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Graphene-Based FRET Aptasensors

Yuko UENO*†

* Department of Applied Chemistry, Faculty of Science and Engineering, Chuo University, 1-13-27 Kasuga, Bunkyo, Tokyo 112-8551, Japan

† To whom correspondence should be addressed.

E-mail: yuko.ueno@kc.chuo-u.ac.jp
Abstract

Graphene-based FRET aptasensors can be realized only by these unique combinations of aptamer that can be freely functionalized by chemical modification, and graphene/graphene oxide that works as an excellent fluorescence acceptor at the same time as aptamer adsorbates. The review describes the principles of the sensor, several applications to microchannel devices, improvement of the sensing performance by molecular design of aptamer and future remarks by introducing recent works and achievements including our paper. The sensor employs DNA that modified with graphene/graphene oxide at the terminal as the molecular probe. This system is supported by the excellent property of DNA that the molecular recognition ability without lost even by a chemical modification at the terminal with huge structure. I hope that this review will be useful for developing research on higher performance of graphene aptasensors in the future.
1. Introduction

DNA forms a predictable three-dimensional structure determined by its base sequence and expresses structure-dependent functions. Artificial synthesis and chemical modification of DNA are easy and has excellent thermal and chemical stability [1]. We can connect various functional groups such as fluorescent labels, polymer chains, and reactive terminal groups such as amino, sulfhydryl and biotin to the ends without changing the properties of the DNA [2]. Taking advantage of these characteristics, applications to molecular probes and molecular recognition devices of DNA have been actively studied [3]. Furthermore, by reversibly controlling the formation of the double helix structure and the dissociation reaction to a single strand, the three-dimensional structure and surface charge can be changed as we required [4-5]. This enables us to use more sophisticated and complex molecular functions of DNA. Among these DNAs as functional molecules, a special DNA having a sequence that selectively forms a complex with a specific target molecule, is called an "aptamer" [1, 6-12, saito AS]. Many aptamers have been reported for various kinds of target, including cells [7] and proteins [8, 9, wakui, wakui], as well as small molecules [10, nakatsuka, tokeshi, wang] and ions [11, 12]. Thus, wide varieties of biosensors including electrochemical and fluorescence detection methods that utilize the molecular recognition function of aptamer has been studied. The materials that conjugate with DNA or aptamer are also various, such as metal nanoparticles [13-17], hydrogels [18, 19] and magnetic beads [20, 21] (Fig. 1). A biosensor that uses aptamer on the graphene surface (graphene aptasensor) has a unique feature because it utilizes a special adsorption interaction called π-π interaction between aptamer and graphene [22-24].

Graphene is an atomically thin two-dimensional sheet consisting of sp² carbon atoms, which exhibits excellent thermal and chemical stability, as well as extremely high
electronic mobility and mechanical strength [25]. The mass production of graphene was difficult when it was first isolated in 2004 [26] but has progressed dramatically in the following years. It is possible to obtain a monolayer of graphene larger than several tens of centimeters square by using synthetic techniques as the chemical vapor deposition process [27]. It is worth mentioning graphene oxide (GO) that is also attracting considerable interest as the most widely utilized chemical derivative of graphene [28-30]. GO is an oxidized form of graphene and relatively easy to mass-produce. The most important merit is that we can chemically synthesize GO by oxidizing graphite powder in strong acid and this yields a large quantity of GO. However, it is difficult to obtain a synthetic GO that is larger than 1 mm square, which is one of the disadvantages for application to devices. Another disadvantage of GO is that many of the bonds between the carbon atoms in graphene are broken and link with oxygen to form carbon-oxygen bonds. Thus, GO contains large number of nanometer-sized graphene-like sp² domains discrete from each other. For these reasons, GO is not suitable as a replacement for graphene in electronic materials that require high mobility but is still applicable to optical devices [31-33].

In order to perform molecular recognition using graphene/GO conjugated with aptamer, using graphene/GO as a converter of an invisible binding reaction between the aptamer and its target molecule into measurable physical energies such as light and electricity. On the surface of graphene/GO, energy transfer occurs when molecules are located close to the surface. As a typical example, graphene/GO works as an excellent acceptor for fluorescence resonance energy transfer (FRET) over the entire visible wavelength region [34, 35]. The yield of the energy transfer depends on the strength of molecular interaction between the adsorbed molecules and the graphene/GO surface. Thus, when a fluorescent molecule is located sufficiently close the graphene/GO surface,
the fluorescence is quenched. By utilizing this function as a fluorescent switch, GO has been widely used for the fabrication of FRET aptasensors in the dispersed state in an aqueous solution. In a recent study, we have reported for the first-time fabrication of a graphene FRET aptasensor on a solid surface with a microchannel device [44]. The sensor uses the aptamer, that is functionalized by graphene/GO fixed on the solid surface at the terminal, is used as the molecular probe.

Several reviews overviewing graphene/GO aptasensors including not only fluorescence detection, but also electrochemical detection have been reported by Gao et al. [36] and Wang et al. [37]. In this paper, I focus on FRET aptasensors, that can be realized only by these unique combinations of aptamer that can be freely functionalized by chemical modification, and graphene/GO that works as an excellent fluorescent acceptor at the same time as aptamer adsorbates. The review describes the principles of the sensor, several applications to microchannel devices, improvement of the sensing performance by molecular design of aptamer and future remarks by introducing recent works and achievements including our paper.

2. GO FRET aptersensor

GO fluorescent aptasensors has been actively studied in various variations. These studies are based on the common principle as follows [36, 37]. First, the dye-labeled aptamer is added to the GO dispersion. Aptamers, which are single-stranded DNA (ssDNA), are physically adsorbed on the GO surface by π-π interaction. As the labeled fluorescence energy is well quenched by GO, the entire solution is dark. When a sample solution containing the target analyte is added here, the aptamer forms a complex by stronger interaction than GO with the target molecule and dissociates from GO. As the aptamer released in the dispersion, recovered fluorescence is observed (Fig. 2) [38].
Using the principle, GO aptasensors operating in a dispersion solution has been successfully demonstrated about many different sizes and types of targets, small molecules such as adenosine [39], proteins such as growth factor-BB (PDGF-BB) [40], glycated human serum albumin [41], and large structures such as bacteria (Salmonella typhimurium) [42].

Inspired by these previous researches, we conducted FRET GO aptasensor that operates on the GO surface supported on a solid surface which enables us to detect biologically important proteins such as cancer markers, selectively and quantitatively [43]. In our GO aptasensor, one end of the aptamer is labeled with a fluorescent dye and the other end is connected to a pyrene linker molecule, which shows a strong affinity to the sp² domains of GO as well as graphene surface (Fig. 3).

An important point to mention is that tethering aptamer on the GO surface utilizes the \(\pi-\pi\) interaction between pyrene to the sp² domain of GO, so this method can be applied directly to pristine graphene. In this manner, we have successfully reported for the first time the fabrication of a graphene FRET aptasensor for detecting a cancer marker protein, namely, prostate specific antigen (PSA) (Fig. 4) [44].

The largest merit of this study is that detection of the target molecules is occurred on the aptamer-tethered GO surface that is fixed on the solid surface. This enables us to understand the molecular recognition by employing local analysis and surface analysis methods which could not be used for dispersion. We used a confocal laser scanning microscope and an atomic force microscope to observe protein detection by aptamer on the surface of a single GO piece fixed to glass or silicon and succeeded in linking the relationship between structural changes and fluorescent recovery. Here, we used thrombin, a protein important for blood coagulation, as a detection target. The creation of aptasensor that operates on this solid surface has made it possible to combine with a
microanalysis technique. I will introduce these studies in the next section.

3. Graphene sensors operating on microdevices

We fabricated a microfluidic sensor chip by attaching a polydimethylsiloxane (PDMS) polymer sheet with multiple microchannels to a glass substrate functionalized with a graphene/GO aptasensor on its surface [45, 46]. By mounting multiple microchannels, it is possible to unify the measurement conditions for responses to many different samples at once. This chip enables us to conduct accurate quantitative analysis by ratiometric comparison between channels, even for fluorescence sensors, which are difficult to make quantitative comparisons in principle. We can fabricate a microchannel with the desired design by using photolithographic techniques. The number of the channels is also variable. Thus, if we form multiple microchannels on a single chip, we can perform a high-throughput analysis of several different measurements in parallel. It is noteworthy that the PDMS microchannel requires no external power or expensive equipment to flow liquid, because the flow in the microchannel is driven by capillary force [47]. Thus, detection of the target protein is one-step, namely, simply by adding a sample smaller than 1 μL to the chip and the fluorescence is observed in about a few second after. A real-time detection is possible by tracking the fluorescence intensity at the microchannels by adding different sample solutions sequentially. We injected DI water and PSA (100 μg·mL⁻¹) in one of the dual microchannel, while DI water was injected in the other channel throughout the measurement as a reference. Initially, the both channels were filled with DI water in which almost no fluorescence was observed. Immediately after injecting the solution containing PSA, bright fluorescence was observed, and when rinsed with DI water, it returned to the same darkness as the reference channel. The same phenomenon was observed each time DI water and PSA
were alternated, and the signal strength was similar. The result indicates that the tethered aptamer maintains on graphene surface and thus the sensor is sufficiently durable for a real-time and/or continuous monitoring of the target proteins under flow conditions.

The strength of microdevices is that they are easy to array. We fabricated a 2×3 linear-array aptasensor by using two different aptamers for different targets, namely thrombin and PSA, which we labeled with red fluorescent TAMRA and green fluorescent FAM, respectively, for ease of distinction. After injecting PSA and thrombin solution into the top and bottom microchannels, respectively, we obtained a color-composite fluorescence image of the sensor (Fig. 5). Bright fluorescence was observed only in the position where the combination of aptamer and target molecule was correct. Thus, a simultaneous multiple protein detection system on a single chip was successfully demonstrated.

Several different kinds of microfluidic GO aptasensor has been studied based on the idea of using tethered aptamer to solid surface. Luo and co-workers fabricated PDMS/paper/glass hybrid microfluidic system integrated with GO aptasensor for simple, one-step, multiplexed pathogen detection (Fig. 6) [48]. Simultaneous detection of two infectious pathogens, S. aureus and S. enterica, were successfully demonstrated. The assay takes 10 min to complete. Wang and co-workers fabricated another paper-based microfluidic GO aptasensor for accurate detection of food allergens and food toxins (Fig. 7) [49]. Here, aptamer-functionalized quantum dots (QDs) were used as a probe instead of aptamer modified with fluorescence dye. The limits of detections (LODs) for the four targets, namely, egg white lysozyme, β-conglutin lupine, okadaic acid, and brevetoxins are 343, 2.5, 0.4, and 0.56 ng·mL⁻¹, respectively. These LODs are superior or comparable to those claimed by the ELISA kits. The required sample volume for the
assay was 10 μL and a single test was completed within 5 min. These pioneering studies successfully show that the GO aptasensor is promising as a sensor platform to realize simple on-site measurement that meets many different requirements such as sufficient sensitivity, selectivity, low cost, and chemical stability.

4. Aptamer design and improvement of sensor performances

Among the characteristics of aptamers, the most attractive property for chemists who handle molecular tools is that the degree of freedom of various chemical modifications including fluorescence modification is very high, and thus the variation of functionalization by molecular design is very wide. As an example of high functionality by molecular design of aptamer modification, we have succeeded in making the GO aptasensor highly sensitive by modifying an aptamer with an ssDNA spacer, so I will introduce this study as an example [50].

The strategy was to increase the distance between the fluorescence dye and the graphene surface, which is crucial for FRET-based sensors, when forming a complex with the target protein. We fabricated a 2×3 linear-array aptasensor by using three different probes and introducing ssDNA spacers with 0, 10, and 20 thymine segments between the aptamer and the dye. The fluorescence intensity increased significantly with increases in the spacer length for detection of thrombin (100 μg·mL⁻¹). The results show that introducing an ssDNA spacer at the correct position is an effective way of enhancing sensor sensitivity (Fig. 8). The limit for thrombin detection was about 1 nM, which corresponds to the in vivo concentration range during blood clotting, by using the probe with 20 thymine segments, the best design in our present study. We also studied another molecular design for enhanced sensitivity. We inserted a double-stranded DNA (dsDNA) as a spacer between a linker and an aptamer sequence to extend the distance
between the fluorescence dye and the graphene surface. Since dsDNA is a rigid polymer and has a weak affinity to graphene surface, the dsDNA can work effectively as a spacer. Moreover, the dsDNA spacer should interfere little with aptamer activity for any DNA sequence, because the structure is completely different from that of the aptamer, which is an ssDNA. We prepared the modified aptamer with 10 and 30 base pair (bp) dsDNA spacers in relation to thrombin and PSA detection by using thrombin- and PSA-binding DNA aptamers, respectively. The 30bp-spacer exhibited a signal about twice as large as that of the 10bp-spacer for both thrombin and PSA detection, indicating that a longer dsDNA spacer has a greater effect on increasing aptasensor sensitivity [51].

Qiu and co-workers reported a GO aptasensor for label-free cocaine detection that uses a hearpin probe (HP) that consists of a cocaine aptamer combined with a complementary ssDNA to the primer of polymerase aided isothermal circular strand-displacement amplification (ICSDA). In the absence of cocaine, HP are maintaining its hairpin structure with a long ssDNA and well absorbed by GO. This is resulting in an efficient quenching of the fluorescence of SYBR Green I (SG I), that binds dsDNA selectively. When cocaine was bound with the HP, it opens into ssDNA and then trigger polymerase elongation, generating a dsDNA and displacing the cocaine. Because of the weak affinity between dsDNA and GO, fluorescence quenching by GO would not occur and thus a strong fluorescence signal of SG I is observed (Fig. 9) [52]. The detection limit of cocaine with this method was 190 nM and application to cocaine quantification in human urine samples was successfully demonstrated. There must be still many possible aptamer designs that could be used to improve GO aptasensor performance.

5. Conclusions
I briefly introduced the common principle of the fluorescence detection type GO aptasensor, its application to microchannels, and the high functionality of the sensor by aptamer probe design. Since there are so many types of target molecules for aptamers and there are many variations in aptamer probe design, I need to decline that there are many studies that I could not introduce in this paper. The performance of the aptamer largely depends on the chemical and physical properties and functions of the aptamer itself. Although ease of synthesis and stability over long-term storage have advantages over antibodies, aptamers often do not reach the level of excellent antibodies in terms of binding constants and selectivity, which are indicators of molecular recognition ability. However, research on aptamers has been actively conducted not only in sensor applications but also in fields such as drug discovery. I thus believe that performance of aptamer will improve greatly by these studies in near future. We, analytical chemists, can use these new aptamer probes to achieve even higher performance sensors.
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**Figure Captions**

Fig. 1  Schematic illustration of several examples of DNA/aptamer conjugated materials. (a) DNA-functionalized gold nanoparticles, Reprinted with permission from Ref. 16, Wiley-VCH. (b) Hydrogel conjugated with AFP-Responsive aptamer, Reprinted with permission from Ref. 19, American Chemical Society. (c) Schematic diagram of the substrate detection by capturing of the target enzyme with aptamer-conjugated magnetic beads (MB). Detection of the fluorescent products arising from the enzymatic conversion of the substrate provides an overall measure of the
active enzyme. Reprinted with permission from Ref. 20, American Chemical Society.

Fig. 2  Schematic illustration of GO FRET aptasensor operating in solution phase for ATP detection in living cells. Reproduced from Ref. 38 by permission of the American Chemical Society.

Fig. 3  Schematic illustration of FRET aptasensor using aptamer-tethered GO fixed on a solid surface. Reproduced from Ref. 43 by permission from The Royal Society of Chemistry.

Fig. 4 Fluorescence images of a graphene aptasensor in different PSA concentrations. The concentrations are (a) 0, (b) 33, (c) 20, (d) 14, (e) 11, and (f) 9 μg·mL⁻¹, respectively. Scale bar: 20 μm. Reproduced from Ref. 44 by permission from The Royal Society of Chemistry.

Fig. 5  (a) Layout of a linear-array GO aptasensor, (b) image of microchannel, and (c) color-composite fluorescence image after injecting PSA solution and thrombin solution into the top and bottom channels. Reproduced from Ref. 45 by permission from Elsevier.

Fig. 6 Schematic of the PDMS/paper hybrid microfluidic system for one-step multiplexed pathogen detection using aptamer-functionalized GO biosensors. Reproduced from Ref. 48 by permission from The Royal Society of Chemistry.

Fig. 7 Schematic and the picture of the paper/PDMS microfluidic chip for food allergens/toxins detection. The overall size of the microfluidic chip is 25 × 75 mm².
Reproduced from Ref. 49 by permission from Wiley-VCH.

Fig. 8  Schematic representation of a technique for increasing fluorescence intensity by modifying an aptamer with a ssDNA spacer(left). Fluorescence image of the 2×3 multichannel linear-array aptasensor patterned with aptamer-probes introducing ssDNA spacers with 0, 10, and 20 thymine segments between the aptamer and the dye from left to right. Reproduced from Ref. 50 by permission from The Royal Society of Chemistry.

Fig. 9  Schematic representation of the label-free fluorescent aptamer-based sensor method for cocaine detection based on GO and ICSDA. Reproduced from Ref. 52 by permission from The Royal Society of Chemistry.
Graphical Index
Fig. 1
Fig. 2
Fig. 3

Thrombin aptamer (TBA):
5'-GGTTGGTGTGGTTGG-3'

Graphene Oxide
Fig. 4
Fig. 5

(a) On-chip aptasensor
Control (without aptamer)
Thrombin aptamer
Graphene/GO
Thrombin
PSA aptamer
PSA

(b) PSA
Thrombin

(c) 500 μm
Fig. 6
Fig. 7
Fig. 8

Components of designed probe:
- Dye: donor
- DNA spacer
- Target protein
- Aptamer
- Linker
- Graphene oxide: acceptor

Longer DNA spacer → Brighter fluorescence

Protein detection on graphene oxide surface
Fig. 9