Covalent and Noncovalent Functionalization of Graphene Oxide with DNA for Smart Sensing

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Interfacing nanomaterials with DNA has resulted in the development of numerous biosensors, optimized for different targets and applications. Of all nanomaterials, graphene oxide (GO) has emerged as a prime sensing platform due to its high specific surface area, good aqueous stability, varied functional groups and desirable surface, and electrical and optical properties. This review starts with an introduction of GO and describes its physical and chemical properties. Then, the general strategies of interfacing DNA and GO to develop sensors are discussed. The trends in GO/DNA biosensor development are organized into classes based on the mode of DNA interaction with GO (physisorbed vs chemisorbed). Due to the intermediate DNA adsorption strength on GO, most of the sensors developed utilize physisorption of DNA to GO. Even within the realm of physisorbed probes, there are multiple sensing methods: direct adsorption, inhibited adsorption, competitive adsorption with the use of blocking agents, and tethered adsorption containing a strongly adsorbing block of DNA. Covalently linked DNA probes are also used to increase the biosensor stability. Each of these sensors has its advantages and disadvantages and the designs are discussed with representative examples in detail.

1. Introduction

Being a single layer of graphite, graphene has a huge specific surface area and interesting physical and chemical properties.[1,2] Aside from its applications in energy, electronics, and catalysis, graphene-based materials are also widely used for adsorbing bio-molecules, and proteins by using DNA aptamers and DNAzymes.[23] DNA offers molecular recognition function not only for complementary nucleic acid but also for many metal ions, small molecules, and proteins by using DNA aptamers and DNAzymes.[22–28] The ease of synthesis and modification of DNA, its stability, and predictable and programmable structures are highly attractive for sensor design. GO is also a strong fluorescence quencher and has moderate electric conductivity, both of which are useful for signal transduction.

To achieve optimal sensor performance, it is important to understand and control the interaction strength between DNA and GO.[13,14,29–33] Depending on the sensor design, both weak and strong interactions can be useful. At an individual nucleotide level, π–π stacking is believed to be the most important force on graphene, leading to purines adsorbed more tightly than pyrimidines.[32] For DNA oligonucleotides, additional hydrogen bonding was also identified,[14] which also involves nucleobases. As a result, double-stranded DNA is adsorbed more weakly than single-stranded due to the shielded DNA bases,[35] and DNA of different sequences is also adsorbed differently.

In the past decade, at least three strategies have been developed to attach DNA to GO for designing sensors, including 1) simple probe adsorption, 2) use of diblock DNA, and 3) covalent conjugation. Each strategy has its own advantages and disadvantages. Although a number of review articles have been published on DNA/GO-based biosensors,[13,16,20,36–38] we summarize the field from a new perspective based on the method of probe attachment. We review each of the methods and related surface science, presenting examples of recent applications.
2. Three Strategies to Interface DNA with GO

The simplest way to form a DNA/GO hybrid is to directly mix a single-stranded DNA oligonucleotide with GO, as shown in Figure 1A. As both DNA and GO are negatively charged, salt (e.g., NaCl or MgCl₂) is required to screen charge repulsion for DNA adsorption. [46,59] The concentration of monovalent cations required to achieve DNA adsorption is much higher than that of divalent cations. DNA adsorption is also facilitated by lowering pH due to protonation of carboxyl groups on GO and some of the divalent cations. [39,59] It is believed that DNA tends to maximize its interaction with GO by adopting an extended conformation, but this interaction is balanced by internal secondary structures of DNA. [40] Adding complementary DNA (cDNA) can induce desorption of the adsorbed DNA. [15,41]

This simple adsorption suffers from two problems. First, DNA adsorption by GO can impede target recognition, thus affecting sensitivity. At the same time, other molecules such as proteins may nonspecifically displace adsorbed DNA, affecting specificity of detection. For comparison, carbon nanotubes (CNTs) have also been studied for their interaction with DNA. CNTs can be single-walled (graphene rolled up forming a tiny tube) or multi-walled. [42] Unlike GO, CNTs do not have such a high oxygen content, and DNA can wrap around CNTs, resulting in a much higher adsorption affinity. [43–45] In this case, its cDNA can hardly induce the desorption of the adsorbed DNA. [46] Similarly, DNA is also very strongly adsorbed on gold surfaces, making such a sensing mechanism difficult. [47] In this regard, GO has an appropriate adsorption strength.

The second strategy takes advantage of sequence-dependent adsorption. As purines are adsorbed on graphene more tightly than pyrimidines, the use of a high affinity polyadenine (poly-A) anchoring sequence in a diblock DNA was performed, as shown in Figure 1B. [18] The promise is that the block in yellow (e.g., a poly-A sequence) has a higher affinity than the rest of the DNA. If the density of DNA is sufficiently high, this high affinity block should displace the other block. This can separate the adsorption function (by the poly-A block) from the molecular recognition function (by the other block). If the DNA density is low, it is likely that most of the nucleotides are adsorbed, similar to that in Figure 1A. This diblock DNA concept has also been applied in functionalization of gold nanoparticles. [48–51] Recently, we noted that the affinity of poly-C DNA on GO is even stronger than poly-A DNA, [52,53] whereas it is generally accepted that poly-T DNA has the weakest interaction with GO at physiological conditions. [40] Nevertheless, as DNA is physisorbed, it may still suffer from nonspecific displacement.

The final strategy is covalent attachment as shown in Figure 1C, which is usually achieved by using an amino-modified DNA [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) mediated chemistry forming an amide bond], although tether molecules (such as biotin/streptavidin) may also be used. [54–56] As the yield of covalent conjugation is unlikely to be 100%, a key step is to wash away noncovalently associated DNA. [54,57] For the covalently linked DNA, it is likely that some DNA bases are still adsorbed by GO, especially under favorable adsorption conditions. Covalently linked DNA fully resists nonspecific displacement. The advantages and disadvantages to each attachment method with respect to biosensor development are shown in Table 1. The rest of this review outlines recent biosensors using these three methods.

3. Biosensors Using Physisorbed DNA Probes

3.1. Sensing Based on Desorption of Probes

In 2009, Yang and coworkers adsorbed a fluorescent DNA and GO yielding quenched fluorescence. [15] When its cDNA was added, the probe DNA desorbed forming a duplex with up to 20-fold fluorescence enhancement in 15 min, and a detection limit of 2.0 nM cDNA was achieved. Adding mismatched DNA produced a significantly lower amount of signal. The simplicity of this method has attracted extensive attention. This idea was previously applied to CNTs, and Strano’s work was a good example, [46] but the kinetics on the nanotubes was too slow to be analytically appealing. [29]

Generally, targets can be any species that can induce specific desorption of the probe DNA from GO, including cDNA as mentioned earlier, [58] and other targets detected by aptamers such as......

Figure 1. Three methods of interfacing a single-stranded DNA with GO and the reaction with the cDNA: A) simple physisorption; B) adsorption with a strongly adsorbing anchoring block (poly-A or poly-C); and C) covalent attachment.
bacterial and cancer cells, the prostate-specific antigen, adenosine triphosphate (ATP), alfatoxin B1, human chorionic gonadotropin, and bisphenol A. While this simple detection scheme has been used for more than a decade, several recent approaches have also emerged. Tomita et al. immobilized several fluorescently labeled DNA probes on GO for detecting ten different proteins (Figure 2A). Each protein would have a different affinity to the probes and/or GO, and therefore would be desorbed from GO at different ratios (Figure 2B). This gave each target protein a specific signature dictated by the amount of probe released. Using this method, protein discrimination was even afforded in a human serum sample matrix.

An interesting variation of the simple probe desorption method was demonstrated by Zhang et al. for the detection of cancer cells. Essentially, two DNA strands were adsorbed on GO: 1) a labeled probe DNA, and 2) a primer DNA that can be extended once desorbed from GO using telomerase in cells. The extension (a TTAGGG repeat sequence) was complementary to the probe DNA, which would result in a fluorescence signal increase due to its subsequent desorption from GO. The purpose of GO was not only to quench the dye-labeled probe, but also to promote internalization of the DNA into the cell. Using this method, they were able to attain a limit of detection (LOD) of 10 and 30 cells for the HeLa (shown in Figure 2C) and A549 cell line, respectively.

While the fluorescence measurement shown earlier is quite sensitive for target detection, it is practically difficult to calibrate fluorescence intensity, which can vary significantly depending on lamp intensity, detector sensitivity, and photobleaching of fluorophores. In this regard, the use of intensity-independent assay methods is desirable, and fluorescence anisotropy/polarization is such a method. Anisotropy and polarization describe the same

Table 1. A comparison of the three methods of interfacing GO and DNA for sensing.

| Method                  | Advantages                                      | Disadvantages                                      |
|-------------------------|-------------------------------------------------|----------------------------------------------------|
| Simple physisorption    | Simple and quick                                | Nonspecific desorption                             |
|                         | Versatile sensor                                | Biosensor sensitivity, selectivity dependant on DNA sequence |
| Adsorbing diblock DNA   | Less probe/GO interaction                       | More expensive due to longer DNA strand            |
|                         | Less nonspecific desorption                     | Anchor strand can still be displaced               |
| Covalent attachment     | Stable, reproducible attachment                 | Extra steps needed                                 |
|                         | Regeneration of sensor                          | Lower signal since full desorption cannot happen   |

Figure 2. A) Scheme of detection of different proteins using DNA probes labeled by different fluorophores. B) Statistical ensemble of different proteins measured using this method with linear discriminant analysis. Reproduced under the terms of a Creative Commons Attribution CC BY 4.0 license. Copyright 2017, The Authors, published by MDPI AG. C) Detection of HeLa cells using the telomerase activity to extend the DNA desorbed from GO. Reproduced with permission. Copyright 2018, The Royal Society of Chemistry.
physical process and are related to each other mathematically. When a fluorophore is excited by polarized light, the emitted light is more depolarized if the fluorophore can tumble faster within the timescale of the fluorescence emission. The extent of depolarization is expressed by anisotropy, $r = (I_\parallel - I_\perp) / (I_\parallel + 2I_\perp)$, where $I_\parallel$ and $I_\perp$ are the intensities of the parallel- and perpendicular-polarized light emitted from the fluorophore, respectively, relative to the excitation light.\(^{[71]}\) As anisotropy is an intensity ratio, it is independent of fluorescence intensity and is therefore immune to small experimental variations mentioned earlier. For a fluorophore-labeled DNA, the measured anisotropy is expected to increase if it is adsorbed on a nanomaterial due to the effectively increased molecular weight and slower tumbling.\(^{[72]}\)

With respect to GO/DNA sensors utilizing anisotropy, it has been seen experimentally that the difference between bound and unbound states is analytically useful.\(^{[73–75]}\) A recent example was shown by Ye et al. for the detection of aflatoxin B$_1$ (AFB$_1$) (Figure 3A).\(^{[76]}\) The carboxyfluorescein (FAM)-labeled AFB$_1$ aptamer was immobilized on GO, resulting in a high measured polarization. In the presence of AFB$_1$, the aptamer desorbed from GO to bind the target and the polarization decreased. The change in polarization reflected the concentration linearly, and a LOD of 0.05 nM was determined, with a linear range of 0.05 to 5.0 nM. Another recent study by Wang et al. utilized this change in anisotropy in conjugation with the signal amplification capability of the T7 exomucin enzyme to detect target DNA down to a LOD of 9.1 pM.\(^{[77]}\)

It should be noted that unlike a typical macromolecule, such as a protein, used for aptamer binding assays,\(^{[78,79]}\) GO has strong fluorescence quenching properties. Therefore, the contributions to the anisotropy of the adsorbed DNA might be much less than the free fraction. Our group recently investigated this interaction in detail using a quenching (GO) and a nonquenching (Y$_2$O$_3$, nanoparticle) surface.\(^{[72]}\) We determined that once DNA is mixed with GO, the measured anisotropy becomes a sum of the adsorbed and nonadsorbed populations (Figure 3B). Analytically, researchers are more concerned the fraction of adsorbed DNA (θ in Figure 3B), but the effect of quenching efficiency δ cannot be overlooked. Only when δ = 0, then the equation reduces to a normal binding curve. For a strong quencher like GO, low concentrations of free, nonquenched DNA heavily dominate the measured anisotropy. In addition, scattering effects would also become important at such low concentrations of free DNA (and thus weak fluorescence). In other words, while the measurement of high anisotropy appeared analytically useful as seen in the aforementioned examples, this might not be due to the adsorption of the fluorophore-labeled probe but due to the low concentration of the free probes in solution and the scattered light from the added GO.

Apart from fluorescence, electrochemical methods have also been used for GO/DNA biosensors. In general, there are two different types of such sensors: 1) amperometry (current)-based and 2) impedance-based. It is important to note that some biosensors with GO and DNA have gold (Au) or gold nanoparticles (AuNP) as an intermediary surface.\(^{[80–81]}\) These will not be discussed here because the DNA did not directly interact with GO.

First, we discuss amperometric methods. The general principle of such a sensor relies on the change in the current flow when a reporter-labeled DNA is adsorbed on GO, which, in turn, is immobilized on an electrode. This reporter is redox active (e.g., labeled with a methylene blue or MB), which allows for a strong electrochemical signal during measurement.\(^{[82]}\) If the DNA is adsorbed on GO, effective electron transfer occurs between the reporter and the GO surface, resulting in a high current. Once the DNA is desorbed and/or the reporter molecule is far enough from the GO surface, this current is reduced, allowing for discrimination between the “on” and “off” states.

An interesting Hg$^{2+}$ sensor was developed by Lu et al.\(^{[83]}\) In this case, GO was immobilized on a gold electrode (Figure 4A). MB-labeled T$_{15}$ DNA was adsorbed on the GO, resulting in an initial high current. However, when Hg$^{2+}$ was added, it mediated the base pairing of two T$_{15}$ strands (via T-Hg$^{2+}$-T base pairs) or intramolecular folding, resulting in DNA desorption from GO.
This alone would cause the current flow to decrease. However, DNase I was added to cleave the desorbed DNA strands, liberating Hg$^{2+}$ for more adsorbed MB-T15, continuing the cycle of amplification. Using this method, the authors reported a LOD of 0.12 nM Hg$^{2+}$ (Figure 4B). Furthermore, high specificity was seen for Hg$^{2+}$, with no other tested metal ions showing any significant decrease in current.

For impedance-based sensors, no covalent electrochemical reporters are needed. Instead, a solution-based probe or mediator is used (e.g., [Fe(CN)$_6$]$_4$$^{3-}$/C$_0$$^{2+}$). If a DNA is adsorbed on GO, then the mediator ions are hindered from approaching the electrode for a redox reaction, leading to a high impedance. An interesting recent application of this method was utilized by Cao et al. for DNA detection (Figure 4C). An enzyme, exonuclease III, was used to cleave the probe strand of the hybridized probe-target duplex, recycling the target DNA to bind to more probe DNA. The impedance decreased when increasing concentrations of target DNA was added (Figure 4D), and the authors claimed a LOD of 10 aM. Furthermore, the method was very specific to cDNA, with a nearly 10-fold difference in signal of a single mismatched target. Similar methods have been used to detect thrombin and lactate dehydrogenase.

It should be noted that researchers in this field have been advocating this method to be applied to many modified systems, especially by changing the substrate material. Although many other materials have been tested, such as MoS$_2$, various metal oxides, and other carbon-based materials, GO was still used as a benchmark to compare the performance.

3.2. The Mechanism of Sensing by cDNA Hybridization

The addition of the cDNA resulted in hybridization with the adsorbed probe DNA and desorption from GO. How does this hybridization reaction take place? It was always assumed that the DNA hybridization reaction occurred on or in close proximity to the GO surface (Figure 5A). In 2013, our group utilized poly-A and poly-T DNA to show that nonspecific displacement generally occurred before hybridization (Figure 5B). This mechanism has a detrimental effect on sensing applications because more than one target DNA would be required to generate a signal. Despite this, GO/DNA-based biosensors without signal amplification still have detection limits in the low nM range likely due to the high sensitivity of fluorescence and low background of stably adsorbed DNA probes.

This was investigated further by Park et al. in 2014 by exploring the concentration dependence of target DNA on GO saturated with probe DNA. Unlike noncomplementary targets, increasing cDNA concentration resulted in higher desorption of probe DNA.
It was proposed that there were two regimes in the desorption of the probe DNA for hybridization with a complementary target. First, if a lower concentration of target DNA is used, the probe DNA is slowly displaced, hybridizing with free target DNA in solution to prevent readsoption. At higher concentrations, increased encounters with probe DNA result in hybridization near the GO surface followed by desorption. With noncomplementary targets, the concentration would have to be significantly higher to desorb the probe by competing adsorption on GO (as shown in Figure 5B, but without hybridization).

With respect to GO concentration and size, a 2016 study by Lee et al. compared large, micron-sized GO (L-GO) and small, nano-sized GO (N-GO). The adsorption and desorption profiles of DNA were similar for both these surfaces. However, there was an optimal GO concentration for desorption; if it was too high, then the added target would adsorb on free GO surface compared to displacement of the probe DNA. If it was too low, then the target would hybridize with the unbound probe rather than displace bound probe. Therefore, for sensing applications, probe DNA should be adsorbed at concentrations close to the capacity of the available GO in solution.

3.3. The Problem of Nonspecific Probe Release

As the DNA probes are only physisorbed in the aforementioned cases, nonspecific displacement of the probe by a high concentration of nucleic acids (i.e., not cDNA), proteins, and even small molecules is likely to take place, especially in complex biological sample matrix. Such nonspecific displacement increases background and causes false positive signals.

One way to deal with this is to use an internal reference DNA labeled with a different fluorophore (Figure 6A). The basic idea is that the interfering reagents would desorb the internal standard to the same degree as the probe DNA. Therefore, comparing the desorption of both fluorophores could more clearly determine how much of the probe desorption was due to target, rather than nonspecific events. An example of this was done by Tan and coworkers for intracellular ATP detection. The ATP aptamer was designed into a molecular beacon to further reduce false positive signals in a cellular environment, which was adsorbed on GO. Simultaneously, a single-stranded control DNA was adsorbed on GO to mitigate the nonspecific displacement by proteins. Using this, they were able to visualize ATP in cells, with sensitivity to treatments (such as Ca$^{2+}$ and etoposide) which increased ATP production (Figure 6B). While this method can decrease the problem, it cannot fully solve it. First, target DNA may also desorb the internal standard (because they are competing for the same binding sites on GO), leading an inflated standard response and a lower sensitivity. Second, the sharing of the GO surface between probe DNA and the internal standard will lead to a narrower range of detection and potentially slower kinetics. When the nonspecific displacement was too high, both probes could be fully desorbed. Finally, the cost was doubled.

Another strategy was the use of blocking agents. As discussed earlier, the GO surface is heterogeneous; there are localized regions of higher/lower hydrophobicity, along with different functional groups. These regions bind to DNA and other molecules in different ways. Therefore, the purpose of blocking agents would be to occupy sites that are not critical to DNA adsorption so that interfering agents cannot effectively displace DNA (Figure 6C). In theory, this would increase sensitivity and reduce nonspecific interactions, and our group systematically studied it in 2016. We found four different agents that could effectively block the GO surface and lead to more sensitive cDNA detection: polyethylene glycol (PEG), poly(styrenesulfonate) (PSS), A$_{15}$ DNA, and Tween-20. An A$_{15}$ blocking strand was then used to improve the sensitivity of the GO/probe DNA system by ten-fold (Figure 6D). A very recent study utilized a nanocellulose scaffold to confine GO (loaded with probe DNA and a blocking protein) to lower the background signal further.

With simple adsorbed DNA, GO might not be the most optimal surface in complex biological media, despite the strategies discussed earlier. This is likely because DNA competes directly with proteins for the same binding sites on GO with similar binding mechanisms. Our group investigated this in more detail by comparing GO with metal oxide nanoparticles (MONPs) DNA interacts with MONPs through their phosphate backbone rather than the bases for GO (Figure 6E). This interaction is often based on strong Lewis acid/base interactions and sometimes with additional electrostatic attraction, compared with the
predominantly π–π stacking mode for GO. Typical proteins (e.g., bovine serum albumin [BSA]) do not interact with MONPs strongly enough to displace DNA (Figure 6F). It should be noted that the MONP/DNA system is susceptible to phosphate ions, which are prevalent in cell culture media,[88] although phosphate ions do not displace DNA from GO. Therefore, the problem of interfering agents in complex media for the simple adsorbed DNA probes needs to be carefully analyzed to ensure high sensitivity and specificity.

3.4. Sensing Based on Inhibited Adsorption of Probe DNA

The sensing methods discussed earlier require the dye-labeled probe to be stably adsorbed on GO before adding the target. The kinetics of sensing is slow and full desorption typically takes >10 min. Since adsorption of single-stranded DNA is much faster than its desorption from GO, one way to alleviate this issue is to eliminate the desorption step completely. Premixing the target with the probe DNA before adding GO would reduce the sensing time to just a few minutes. This was shown experimentally in the pioneering work of He et al. to detect cDNA.[35] The reported LOD was 0.1 nM cDNA, lower than the conventional sensing where probe DNA and GO were mixed first.

This method was also applied to simultaneously detect Cu²⁺, Mg²⁺, and Pb²⁺ (Figure 7A).[39] A three-way junction was synthesized containing three different RNA-cleaving DNAzymes which are, respectively, dependant on the three ions. For each enzyme strand, the corresponding substrate strand was labeled with a different fluorophore to discriminate between the ions. In the presence of a target, the substrate strand was cleaved, releasing the fluorophore-labeled fragment. This single-stranded DNA adsorbed on GO, quenching the fluorescence. The LOD was determined to be 1, 200, and 0.3 nM for Cu²⁺, Mg²⁺, and Pb²⁺, respectively, without interference from other ions (Figure 7B). The performance of this sensor was directly related to the DNAzyme activity.

Measuring fluorescence anisotropy can also be done with this method, and a representative sensor was developed by Zhen et al. to detect K⁺ ions.[94] In this case, the inhibited adsorption was due to the formation of the G-quadruplex upon binding K⁺ and resulting in a low affinity to GO. To acquire a signal, acridine orange (AO) dye was added and the fluorescence anisotropy was measured. If K⁺ was present, AO partitioned within the G-quadruplex, and the anisotropy remained high even when GO was added (Figure 7C). In the absence of K⁺, the single-stranded DNA and the dye were both adsorbed on GO showing increased anisotropy, although the effect of fluorescence quenching on anisotropy was not considered. The LOD was determined to be 1 μM with a range of detection between 10 μM and 2 mM (Figure 7D).

Similar sensors have also been developed for other targets, such as C-reactive protein, tropomyosin, and Hg²⁺, with the latter being implemented in a “signal on” variation.[27,95,96]
4. Sensing Using Diblock DNA

All the aforementioned examples involved the adsorption of probe DNA, whose function was just to recognize target analytes. Therefore, no further design of probe sequence was needed in most cases, and probe DNAs are assumed to randomly adsorb on GO. Another category of biosensors only utilizes partial adsorption of DNA on GO, with the rest serving as the probe for binding target. The simplest design is a diblock DNA, where one block has strong adsorption affinity and the other block is the probe (Figure 1B). This design has the advantage of decreased probe interaction with the GO surface. An early example of this was shown by Tang et al., where they designed two strands of DNA that were only partially complementary to a target DNA and adsorbed them on two separate batches of GO. Mixing these two GO samples in the presence of target DNA resulted in hybridization with the target, linking GO sheets together. The hybridization was reversed by increasing temperature beyond the melting point of the DNA. Ultrasensitive detection of target DNA was achieved down to a LOD of 1 pM. A more recent study utilized electrochemiluminescence to detect microRNA by using DNA intercalating dyes for signal amplification. One way to mitigate this is to use an anchoring strand that keeps the fluorophore far enough away from GO so that fluorescence is not fully quenched. A good example of this is a sensor designed by Xiao et al. for the detection of ricin B-chain (Figure 8A). Essentially, a magnetic bead was conjugated with the ricin B-chain aptamer (partially hybridized with a blocking strand) through biotin-streptavidin interactions. In addition, an anchoring DNA partially hybridized with probe DNA was adsorbed on GO via a poly-A block. As the distance between the fluorophore and GO was long, quenching was inefficient, and the measured anisotropy was high due to immobilization. Upon addition of ricin B-chain, the aptamer bound to it, releasing the blocking strand. Then, the blocking strand complementary to the probe DNA displaced the probe DNA from the GO surface to form a free duplex. Exonuclease III was then used to cleave the probe DNA from the blocking strand, resulting in essentially a free fluorophore with low anisotropy. The blocking strand was recycled to displace another probe strand for signal amplification. Another report from this group also detected cDNA (LOD: 4.6 nM) and adenosine (LOD: 22.0 μM) using a simpler method without amplification. A later work for microRNA detection used a DNA displacement amplification mechanism, which achieves a LOD of 47 pM microRNA. One critical aspect of this system is the anchoring sequence. Although the perception is that purines adsorb on graphene more strongly compared with pyrimidines, and polyadenine...
(poly-A) DNA was the most often used anchoring sequence, our group investigated the strength of the anchor by using a fluorescence-based displacement assay. Interestingly, polycytosine (poly-C) DNA was found to have the highest affinity for GO (even higher than polyadenosine DNA), making it the most favorable anchor. Diblock DNA was systematically studied; one block was anchoring (poly-A or poly-C) and the other block was targeting (Figure 8C). Furthermore, the use of poly-C as an anchor led to a significantly higher target-specific hybridization fraction compared with poly-A in the presence of interfering agents (Figure 8D).

We also studied the effect of anchor length and found that, in the case of poly-C, the 15-mer (i.e., C15) DNA was an efficient anchor in terms of stability and hybridization efficiency. Interestingly, when it comes to poly-C, the role of the labeled fluorophore was recently found to be important for adsorption affinity. We investigated the FAM-labeled C15 and found that its fluorescence intensity was much lower at neutral pH compared with other FAM-labeled 15-mers. Once heated, the intensity reverted to that of the other strands. Through circular dichroism and melting temperature experiments, we determined that FAM could stabilize the C15 i-motif formation at neutral pH, which was irreversibly disrupted by heating. Furthermore, the heated FAM-C15 adsorbed more strongly on GO compared with unheated, presumably due to more stacking of the bases with GO. Therefore, a poly-C DNA adsorbs strongly on GO is not due to its tendency to form i-motif.

5. Covalently Linked and Tethered Probe DNA

Although using a high affinity anchoring block can be more resistant to nonspecific displacement of probe DNA, such interference cannot be fully eliminated. Apart from physisorption, DNA can also be attached to GO through covalent bonds. Covalent DNA probes to GO also allows for simple regeneration and long-term reversible monitoring of samples. Ultimately, this would lend itself better to devices which require continuous operation and monitoring. Furthermore, this method eliminates nonspecific displacement of probe DNA, allowing for better specificity. Many sensors used gold nanoparticles as intermediate substrates for DNA attachment, and they will not be discussed here if DNA is linked to gold instead of GO.

The most popular strategy of linking a DNA and GO is through the EDC chemistry using amine-functionalized DNA to couple with the carboxyl groups on GO. GO has abundant carboxyl groups on its edges which can be activated by EDC to a reactive intermediate and further linked to amine-functionalized DNA forming a peptide bond (Figure 1C). Despite this, conjugation efficiencies are typically only 50% or lower, and unreacted DNA must be washed off using centrifugation or filtration. Otherwise, detection signals from both covalent and physisorbed probes would be produced. Another method involves using a tether molecule which can bind to GO more strongly than DNA. These can be aromatic/hydrophobic molecules (interacting with hydrophobic/aromatic regions on GO), positively charged groups (interacting with negatively charged carboxyl groups on GO), or biotin/streptavidin interactions.

Early work in biosensor development using covalent conjugation was mainly for electrochemistry. The general idea is that current is enhanced once the tethered DNA is hybridized with its cDNA. For example, Mohanty and Berry used DNA tethered on GO to design a biotransistor that allowed increased current flow when cDNA was hybridized. A similar kind of
sensor was designed a few years later by Xu et al., instead using methylene blue as a redox label to enhance the electrochemical signal. They tethered pyrene-labeled aptamers specific to either K\(^+\) or Pb\(^{2+}\) ions, which could help stabilize a G-quadruplex structure. The formation of the G-quadruplex brought the methylene blue label closer to the GO surface to increase the measured current. A sensitivity of 0.09 \(\mu\)A M\(^{-1}\) (K\(^+\)) and 1.4 \(\mu\)A M\(^{-1}\) (Pb\(^{2+}\)) was achieved. A more recent example from Sharma et al. utilized positively charged polyethyleneimine (PEI)-modified GO to tether DNA for the detection of cardiac myoglobin (Figure 9A). As a covalent modification on GO was made, we put these examples here. As the target molecule myoglobin was already redox active (Fe\(^2+/3^+\)), the aptamer was not labeled. The LOD was 0.97 pg mL\(^{-1}\) with a wide range of detection (0.001–1000 ng mL\(^{-1}\)), though the sensitivity was low due to such a wide dynamic range. Other electrochemical sensors utilizing covalently bound or tethered DNA probes have also been recently investigated for detecting HIV DNA, circulating tumor DNA, biotin-streptavidin interactions, and cardiac troponin I.

Biosensors using covalently linked strands are not limited to electrochemical methods. For fluorescent detection, one of the earliest reports came from our group in 2012. Using EDC, we conjugated a fluorophore-labeled probe to GO for cDNA detection. A major challenge was to remove physisorbed DNA (i.e., DNA that did not participate in the coupling reaction) from the GO surface. Ultimately, this was achieved by adding excess cDNA and washing. The detection limit was 2.2 nM cDNA, only slightly higher than typical sensors based on physisorption (=1 nM). Later, we proved that aptamers can also be covalently linked to GO and used for target detection in a similar way. In this case, the ATP aptamer was used as a model, and the LOD was 0.019 mM. In addition, intracellular detection of ATP was achieved using fluorescence microscopy.

Our group also compared Hg\(^{2+}\) detection using both physisorbed and chemisorbed aptamers on GO (Figure 9B). The method was similar, but a major difference was the position of the fluorescent label. Due to the folding of the Hg\(^{2+}\) aptamer upon target binding, the label was placed in the middle of the aptamer sequence, rather than the end. This ensured that the fluorophore was furthest away from GO once Hg\(^{2+}\) was bound. The sensitivities of the covalent and noncovalent sensors were similar with the former having a slightly smaller LOD (16.3 vs 20.6 nM). The specificity was also compared with both showing a similar profile: highest response from Hg\(^{2+}\), a moderate response from Ag\(^{+}\), and no response from the other tested ions. This likely reflected the nonspecificity of the aptamer itself, rather than the method used for detection.

He et al. sought to combine both physisorbed and chemisorbed DNA to incorporate the advantages of both for intracellular pH sensing (Figure 9C). They directly linked a Cy5-labeled sequence (AR) to GO, and then added a Cy3-labeled strand (an i-motif forming sequence [IFO]) which was partially complementary to the AR sequence such that the Cy3 was close enough
to be moderately quenched by GO. Lastly, herring sperm DNA was added to passivate the surface of GO to prevent non-specific adsorption in cells. At high pH (≥pH 7.4), the fluorescence of Cy5 would dominate over Cy3. At low pH (≥pH 5), the IFO dehybridized due to the protonation of the cytosine bases and formed an i-motif, increasing the Cy3 fluorescence. Simultaneously, the single-stranded AR adsorbed on GO, quenching the Cy5 fluorescence. They were able to observe these specific changes in intracellular pH in both HeLa and MCF-7 cells using confocal microscopy (the former shown in Figure 9D). Furthermore, at pH 5.8, both fluorescent populations were seen, and the relative intensities could be used to estimate the pH.

6. Conclusions and Future Perspectives

This review summarizes different ways to attach DNA probes to GO. GO can adsorb DNA with an intermediate affinity, yet different DNA sequences still have quite different affinities owing to the base composition and secondary structures. While simple physisorption is most convenient and a high sensitivity can be achieved, nonspecific displacement of probe DNA makes it difficult to be practically useful in complex biological sample matrix due to nonspecific displacement by proteins and other nucleic acids. Covalent attachment can largely solve this problem, but it takes additional steps to prepare the sensors and removing noncovalently attached probes is also difficult. Using the diblock design is an alternative, which has the merit of both previous methods, although the nonspecific displacement problem is not being fully solved. In this review, we focused on fluorescent and electrochemical detection, but the DNA/GO system can certainly be used for other signaling mechanisms such as those based on color and mass change (e.g., QCM).[116,117] Given the progress so far, we believe the following developments to be made to further grow the field.

The diblock DNA idea can be extended to a multiblock design to further increase the affinity of adsorption. For example, by immobilizing DNA on gold nanoparticles to form a spherical DNA, with multiple DNA strands on each gold, the polyvalent interaction can be very strong.[118] In this case, noncovalent adsorption can rival the strength of a covalent bond, although the DNA/GO interaction is no longer a major factor for sensing. Tuning the interaction force between GO and DNA can also be achieved by varying the surface chemistry of GO, for example, by introducing more reduced carbon-rich areas that have higher affinity with DNA (e.g., using rGO).[14] pushing DNA strands to the high affinity regions, and future optimization of DNA sequences.

Another area that can be expanded is signal amplification. Enzymes were used to correlate one target/probe binding event with multiple signal reporters. Enzymes, while very efficient, are stable only in carefully controlled environments.[119] One avenue that can be used is liposome encapsulation or inorganic nanozymes. Enzymes were used to correlate one target/probe binding event with multiple signal reporters. Enzymes, while very efficient, are stable only in carefully controlled environments.[119] One avenue that can be used is liposome encapsulation or inorganic nanzymes. While a DNA typically has one fluorophore label, a liposome can hold several hundred fluorophores. If DNA desorption from GO can trigger the rupture of a lipid membrane, then significant signal amplification could be achieved. Other similar systems could be loaded polymersomes or porous nanoparticles.

Using nanozymes can also provide more robust catalytic activity.[121,122]

Finally, more research needs to be done in the development of devices, and this is related to large-scale translation of this technology to practical use. GO and graphene are ideal for designing sensing devices taking advantage of their optical and electric properties. Most of the work outlined earlier was performed in simple, solution-based environments which, in practice, would be difficult to implement on a large scale. The exception to this is electrochemical sensors because their working principle relies on designing electrodes. However, virtually no device has been made for fluorescent-based detection methods, despite the significant research efforts. An example of such a sensor could be very simple: immobilizing GO/probe DNA on paper, wicking sample to induce flow through paper, and finally detection by visual observation (e.g., using a UV lamp). Such methods have been used for antibody-based paper sensors,[123,124] and can be applied for DNA/GO systems.[60]

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Conflict of Interest

The authors declare no conflict of interest.

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