

Chapter 2

Graphene for DNA Biosensing

Abstract Graphene (or GO) is an excellent candidate for biomolecules anchoring and detection due to its large surface area (up to 2,630 m²/g) and unique sp² (sp²/sp³)-bonded network. According to the binding affinity difference between single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) to graphene sheet, GO has been successfully adopted as a platform to discriminate DNA sequences. Fluorescent, electrochemical, electrical, surface-enhanced Raman scattering (SERS) and other methods have been utilized to achieve the sensitive, selective, and accurate DNA recognition. Both theoretical and experimental results illustrate that ssDNA sequences are adsorbed on the surface of graphene sheet with all nucleobases lying nearly flat. DNA or RNA sequencing through graphene nanopore, nanogap, and nanoribbon has also attracted much interest because it is a label-free, amplification-free, and single-molecule approach that can be scaled up for high-throughput DNA or RNA analysis. Meanwhile, miRNA detection is achieved by forming DNA–miRNA duplex helices, and strong emission is observed due to the poor interaction between the helix and GO.

Keywords DNA sequencing • miRNA detection • Fluorescent spectroscopy • Electrochemical method • Electrical method • Graphene nanopore • Graphene nanogap • Graphene nanoribbon

2.1 Investigation of DNA and Graphene Binding Interaction

The large surface area (up to 2,630 m²/g) and unique sp² (sp²/sp³)-bonded network make graphene (GO) an excellent candidate for biomolecules anchoring and detection. Taking DNA for the first example, various strategies have been adopted to nucleic acid sequence recognition [1–10]. To better illustrate the interaction, how nucleobases interact with graphene should be addressed, which may be inspired by the interaction of nucleobases with CNTs [11–13] and highly oriented pyrolytic graphite (HOPG) [14].

Single-stranded DNA (ssDNA) strand is unwound double-stranded helix (dsDNA), which is held together by hydrogen bonds between the bases linked to two ssDNA strands. It consists of one long polymer of simple units called nucleotides, with backbones made of alternating sugars (deoxyribose) and phosphate groups, with the nucleobases (G, T, A, C abbreviated for guanine, adenine, thymine, cytosine, respectively) attached to the sugars. Employing various strategies and techniques, researchers tried to illustrate the interactions between DNA and graphene. Molecular dynamics (MD) simulations of ssDNA and dsDNA anchored via an aliphatic linker to a graphene surface were performed in order to investigate the role of the surface charge density in the structure and orientation of attached DNA [15]. A considerable difference in the behavior of the ssDNA and dsDNA anchored to graphene layer was observed. The rigid dsDNA kept its geometry close to the canonical B-DNA form and interacted with graphene surface by the nearest base pairs. The flexible ssDNA intended to maximize the attraction to graphene surface by exploiting more bases for the interaction, namely 'lying' on the surface with bases as the anchor. This interaction was also investigated by isothermal titration calorimetry (ITC) [16]. ITC is an ultrasensitive tool to study binding interactions in aqueous solutions. Experimental results showed the relative interaction energies of nucleobases decreasing in the order of $G > A > T > C$, with the position of T and C interchangeable. Theoretical calculations including van der Waals interaction and solvation energies gave the similar trend $G > A \sim T > C$. Using first-principles investigation, the calculated binding energy was found to be $G > A \sim T \sim C > U$, where U represents uracil in RNA [17]. And the stabilizing factor in the interaction between the base molecules and graphene sheet is dominated by the molecular polarizability that allows a weakly attractive dispersion force to be induced between them. These interaction magnitudes are similar to those found between bases with SWCNTs [18].

Based on the binding energy difference, the anchoring of DNA sequences on graphene may present as different morphologies. Recently, Postma and coworker demonstrated that DNA assembled into two distinct patterns, small spherical particles and elongated networks, on graphene surface [19]. According to the AFM results of poly-A, C, T, G assembling on graphene (Fig. 2.1a), they found that these common mechanisms, e.g., hydrogen bonding, base stacking, electrostatic, van der Waals, and hydrophobic interactions, could not explain these observations. Alternatively, they concluded a different mechanism (Fig. 2.1b), where E_b and E_g represented the interbase binding energy and base-graphene binding energy, respectively. Hence, they argued that the root cause of DNA assembling in distinct patterns is a crossover in the balance between the two energies, i.e., $E_b > E_g$ or $E_b < E_g$. When $E_b > E_g$, meaning that the interbase coupling is more energetically favorable than binding to graphene, the DNA (A, C) assembled into spheres to maximize E_b (Fig. 2.1b A). When $E_b < E_g$, meaning that the base-graphene interaction is more energetically favorable than interbase, the DNA (G, T) stretched out across graphene surface to maximize the energy and resulted in network formation (Fig. 2.1b B). This hypothesis was supported by the fact that $E_g(G) > E_g(A) \sim E_g(T) \sim E_g(C)$ and $E_b(G) \sim E_b(T) > E_b(A) \sim E_b(C)$. They also

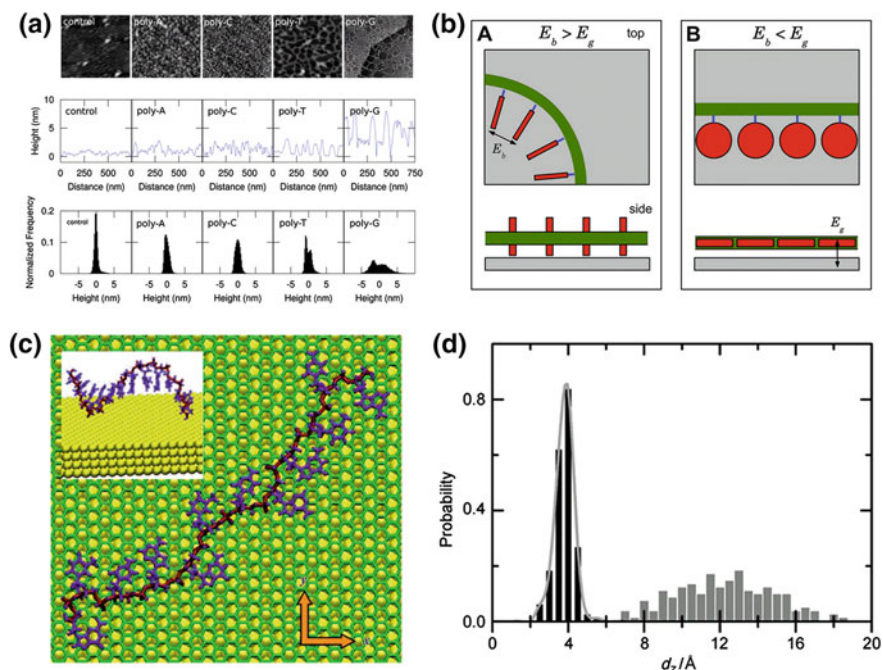


Fig. 2.1 **a** Atomic force micrographs of graphene with and without ssDNA, line scans, and histograms. **b** DNA assembles onto graphene (gray) into spheres (A) or networks (B). The nucleotides (red) can rotate around the link (blue) to the sugar phosphate backbone (green) to either maximize the interbase binding energy E_b (A) or the base-graphene binding energy E_g (B). Reprinted from Ref. [19]. **c** Schematic representation of an ssDNA nucleobase on the surface of the graphene-on-Au(111) material. The nucleobases and the backbone of the ssDNA are shown in violet and dark red, respectively. The graphene sheet is shown in green. The Au(111) surface is indicated by yellow spheres and sticks. The inset is a typical conformation of ssDNA on Au(111). **d** Probability of finding the atoms in the nucleobases of ssDNA at a distance d_z from the graphene-on-Au(111) surface (black columns) and the Au(111) surface (gray columns), respectively. Reprinted from Ref. [20] by permission of John Wiley & Sons Ltd.

estimated that the critical interbase interaction strength that separated network formation from sphere assembly was $\sim 0.3\text{--}0.5$ eV.

Both above-mentioned theoretical and experimental results illustrate that ssDNA sequences are adsorbed on graphene sheet surface with all nucleobases lying nearly flat (Fig. 2.1c). To more specifically and directly elucidate the interaction, MD simulations were carried out [20]. A clear distance of ~ 4.0 Å was observed in Fig. 2.1d (black). This distance was quite close to the van der Waals distance between a carbon atom in graphene and carbon/oxygen/nitrogen atoms, which are the main elements in nucleobases. This showed that most of the atoms in the nucleobases were directly adsorbed by graphene, which was strong enough to destroy the helical structure of ssDNA. A comparison was also carried out, in which the same ssDNA was adsorbed on Au(111) surface (Fig. 2.1d [gray]). The distance

had a wide distribution from 7.0 to ~ 18.5 Å. Hence, ssDNA still retained its helical structure on the Au(111) surface (Fig. 2.1c inset) as the segment with six nucleobases in bulk water. Besides, the developed graphene-on-Au(111) composite was very stable, and the conductivity of this material was excellent, which resulted in a very high signal-to-noise ratio of the resonant currents through different nucleobases. Then, this hybrid held the potential for single-molecule DNA sequencing via AFM or STM.

Using scanning tunneling spectroscopy, Ahmed et al. [21] calculated the tunneling conductance and electronic local density of states (LDOS) for DNA nucleobases at various orientations on graphene surface and showed that the different nucleobases had significantly different LDOS peaks (fingerprints), allowing differentiation via local tunneling conductance. Besides, a plane orientation of DNA nucleobases with respect to graphene surface was the most stable state, which favored π - π stacking between the bases and aromatic carbons in graphene. This conclusion is in consistent with above-mentioned research results.

2.2 DNA Biosensing Employing Graphene Platform

Although the nucleobases-graphene binding energy is slightly different via various strategies and equipments, one might be certain that ssDNA could be adsorbed on graphene sheet surface coupling crossover of several interaction forces and employing nucleobases as the anchors. This is also the root cause that ssDNA binds more strongly to graphene than dsDNA does [19], in which nucleobases are entrapped and shielded by the phosphate-deoxyribose backbones. According to the binding affinity difference between ssDNA and dsDNA to graphene sheet, graphene (GO) has been successfully adopted as a platform to discriminate DNA sequences. Several methods, such as fluorescent, electrochemical, electrical, and SERS, have been utilized to achieve the sensitive, selective, and accurate DNA recognition.

2.2.1 Fluorescent Assay

In a typical fluorescent manner (Fig. 2.2a) [4], fluorescent dye-labeled ssDNA was adsorbed on graphene surface. Then, the fluorescence of dye-labeled ssDNA was completely quenched due to the fluorescent resonance energy transfer (FRET). In the presence of a target, the hybridization between dye-labeled ssDNA and target sequence altered the conformation of ssDNA and disturbed the binding between the dye-labeled ssDNA and graphene. This behavior might originate from the fact that DNA nucleobases matching is more energetically favorable than binding with graphene. The formed dye-labeled dsDNA left from graphene surface, resulting in the restoration of dye fluorescence. Based on the mechanism, both hairpin DNA and molecular beacon (MB) were designed for homogeneous DNA detection [5, 6].

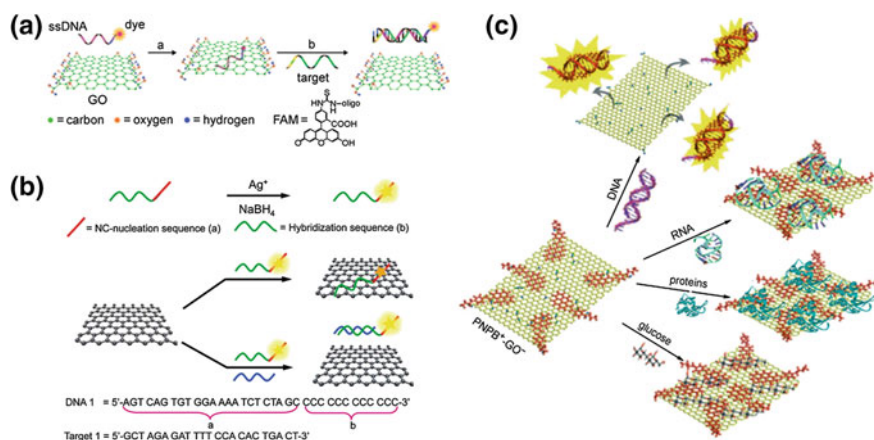


Fig. 2.2 **a** Schematic representation of the target-induced fluorescence change of the ssDNA-FAM-GO complex. FAM is the fluorescein-based fluorescent dye. Reprinted from Ref. [4] by permission of John Wiley & Sons Ltd. **b** Schematic representation of the preparation of silver nanoclusters on DNA scaffolds in aqueous solution and the assay for DNA detection using AgNCs-GO nanohybrid materials. Reprinted from Ref. [31] by permission of The Royal Society of Chemistry. **c** Sensing by PNP⁺GO⁻. DNA can complex efficiently with PNP⁺ to form ionic complex PNP⁺DNA⁻ and thus switches on the fluorescence. Other biomolecules undergo π - π stacking on GO but do not remove PNP⁺ from GO, and thus, fluorescence remains quenched. Reprinted from Ref. [33] by permission from John Wiley & Sons Ltd.

dsDNA was also detected by the formation of triplex DNA with dye-labeled ssDNA [22]. As a nanoquencher, graphene shows super-quenching capacity with a wide energy transfer range, which may quench the fluorescence of different dyes simultaneously. Fan and coworkers devised a multicolor fluorescent DNA analysis manner [1], in which multiple ssDNA sequences labeled with different dyes were anchored on graphene. Hybridization to multiple DNA targets in the same solution led to fluorescence recovery of different dyes. The multicolor biosensor might also be suitable for the detection of a range of analytes.

Due to the different graphene fabrication methods, the carbon-to-oxygen (C/O) ratios in GO might differ at a large level, also the sp^3/sp^2 bonded network structure. This may have a great impact on GO's ability for fluorescence quenching of adsorbed dyes and the binding interactions to ssDNA, resulting in a broad range of DNA detection sensitivity. In fact, rGO, in which the oxygenic groups are partially or totally reduced, might not be directly used in the detection due to the strong tendency to aggregate [23]. To get a deeper insight into this, properties of GO samples with tuned C/O ratios (1.1, 1.3, 1.6, and 1.9) were fairly investigated [24]. Results showed that GO with high C/O ratio bound more strongly to ssDNA and quenched the fluorescence of organic chromophores more effectively than that with low C/O ratio. The loading amount of fluorophore-labeled ssDNA at least oxidized GO (C/O ratio = 1.9) was four times than that at GO with C/O ratio of 1.1. With the tunable chemical compositions, GO nanosheets offer a broad range of materials with

different fluorescence quenching efficiencies and binding affinity with ssDNAs, which exhibit the great potential in biological applications.

Importantly, the binding of ssDNA to graphene in solution may be influenced by other elements, such as ssDNA length, ions, pH, organic solvent, and temperature, which then contribute to the hybridization accuracy and efficiency. Experiments were carried out by Liu and coworkers to study these impacts on DNA adsorption and desorption [25]. By using dye-labeled ssDNA, they found that shorter ssDNA bound to graphene with higher kinetics and higher adsorption efficiency. Adsorption was also favored by low pH value and high ionic strength. Desorption could occur by adding the complementary DNA to form dsDNA, adding the same ssDNA to exchange, and increasing temperature. These findings are important for further understanding the interactions between DNA and graphene and optimizing DNA and graphene-based devices and sensors. On the other hand, the self-assembly of graphene in aqueous solution can be tuned with ions to form colloidal stacked graphene, which may be powerful platforms for capturing dsDNA [26, 27].

Besides taking fluorescent dye as label, FRET could also happen between quantum dots (QDs) and GO. Dong et al. [28] designed MB-modified QDs to recognize target. The strong interaction between MB and GO resulted in the fluorescence quenching of QDs. After hybridization with target, the interaction between target-bound MB and GO was weakened, leading to the restoration of QD fluorescence. Due to the ultrasmall size (composed of a few to hundred atoms), good biocompatibility, and excellent photostability, fluorescent metal nanoclusters, e.g., Au and Ag, have attracted much attention and been widely used in biological applications [29, 30]. By taking advantage of Ag nanoclusters' optical features and GO's super-quenching capacity, a kind of sensitive fluorescent DNA detection manner was proposed [31]. ssDNA consisted of hybridization sequence, which was for target detection, and nucleation sequence, which was for Ag nanocluster formation (Fig. 2.2b). The fluorescence of ssDNA–Ag nanoclusters was quenched by GO and recovered after hybridization with target sequence, which was similar to the above fluorophore-labeled detection manner. This strategy avoided the direct labeling process of probe DNA or target DNA; however, the nucleation process needs to be precisely controlled. Moreover, due to the tunable fluorescence of Ag nanoclusters, multiple and simultaneous DNA detection was achieved, which was an obvious improvement in the present study.

These labeling manners possess high sensitivity, good selectivity, etc., while at the mean time, they need DNA sequences to be previously fluorophore-labeled. Labeling step incorporated into nucleic acid assay has shortcomings of limited labeling efficiency, complex multistep analysis, and contamination to samples [32]. Eliminating the tedious labeling process, a GO–organic dye charge transfer complex was fabricated by Loh and coworkers [33]. The positively charged and fluorescent pyrene derivative, namely 4-(1-pyrenylvinyl)-*N*-butylpyridinium bromide (PNPB), could interact with negatively charged GO to form PNP^+GO^- complex. The fluorescence of PNP^+ was quenched due to the charge–transfer interactions between PNP^+ and GO. Negatively charged dsDNA bound to PNP^+ through ion-exchange process, resulting in the release of PNP^+ -dsDNA from GO and a

fluorescence ‘turn-on’ manner (Fig. 2.2c). This simple, efficient ion-exchange strategy could distinguish dsDNA from other biomolecules, such as protein, RNA, glucose, and surfactants, while one issue concerning it might be the selectivity toward ssDNA, or the capability to target DNA sequence hybridization detection. Because of the different binding ability of graphene (GO) to ssDNA and dsDNA, the current concept might be extended to label-free fluorescent DNA detection.

With the aid of DNA ligase, fluorescent DNA single-nucleotide polymorphism (SNP) detection was achieved based on the quenching capacity of graphene [34]. By using in situ-generated hydroxyl radical ($\cdot\text{OH}$), which caused DNA damage by abstracting an H atom from phosphate backbone, the interaction between ssDNA and graphene was regulated. It also probed the $\cdot\text{OH}$ -induced ssDNA damage and exhibited the potential in screening antioxidants in DNA repair and anticancer therapy [35].

2.2.2 Electrochemical Assay

Recently, there has been an increasing interest in electrochemical devices for DNA biosensing since they allow a simple, fast, and sensitive analysis without usage of expensive equipment. Among these, electrochemical impedance spectroscopy (EIS) is often used due to its truly label-free manner and high sensitivity. EIS is a powerful method to analyze the complex electrical resistance of a system and is also sensitive to surface phenomena and changes of bulk properties. Thus, in the field of biosensing, it is particularly suited to the detection of binding events and sensitive to changes in interfacial impedance upon biorecognition events occurring at the surface/electrolyte interface. In addition, it is also a valuable tool in characterizing surface modifications, e.g., on transducer devices during the immobilization procedure of recognition elements. A thorough introduction to EIS theory, experiment, and applications could be found in a book [36] and reviews concerning EIS biosensing [37–40].

Graphene’s unique 2D structure and properties, such as rapid heterogeneous electron transfer, make it an excellent candidate in EIS DNA biosensing. It provides the essential elements that an electrochemical substrate needs, e.g., large surface area, various functional groups ($-\text{OH}$, $-\text{COOH}$, $-\text{C}=\text{O}$, epoxide, etc.), good conductivity, and high affinity for molecule binding. A review on graphene for impedimetric biosensing has been published recently [41], while at this part, we mainly focus on the comparative studies of these strategies.

Based on the unique structure of ssDNA and graphene, physical adsorption, especially π – π stacking, dominates their direct interactions between the aromatic rings of nucleobases and the hexagonal cells of graphene sheets. Both linear and hairpin DNA probes had been adsorbed on graphene. After hybridization with cDNA, an impedance ‘signal-off’ assay was observed due to the detachment of dsDNA from graphene surface (employing $[\text{Fe}(\text{CN})_6]^{3-/4-}$ as the redox indicator) [3, 42]. To keep dsDNA on graphene surface and gain ‘signal-on’ process, covalent

immobilization of ssDNA was often adopted using graphene's own oxygenic groups, such as $-\text{COOH}$ [43]. There are extensive nuclear magnetic resonance, infrared spectroscopic, and electron diffraction evidences for the presence of $-\text{COOH}$, $-\text{OH}$, and $-\text{C}=\text{O}$ groups at the edge of the GO sheet, while the basal plane is covered with mostly epoxide and $-\text{OH}$ groups [44]. Hence, it would be necessary to decorate the basal plane with $-\text{COOH}$ for covalent binding reactions. Recently, Loh and coworkers fabricated anodized epitaxial graphene (EG) platform, which contained large amount of $-\text{COOH}$ groups [8]. After activation by EDC and NHS, NH_2 -ssDNA could be covalently immobilized on graphene and hybridization detection was carried out in phosphate buffer solution. Wide dynamic range and more sensitive response than π - π stacking immobilization manner were observed. However, one main obstacle for applying this strategy might be the preparation of anodized EG, which depends highly on the equipment. Our group proposed a very easy way to introduce $-\text{COOH}$ on graphene for DNA biosensing [7]. 3,4,9,10-Perylene tetracarboxylic acid (PTCA), bearing π -conjugated perylene ring and carboxylic acid groups, was used to decorate graphene. Based on the π - π stacking and hydrophobic interactions between the perylene ring and the basal plane of graphene, PTCA molecules separated graphene sheets efficiently and decorated them with plenty of $-\text{COOH}$. This method resolved the graphene aggregation problem, and the resulting hybrids dispersed well in aqueous solution. After activation, NH_2 -ssDNA was covalently immobilized on the hybrid, which presented as 'lying' structure (Fig. 2.3a). Hybridization to cDNA induced the 'lying' ssDNA to 'standing' dsDNA, which caused the graphene interfacial property changes, e.g., negative charge and conformation. Employing $[\text{Fe}(\text{CN})_6]^{3-/4-}$, a 'turn-on' impedance signal was observed with the increment of cDNA concentration (Fig. 2.3b). In a recent research, we optimized the experimental procedure [9]. ssDNA was designed to consist of two parts, the immobilization sequence and the probe sequence. The immobilization part was for anchoring ssDNA and dsDNA on graphene, which eliminated the previous graphene decoration steps and simplified the procedure efficiently. The hybridization between probe and target induced the formation of dsDNA, which stood on graphene surface due to the anchoring by the immobilization sequence.

Instead of occupying the π - π stacking between DNA nucleobases and graphene, an optional way might be through the negatively charged phosphate backbones of DNA. In this manner, the nucleobases are free for efficient hybridization. Onto the PTCA-modified graphene surface, Au NPs were deposited using amine-terminated ionic liquid (NH_2 -IL) as the reductant and capping agent [45]. Au NPs with mean diameter of 3 nm dispersed uniformly on graphene, and their outer layer was positively charged imidazole termini, which increased the surface area of graphene and provided large amount of attachment points for ssDNA immobilization. Based on the electrostatic interaction and adsorption effect, ssDNA was conveniently immobilized and its nucleobases were free to hybridize with cDNA. The HIV-1 pol gene sequences were satisfactorily detected with low detection limit of 3.4×10^{-14} M. In a similar manner, we designed a positively charged perylene derivative PDI, *N,N*-bis-(1-aminopropyl-3-propylimidazol salt)-3,4,9,10-peryrene

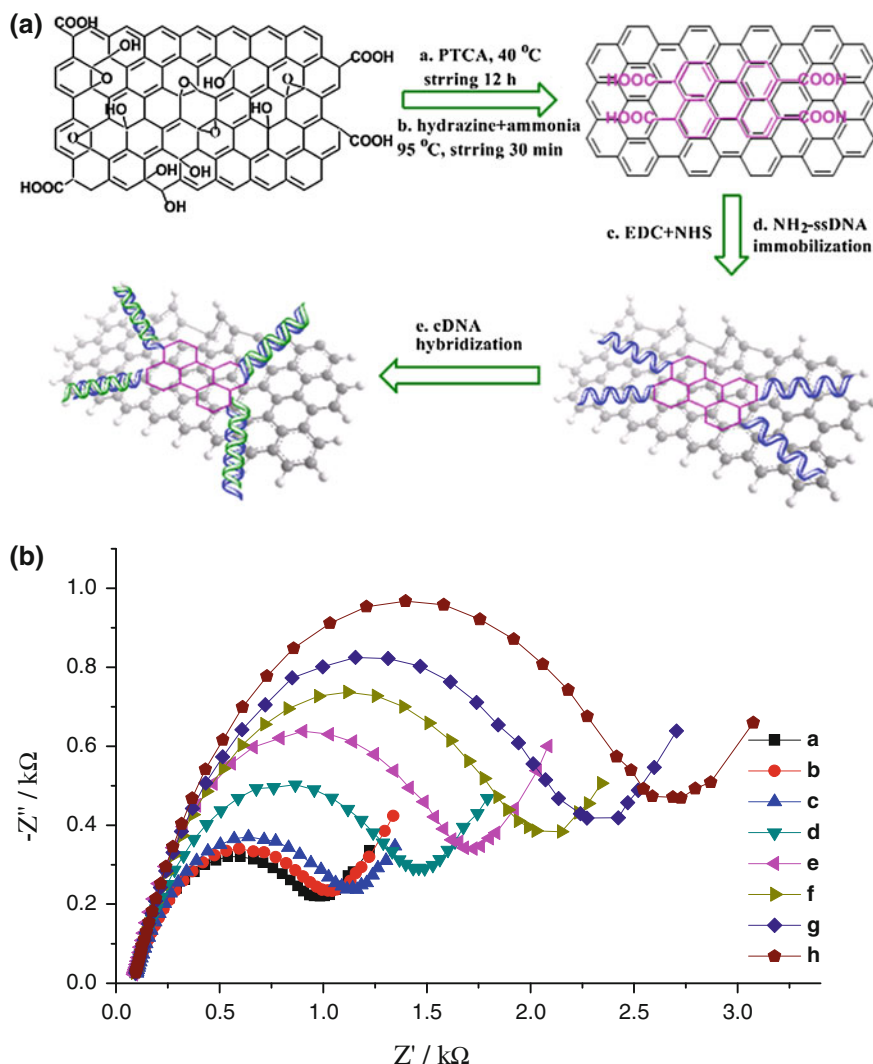


Fig. 2.3 **a** Schematic representation of graphene functionalization with PTCA, ssDNA immobilization, and hybridization. **b** Nyquist diagrams recorded at ssDNA immobilized PTCA/graphene/GCE (*a*) and after hybridization with its complementary human immunodeficiency virus 1 (HIV-1) pol gene sequences of different concentrations from 1.0×10^{-12} to 1.0×10^{-6} M (*b*–*h*). Reprinted from Ref. [7] by permission from The Royal Society of Chemistry

tetracarboxylic acid diimide, to decorate graphene [46]. UV-vis and TEM characterizations illustrated that PDIs self-assembled into pentamer or hexamer, which were uniformly scattered on graphene sheets and beneficial for electrostatic ssDNA immobilization. After hybridization, the formed dsDNA stayed on PDI/graphene, causing an increase of R_{et} value. The ‘signal-on’ process exhibited high sensitivity

and reproducibility. More importantly, the rational design, tailoring, and decoration of graphene endow it with desired properties (dispersive, structural, photoelectrical, and conductive, etc.), which may hold great potential applications in electronics, photovoltaics, and nanodevices. Another impedimetric DNA detection based on reduced GO-modified electrode could be found in Ref. [47].

Another electrochemical label-free DNA detection method is through the direct oxidation of the four nucleobases, without the need of hybridization or labeling molecules. At some point, this strategy is the simplest, while most electrode materials' narrow potential window, high background or slow electron transfer rate restrict their applications [48, 49]. A comparative study of different carbon electrodes for DNA oxidation is illustrated in Ref. [50]. Recently, a nanocarbon film electrode with wide potential window and high activity was successfully used for DNA SNP detection [51]. As an alternative, graphene also fits the requirements well. For instance, Zhou et al. [52] used chemically reduced graphene oxide (CR-GO)-modified GCE for direct nucleobase detection. Comparing with GCE or graphite-modified GCE, negatively moved oxidation potentials and enhanced peak currents were obtained at graphene/GCE. Besides, the quantitative SNP detection at physiological pH was well accomplished. Meanwhile, Pumera and coworkers showed that the oxidation signals of four nucleobases at stacked graphene nanofibers were two to four times higher than those on CNT-based electrode, also higher than edge-plane pyrolytic graphite, glassy carbon, or graphite microparticle-based electrodes [53]. Also, anodized epitaxial graphene was found to exhibit superior electroanalytical performances for nucleobase oxidation compared to carbon electrodes, such as boron-doped diamond, carbon nanotubes, and glassy carbon [54]. Much progress was achieved by Akhavan et al. [55] by using reduced graphene nanowalls (RGNWs), which had a very large surface area and edge-plane defects. To the electrochemical detection of DNA bases (as the free form or in ssDNA and dsDNA), the RGNW electrode exhibited higher current and lower anodic potential as compared to other electrodes (Fig. 2.4a). The high electrochemical activity of the RGNWs could be assigned to better electron exchange between the four bases and the edge-plane-like defective sites of the sheets as active sites for oxidation of DNA bases. The linear dynamic detection range for dsDNA at RGNWs was from 0.1 fM to 10 mM (detection limit to be 9.4 zM, ~ 5 dsDNA/mL), while it was from 2.0 pM to <10 mM (detection limit to be 5.4 fM) at reduced graphene nanosheets (RGNSs). The RGNWs was also efficient in label-free detection of SNPs of 20 zM oligonucleotides (~ 10 DNA/mL) with a specific sequence (Fig. 2.4b). This is the first report on electrochemical sensing of nucleic acids at single-DNA levels.

Based on the direct oxidation of guanines, another aspect would be the detection of DNA damage. A graphene-ionic liquid-Nafion/(horseradish peroxidase (HRP)/dsDNA)_n film was firstly constructed on pyrolytic graphite electrode. DNA damage was induced by incubating the modified electrode in acrylamide (AA) or AA + H₂O₂ solution at 37 °C. AA or its epoxide, glycidamide, could form adduct with dsDNA, which disrupted the double helix structure and made guanines exposed. The DPV signals of guanines represented the degree of dsDNA damage [56].

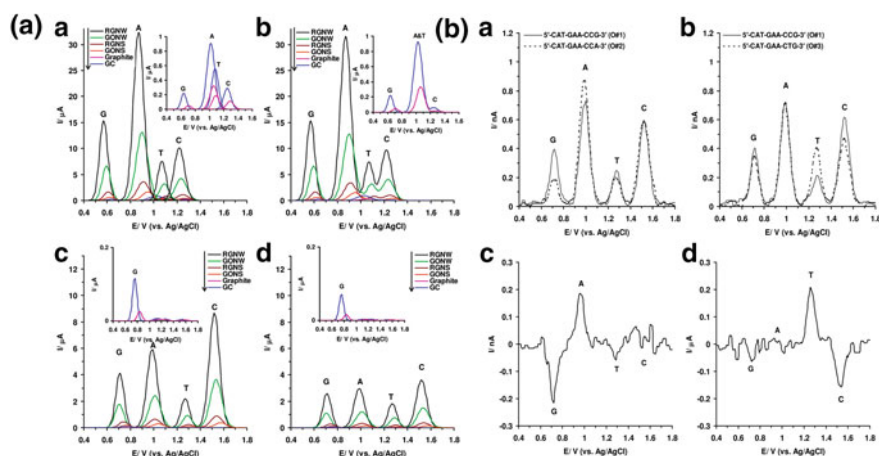


Fig. 2.4 **a** Differential pulse voltammetry (DPV) profiles of the RGNW, GONW, RGNS, and GONS electrodes as compared to the graphite and GC electrodes for the detection of (a) the four free bases of DNA (G, A, T, and C) separately, (b) an equimolar mixture of G, A, T, and C, (c) ssDNA, and (d) dsDNA, with a concentration of $0.1 \mu\text{M}$ for all of the species applied in (a)–(d) in 0.1 M PBS as supporting electrolyte at pH 7.0. **b** Detection of SNPs of oligonucleotides by using DPVs of the single-base mismatched oligonucleotides obtained through (a) G $\rightarrow$ A mutation (O#2) and (b) C $\rightarrow$ T mutation (O#3), as compared to the base oligonucleotide (O#1) at the surface of the RGNW electrode. (c), (d) present subtraction of the DPVs shown in (a) and (b), respectively. Concentration of the different oligonucleotides was 20 zM in 0.1 mM PBS at pH 7.0. Reprinted with permission from Ref. [55] Copyright 2012 American Chemical Society

As mentioned above, plenty of oxygenic groups, e.g., hydroxyl, carboxy, epoxide, carbonyl, aldehyde, peroxide, ether, and ester, present at the basal planes or edges of GO sheets. Using electrochemical methods, GO can be reduced with an irreversibly cathodic peak at negative potentials [57, 58]. According to the researches [59, 60], the hydroxy is not able to be further electrochemically reduced. The reduction of epoxide, aldehyde, and peroxide takes place at potentials around -0.9 to -1.5 V (vs. Ag/AgCl) in neutral buffers. And the reduction of carbonyl, carboxy, and ester groups takes place at potentials around -2.1 V in strongly acidic media. Hence, based on the electrochemical reduction signal, it is feasible to selectively reduce oxygenic groups and subsequently quantify the amount, which makes the composition and property of GO tunable. More importantly, the inherent redox activity can be utilized in electrochemical investigation. Recently, GO nanoplatelets (size value $\sim 37 \text{ nm}$) were employed as the electrochemical label for DNA analysis [61]. According to the different binding ability of GO nanoplatelets to fully matched dsDNA, one-base-mismatched dsDNA, and unhybridized sequences, GO reduction signals with varying intensities were observed. This allowed the discrimination of SNPs related to Alzheimer's disease. Though the electrochemical reduction mechanism of GO is not yet thoroughly investigated, one might be certain that hydrogen ions participate in the reduction process. It may get a hint from the thermal reduction of GO in deionized water [62].

Differential pulse voltammetry (DPV) also represents a sensitive method for DNA detection based on the electrochemical signals of intercalators or tags, such as adriamycin [63], daunomycin [64, 65], methylene blue [66], and Au [67]. Without these intercalators or tags, an ultrasensitive and selective DNA detection by using strand-displacement DNA polymerization and parallel-motif DNA triplex system as dual amplifications was realized on ferrocene-appended poly(ethyleneimine)/graphene–mesoporous silica–gold nanoparticle hybrids (Fc-PEI/GSGHs)_n [68]. The detection limit reached as low as 10 fM, and it discriminated the SNP.

2.2.3 Electrical Assay

Above, we mentioned the optical and electrochemical DNA detection employing graphene. As an attractive alternative, electrical detection also possesses real-time, label-free, sensitive, and rapid measurement qualities [69–71]. Graphene's large surface area, biocompatibility, and exceptional and unique electronic properties, such as ultrahigh mobility, low charge scattering, and ambipolar field effect, might make it superior than CNTs for field-effect transistors (FETs) [69]. In FETs, ssDNA fragments acted as negative potential gating agents, resulting in an increase of the hole density in graphene layers, to be $1.8 \times 10^{12} \text{ cm}^{-2}$ [71]. This increased density was consistent with the Raman frequency shifts in the G peak and 2D band positions and the corresponding changes in the G peak full width at half maximum. Ab initio calculations using density functional theory rule out significant charge transfer or modification of the graphene band structure in the presence of ssDNA fragments. The sensitivity of electrical DNA detection strongly depends on the size and shape of graphene, wrinkles on graphene surface, and the oxidation degree of graphene. Using CVD-grown, large-sized graphene films, Dong et al. [69] constructed FETs for DNA hybridization with single-base specificity. The $V_{g,\min}$ was significantly left-shifted with the addition of probe DNA sequences, suggesting that DNA molecules *n*-doped the graphene film. The left shift in $V_{g,\min}$ increased with the increasing concentration of the complementary DNA, especially in the 0.01–10 nM range. As for the one-base mismatched DNA detection, left shift in $V_{g,\min}$ was less noticeable. Results illustrated that $V_{g,\min}$ shift appeared to be a good indicator for DNA detection with a concentration as low as 0.01 nM and for detection of single-base mutation, which is thought as the key to the diagnosis of genetic diseases and realization of personalized medicine. Furthermore, adding nanoparticles, such as Au and Pt, could increase the loading amount of ssDNA and extend dynamic detection range [69, 72].

Usually, the CVD-grown graphene is compromised by high cost and suffers from alignment and reproducibility issues when integrated into prefabricated circuitry. Chemically modified graphenes with adjustable surface chemistry have offered competitive results in biological FETs [72–74]. In the label-free detection, one of the pressing problems was the response to non-specific binding. To mitigate the non-specific effect, Stine and coworkers designed rGO-based bioFET devices,

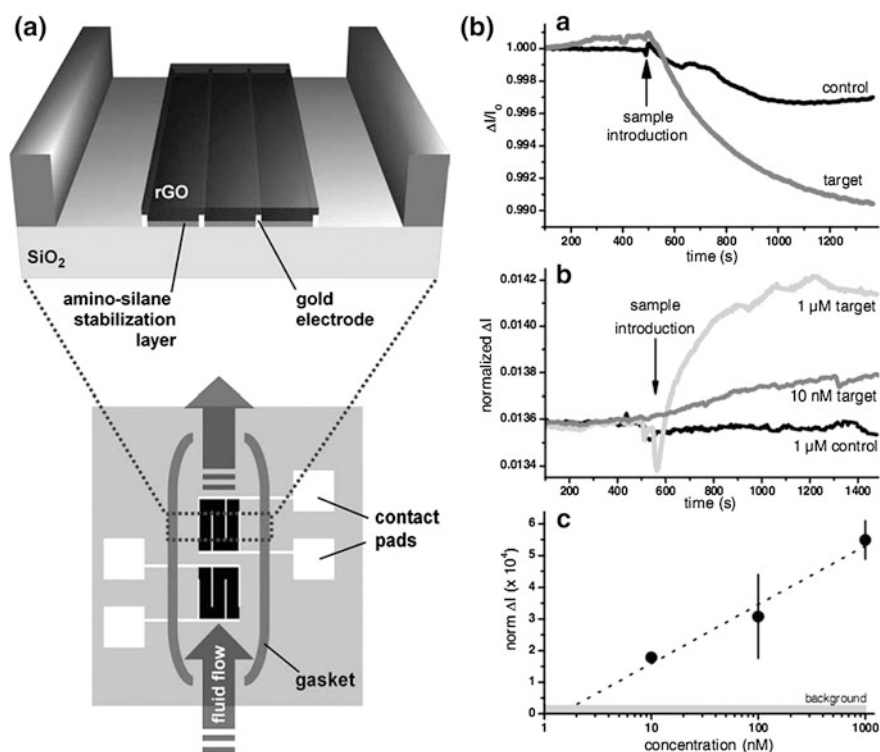


Fig. 2.5 **a** rGO-based FET sensor schematic. The two devices are isolated inside the gasket of the flow cell, with one acting as a reference device to eliminate interference from non-specific biological adhesion (*bottom*). The enlarged area (not to scale) shows the GO deposited on top of the prefabricated electrodes. **b** Functionalized rGO FET devices for DNA detection. (*a*) Response of single FET devices after exposure to both target and non-complementary control DNA solutions. (*b*) Response of differential measurements using one of the FET devices as an internal reference. (*c*) Signal versus target concentration. Reprinted from Ref. [73] by permission from John Wiley & Sons Ltd.

in which the side-by-side devices were exposed to the same sample solution with one device acting as an internal reference for subtracting common sources of interferences, such as pH changes and non-specific biological adhesion (Fig. 2.5a) [73]. The performance of a single isolated FET device (without a reference FET) was shown in Fig. 2.5b a. Though a larger change in current was seen for the target solution due to preferential binding of the complementary strand, the sensor clearly responded to both complementary and non-complementary DNA exposure. In the usage of a second FET as a reference, the limitation was overcome (Fig. 2.5b b). The electrical signal was a differential change between this reference device and the device functionalized with the probe DNA. Clear responses were seen when switching from pure buffer to the two solutions containing the complementary target, but introduction of the non-complementary strand showed no significant

response, with the signal returning to the baseline after the noise caused by switching the feed line. Limit of detection fell near 2 nM (Fig. 2.5b c), placing it in the upper range of the results seen for nanowire/nanotube sensors in similar salt solutions. Moreover, the ability to incorporate a reference sensor by spin coating identical side-by-side devices offers a distinct advantage over similar CNT FETs, as the variability between nanotubes and the bottom-up construction of such devices makes this difficult.

In graphene-based biological FETs, several factors could be optimized, including (a) tailoring the device geometry to maximize the effect of bound targets on conduction pathways, (b) manipulating graphene conductivity, surface groups, and morphology to control the device selectivity and sensitivity, (c) optimizing the density of biological probes to obtain the maximum number of bound targets, and (d) switching the binding affinity or polarity specificity for versatile probe immobilization and target detection.

2.2.4 Other Detection Manners

Based on the unique structure of graphene and its π - π interaction with DNA, other methods, such as colorimetry [75–77], SERS [78], chemiluminescence [79], and mass spectrometry (MS) [80], have been applied to sensitive and multiplex DNA detection. Taking SERS for instance, researches have been carried out to study the Raman spectrum of graphene [81, 82] and its role in Raman enhancement [83]. It is found that the Raman signals of molecules on monolayer graphene are stronger than those on the SiO₂/Si substrate, and the intensity decreases roughly with the increase of the number of graphene layers. Based on the charge transfer of graphene and chemical enhancement mechanism, graphene leads to an enhancement factor of 2–17 [83]. Though it is not clear for the precise origin and further investigation is still needed, one might expand these properties for practical application. Fabricating high-quality graphene with CVD and combining the electromagnetic enhancement of Au nanoparticles, He and coworkers developed the gold-decorated graphene, serving as a highly efficient SERS-active substrate, to facilitate the immobilization of dye-labeled DNAs [78]. A low detection limit of 10 pM was obtained. Also, it realized the simultaneously detection of more types of target DNAs on the same substrate with single-laser excitation.

Due to its double helix structure and the base pairing, DNA-mediated assembled structures can be designed and controlled as a desirable and reversible route. For the first time, DNA hybridization-induced controllable assembly of GO nanosheets was illustrated by Tang et al. [84]. Two single-stranded sequences, DNA₁ and DNA₂, were separately added to GO solutions to form DNA₁-GO and DNA₂-GO complexes, respectively. DNA₁ had a target-specific sequence complementary to target DNA (T-DNA) and a d(GT)₁₅ tail at the 5' terminus to facilitate the assembly of DNA₁ on GO nanosheets. DNA₂ also had a complementary sequence to T-DNA with a d(GT)₁₅ tail at the 3' terminus. In the mixture of the two DNA₁-GO and

DNA₂–GO complexes, T-DNA hybridized with both DNA₁ and DNA₂, which resulted in an assembly of GO nanosheets. Such assembly process was expected to produce the layered structure with tunable interlayer spacing. In particular, it was observed that the size of the GO assembly was concentration dependent and base mismatch sensitive to target DNA, as revealed by dynamic light scattering (DLS) technique. A readily achieved detection limit of 1 pM was observed, and single-base-mismatched DNA was directly discriminated from the perfectly complementary ones.

2.2.5 DNA Sequencing Through Graphene Nanopore, Nanogap, and Nanoribbon

DNA or RNA sequencing through nanopores has attracted much interest because it is a label-free, amplification-free, and single-molecule approach that can be scaled for high-throughput DNA or RNA analysis [85–95]. The concept is simple, which is based on the resistive pulse technique, or Coulter Counter method. A single nanopore is placed in an electrochemical system with electrolytes. Ions are driven through the pore by an applied voltage, resulting in an ionic current. DNA could also be driven through the pore, influencing the flow of ions. As the pore diameter is sufficiently small, DNA is forced to pass through as a linear strand (opposite to the randomly coiled configuration in free solution), then its properties, length, and, ultimately, nucleobase sequence could be determined based on the duration of current blockage and variations in its magnitude [86, 89]. To make this approach practical and reliable, some issues should be resolved, for example, suppressing stochastic nucleobase motions, controlling DNA translocation rate, and resolving the signal overlap between different nucleobases [91]. Among these nanopore materials (e.g., α -hemolysin, SiN, Al₂O₃), graphene is particularly suitable for electronic DNA sequencing because the thickness of a single layer is comparable to the spacing between nucleotides in ssDNA (0.32–0.52 nm) [85]. For instance, Garaj et al. [90], Schneider et al. [92], and Merchant et al. [94] all succeeded in detecting individual dsDNA using graphene nanopores (Fig. 2.6a). A multilayered graphene–Al₂O₃ nanopore was also fabricated, which exhibited low electrical noise (lower than pure graphene nanopore), and was sensitive to electrolyte pH at low KCl concentration [96]. The folded and unfolded transport of single-DNA molecules and RecA-coated DNA complexes could be discerned with high temporal resolution, while a question still remains: Is single-base resolution with a graphene nanopore feasible? To achieve this, one might need to slow down the translocation rate of DNA through nanopores. Also, the nucleotides' geometric fluctuation-induced conductance fluctuations should be reduced. Theoretical investigation suggests that using transverse-conductance-based sequencing in the proper geometry could realize DNA sequencing with an error rate of 0 % [93]. Recently, MD simulations in conjunction with electronic transport calculations revealed that edge-

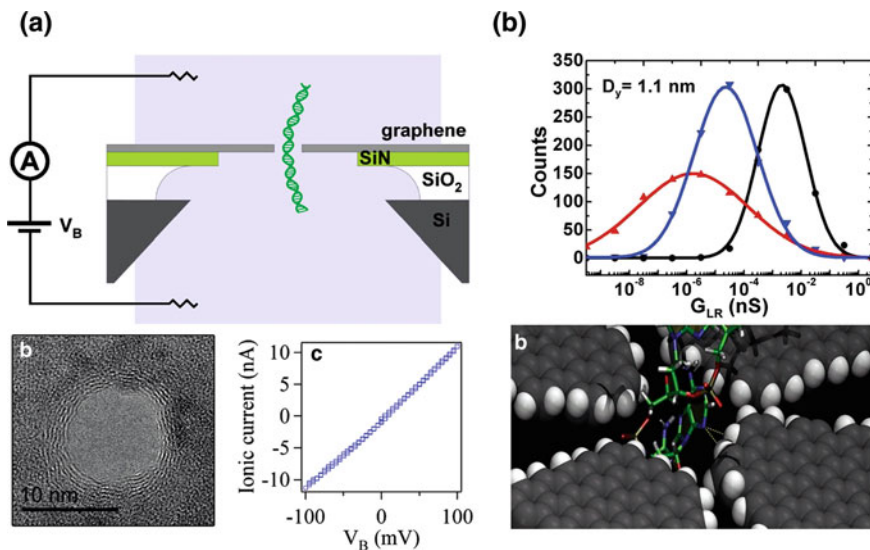


Fig. 2.6 **a** Graphene nanopore devices. *(a)* Device schematic. Few-layer graphene (1–5 nm thick) is suspended over a 1- μm -diameter hole in a 40-nm-thick SiN membrane. The SiN membrane is suspended over an approximately $50 \times 50 \mu\text{m}^2$ aperture in a silicon chip coated with a 5- μm SiO_2 layer. The device is inserted into a PDMS measurement cell with microfluidic channels that form reservoirs in contact with either side of the chip. A bias voltage, V_B , is applied between the reservoirs to drive DNA through the nanopore. *(b)* TEM image of a nanopore in a graphene membrane. Scale bar is 10 nm. The rings surrounding the pore are indicative of the number of graphene layers that form the membrane. *(c)* Ionic current–voltage measurement for this 10-nm graphene nanopore device in 1 M KCl, pH 9. Reprinted with permission from Ref. [94] Copyright 2010 American Chemical Society. **b** Edge-hydrogenated graphene nanopore. *(a)* Transverse differential conductance (G_d) distribution curves of poly(dA)₃₀ translocating through a nanopore using edge-hydrogenated graphene electrodes (*black line*), unhydrogenated electrodes (*red line*), and pseudo-hydrogenated electrodes (*blue line*). The nanopore inner diameter (D_y) amounts to 1.1 nm, the longitudinal driving field has a strength of $E_x = 5 \text{ kcal mol}^{-1} \text{ \AA}^{-1}$, and the transverse bias voltage V_0 is set at 3.2 V, which is near the position of a characteristic eigen level of adenine. *(b)* A snapshot extracted from the molecular dynamics simulation of the DNA translocation through the nanopore, showing a moment when two H bonds (*dotted yellow lines*) are formed simultaneously between the nitrogen atom of a DNA nucleobase and two H atoms attached to the graphene edge. For the sake of clarity, only relevant atoms from the edge-hydrogenated graphene electrodes and the DNA molecule have been visualized, omitting water molecules, counter ions, and the SiN membrane. Reprinted from Ref. [97] by permission from John Wiley & Sons Ltd.

hydrogenated graphene nanopore could facilitate the temporary formation of H bonds with suitable atomic sites in the translocating DNA molecule (Fig. 2.6b) [97]. As a consequence, it reduced the translocation speed of DNA and the average conductivity was drastically raised by about 3 orders of magnitude while exhibiting significantly reduced statistical variance. At the same time, the variation in the conductance was significantly reduced, leading to a faster and more reliable identification of the four nucleotide types. The piconsiemens tunneling conductance

facilitated reading the nucleotide sequence at a much greater speed than what was possible with only femtoampere tunneling currents. This strategy makes an improvement to the fast and reliable nucleotide identification.

Besides the nanopores, graphene nanogaps and nanoribbons are theoretically investigated in DNA sequencing [93, 98, 99]. In the graphene nanoribbons manner, nucleobases interacted with graphene nanoribbons through π - π stacking. As a DNA strand passed through the nanochannel, the distinct conductance characteristics of the graphene nanoribbons (calculated using a method based on density functional theory coupled to non-equilibrium green function theory) allowed the different nucleotides to be distinguished using a data mining technique and a two-dimensional transient autocorrelation analysis [99]. It is believed that further effort and development would be needed to realize the direct label-free DNA sequencing in real time and high resolution.

2.2.6 DNA Cleavage

The performances of above-mentioned graphene are greatly dependent on its large surface area and the few layers' presence, especially the monodispersed single layers. The unique properties of graphene are highly associated with the single-layer existence. When the lateral size of graphene becomes relatively small, e.g., graphene quantum dots (GQDs), the interaction with DNA might change as well. Different from the poor interaction affinity between dsDNA and large graphene sheets, nanosized graphene sheets intercalate and stack into the planes between the base pairs of dsDNA, which induces dsDNA cleavage. Combining with Cu^{2+} ions, which showed high affinity to DNA, GO/ Cu^{2+} system exhibited improved cleavage of dsDNA. The specific interaction mechanism could be found in Ref. [100]. As it is illustrated, the cleavage efficiency was still low and the concentration of Cu^{2+} was high because the size of GO sheets was not well tailored. In a followed study, GQDs were largely produced with photo-Fenton reaction ($\text{Fe}^{2+}/\text{Fe}^{3+}/\text{H}_2\text{O}_2$ reagent under UV irradiation) [101]. Comparing with the micrometer-sized GO sheets, the GQDs with smaller lateral size performed as a better intercalator to DNA. This exhibits the potential applications of GQDs in biological and medical research.

2.3 RNA Detection

Like DNA, RNA is assembled as a chain of nucleotides (G, A, U, C), but is usually single stranded. MicroRNAs (miRNAs) are small, endogenous, non-coding RNAs (approximately 18–25 nucleotides), which play crucial roles in cell proliferation, differentiation, and apoptosis. The sensitive, rapid, and multiple miRNA detection is of great importance. Similar to the above-mentioned fluorescent DNA detection strategies, firstly, ssDNAs labeled with different dyes are adsorbed on GO, where

minimal fluorescence is observed. Upon the recognition of specific target miRNA, DNA–miRNA duplex helices form and strong emission is observed due to the poor interaction between the helix and GO [102]. Employing isothermal strand-displacement polymerase reaction (ISDPR) to amplify the detection signal, a low detection limit of 2.1 fM with a linear range of four orders of magnitude was realized. In a similar manner, DNase I was used to cleave DNA and release miRNA. It acted on ssDNA and dsDNA, chromatin, and DNA in DNA/RNA complex, while it was not active on RNA. DNase I digested the formed DNA/miRNA duplexes and released miRNA to bind another probe DNA on GO, which formed a cyclic reaction and resulted in fluorescent signal amplification [103]. Adopting polyethyleneimine-grafted graphene nanoribbon (PEI-g-GNR) as an effective gene vector and MB, the fluorescent miRNA detection in Hela cells was developed [104]. Recently, an electrochemical miRNA detection manner was reported [105]. Using graphene-dendritic Au nanostructure, multifunctional encoded Au with biotin, and streptavidin-HRP as the tri-amplifications, a detection limit toward miRNA of 0.06 pM (S/N = 3) was realized.

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