP-binding aptamer were hybridized into the sample and fluorescence signal was observed indicating the conformational change of duplex structure. When ATP is introduced into the sample, the labeled ATP-binding aptamer plays an important role to conjugate with ATP and disintegrated from the probe DNA, which was confirmed with the decreased in the fluorescence signal. These methods enabled accurate detection of PDGF and ATP existing in the sample with a low signal-to-noise background caused by physical adsorption of both ATP and PDGF themselves.

Aptasensor performance on sensitivity, selectivity and reusability for PDGF-BB detection.— Figure 4 shows a relationship between the relative fluorescence signal and PDGF-BB concentration. The normalized distributions of the PDGF concentration were plotted in the range of 100 fM to 100 nM. When the concentration of PDGF-BB decreased to 100 fM, the fluorescence signal intensity also decreased accordingly. The volume of probe aptamer and intercalater were constant at 20 μM and 10 μM, respectively. The fluorescence signal intensity is found to be related with the effective number of the conjugated structures, formed by probe aptamer and PDGF-BB protein as well as intercalaters with quite high affinity. The detection limit of this aptasensor for PDGF-BB was experimentally determined to be 0.2 nM based on signal-to-noise >3 while the limit of quantification (LOQ) was determined to be 0.7 nM. This sensitivity is apparently better than that previously reported for PDGF detection with different methods.[23,24] The achievement of this detection limit will be sufficient to be used for wide application in preliminary diagnosis cancer.

A selectivity of PDGF-BB aptasensor on diamond surface was investigated against other biomolecules such as BSA, Calmodulin, Glucose oxidase, Urease, ATP, and PDGF isoforms of PDGF-AB and PDGF-AA. As shown in Fig. 5a only PDGF-BB caused a marked reduction in fluorescence signal, while all the other biomolecules failed to cause any significant reduction in fluorescence signal. The fluorescence change of the aptamer with intercalater is highly selective due to the inherent specificity of the aptamer toward its target protein. However, the selectivity toward its isoforms, PDGF-AB revealed that a reduction in fluorescence signal compared to PDGF-BB was observed in about half of the signal intensity. This is because the similarity of amino acid sequence of PDGF-AB with PDGF-BB is only about 60%. On the other hand, the PDGF-AA can also binds to the aptamer but the affinity caused by PDGF-AA was clearly lower than

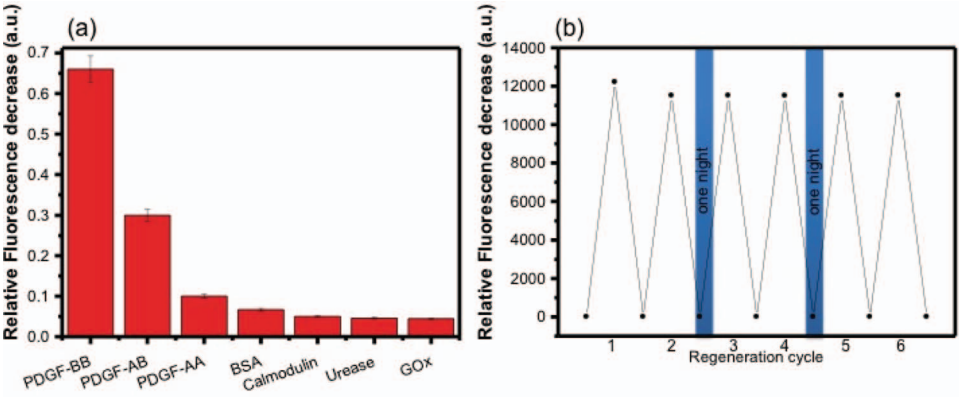

Figure 5. (a) Comparison of binding capability with other biomolecules such as BSA, calmodulin, urease, glucose oxidase and isoforms like PDGF-AB and PDGF-AA. The concentrations of all biomolecules and isoforms were 100 nM. (b) Reusable of the aptasensor on PDGF-BB detection was observed for three days over three cycles.

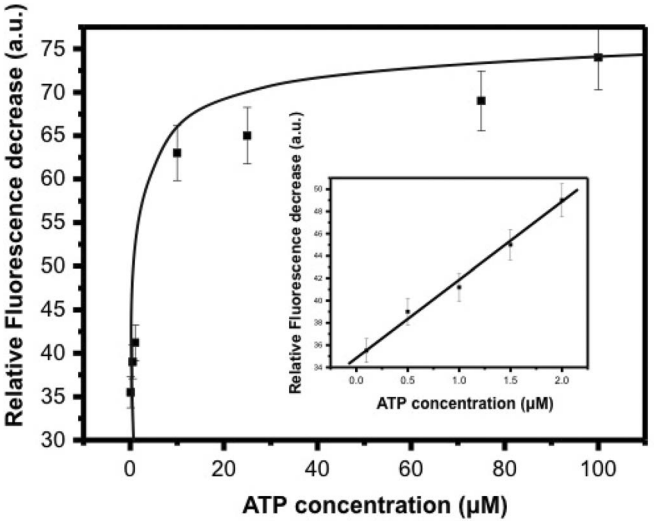

Figure 6. A response of aptasensor for ATP concentration from 0.1 μM to 100 nM. The inset shows the detection limit of ATP concentration from 0.1 μM to 2 μM. The limit detection of ATP was down to 0.2 μM. The concentrations of the complementary strand of aptamer and labeled ATP-binding aptamer were 20 μM and 1 μM, respectively.

PDGF-AB. Thus, this aptasensor can distinguish isoforms with high selectivity.

To examine the reusability of aptasensor toward PDGF-BB on directly aminated diamond surface, we investigate the repetitive cycles involving the binding and denaturing of PDGF-BB using 10% sodium dodecyl sulfate (SDS) treatment for 20 min in three cycles over three days. Fig. 5b indicates that the relative fluorescence is maintained when the binding-denaturing cycles was performed in three cycles. This result confirmed the reusability of the aptasensor, where the aptamer only bind selectively to PDGF-BB.

Sensitivity, selectivity and reusability of aptasensor for ATP detection.— The presence of ATP at different concentration led to a different fluorescence signal intensity, depending on the amount of the released aptamer in the solution. In order to evaluate the performance of the sensor, a series of ATP detection on aminated diamond surface was investigated from 0.1 μM to 100 μM. The difference in fluorescence signal intensity was used to evaluate the fluorescence signal response of ATP. As shown in Figure 6, the reduction of fluorescence signal intensity is bigger with increasing ATP concentrations. The sensor showed linear relationship between the relative fluorescence decrease and ATP concentration from 0.1 μM to 2.0 μM with the correlation coefficient of 0.986 (Figure 6 inset). The limit of detection (LOD) and lower limits of quantification for ATP was experimentally determined to be 0.2 μM and 0.7 μM, respectively, based on a signal-to-noise ratio of 3, which is lower than that in the previous reports.[21,25,26]

On the other hands, the selectivity of aptamer toward ATP was very high as shown in Figure 7a. We examined the selectivity of ATP and NTPs in lower concentration of 1 μM. It is found that this aptamer showed high specificity on ATP as compared with other related group of ATP biomolecules such as NTPs (UTPs, CTPs and GTPs). This is because these NTPs failed to cause any significant signal. These results indicate that this aptasensor can control the selectivity of ATP in different kinds of concentrations by controlling the ATP-binding aptamer concentrations.

Four repetitions of each standard ATP solution were carried out to evaluate the reproducibility and precision of the method. The results indicate that the aptasensor can be reused as shown in Figure 7b, which shows its potential as reusable biosensor. The higher intensity of the first cycle's pre-hybridization was negligible as it is caused by physical adsorption of the aptamers onto the diamond surface. These physical adsorption can be reduced easily by chemical functionalization, such as oxidization or fluorination, in the same way as we decreased the physical adsorption in our previous study.[16,17,27] The reusability and stability of probe DNAs obtained are due to the strong covalent binding of the chemically stabile amino bonds introduced via photochemical amination, which is of great advantage on the sp^3 carbon based diamond surface.

Biological samples.— To test the practicality of this present method, the PDGF-BB has been applied to the potential interference such as bovine serum albumin samples directly. Two samples were used which are of undiluted bovine serum and of a contaminated-ridden sample of 50% BSA. As shown in Figure 8, the feasibility of signal-off method can still be observed in the PDGF-BB concentration range from 0.01 nM ∼ 100 nM. Clearly, the sensitivity for the detection with bovine serum albumin sample cannot be as high as that for the pure one due to adsorption of the complex matrices and non-target proteins such as human serum albumin. Nevertheless, this signal-off method could still be used to detect the PDGF in biological samples for early diagnosis of clinical samples. However, the complex matrices for ATP are still in progress for further investigation.

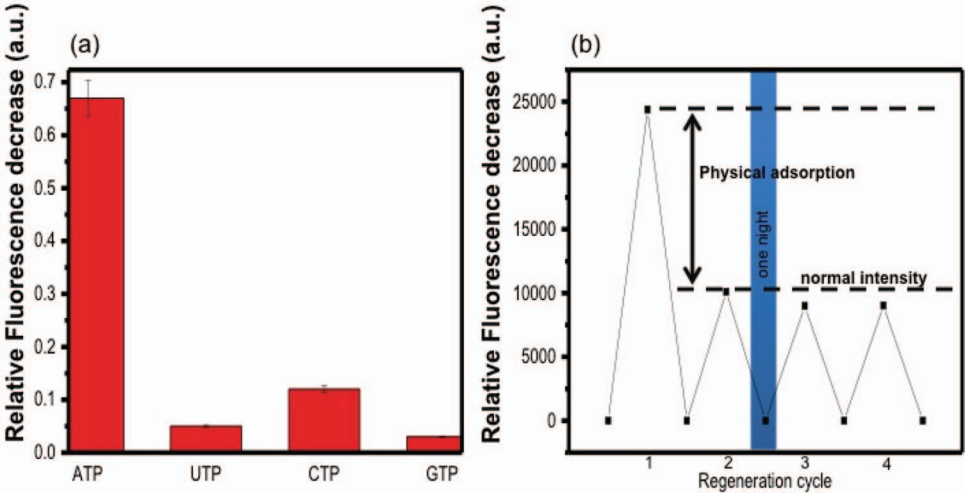

Figure 7. The selectivity and reusability of ATP on aminated diamond surface was performed. (a) A comparison of selectivity on ATP with other related group such as UTP, CTP and GTP. The concentrations of all the biomolecules were 1 μM. (b) The reusability of aptasensor was observed over four cycles in two days.

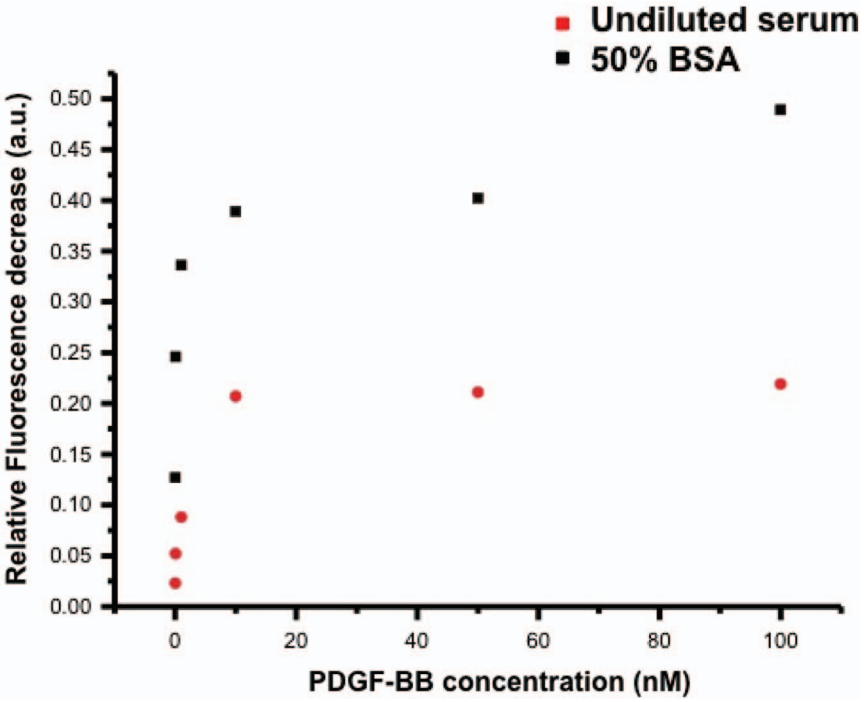

Figure 8. The sensitivity of aptasensor was investigated in biological sample with the concentration of PDGF-BB dissolved in (red square) undiluted BSA and (black square) 50% BSA.

Furthermore, the evaluation results of PDGF-BB in serum albumin can give a model for other biomolecules on diamond as a solid support.

Conclusions

In conclusion, the detection of PDGF and ATP on aptasensor-based aminated diamond surface has been performed successfully in terms of sensitivity, selectivity and reusability. This study provides clear evidence that the aptamer directed immobilization approach could be applied as a simple method to assemble sensors with high specificity. The low signal-to-noise background of PDGF and ATP detection attest a high sensitivity sensor within a repeatable binding-denaturing cycles and high selectivity against other biomolecules. In aptamer technology, simplicity and specificity are the important features and thus, this finding holds a great potential for future medical diagnosis.

Acknowledgments

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