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Short communication

Graphene oxide arrays for detecting specific DNA hybridization by fluorescence resonance energy transfer

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ABSTRACT

The unique properties of graphene oxides (GO) such as water dispersibility, versatile surface modification, and photoluminescence make them suitable for biological applications. In this study, we explored the use of GO sheets as a novel DNA biosensor by applying the GO in an array format to recognize specific DNA–DNA hybridization interaction. When the probe DNA linked to the surface of GO by using carbodiimide chemistry is hybridized with a gold nanoparticle (Au NP) labeled complementary DNA strand, the fluorescence emission intensity of the GO array is drastically reduced. TEM data reveal that the Au NPs are dispersed on the GO surface, particularly at edges and folded structures upon hybridization with a density of ~80 Au NPs per μm^2. This leads to *ca.* 87% fluorescence quenching as a consequence of fluorescence energy transfer between Au NPs and the GO sheets. These results suggest that the GO nanomaterials, which are readily synthesized on a large scale from a cheap graphite source, could have a wide range of bioapplications in the fields of biosensors, molecular imaging and nanobiotechnology.

1. Introduction

Graphene, a single-atom-thick and two-dimensional sp^2 carbon networking material, has recently attracted great attention owing to its remarkable electronic, mechanical, and thermal properties (Novoselov et al., 2004). The unique characteristics of graphene that include ultra-high electron mobility, transparency, and super thermal and mechanical strength are being used advantageously in the development of advanced batteries (Cassagneau and Fendler, 1998), field-effect transistors (Gilje et al., 2007), ultrasensitive sensors (Schedin et al., 2007) and electromechanical resonators (Bunch et al., 2007). Despite growing use of graphene in the fields of physics, nanomechanics and nanoelectronics, only a limited number of applications to biological systems have been explored. Considering that carbon nanotubes (CNTs), scrolled 2D graphene sheets, have been extensively investigated for in vitro and in vivo biological applications (Kam et al., 2004; Liu et al., 2008), graphene and its derivatives should have equivalent or superior properties to be applied for biodevices and biosensors.

In particular, graphene oxide (GO), a precursor for graphene through chemical or thermal reduction process, has unique properties that are suitable for bioapplications. These include its facile synthesis, high water dispersibility, capability of installing biomolecular linkages on the surface, and fluorescence labels (Mohanty and Berry, 2008). Simple treatment of cheap graphites with strong acids and oxidizing agents produces GO sheets as two-dimensional, impurity free crystalline materials on a large scale. The presence of oxygen-containing groups on GO causes it to be strongly hydrophilic and water soluble (Park and Ruoff, 2009). In addition, this functionality serves as sites for chemical modification of graphene using well-known chemistry, which can be employed to immobilize various biomolecules through covalent bonds. In 2008, Liu et al. described the synthesis and functionalization of nanoscale GO sheets for the delivery of water-insoluble cancer drugs. Other studies have made use of covalent and non-covalent binding of DNA to GO sheets in the design of sensitive electrochemical biosensing systems. Interestingly, the chemical oxidization process causes a change in the two-dimensional crystal structure of graphene that leads to a fluorescent emission signal at *ca.* 546 nm. The photoluminescence of GO is mainly due to the recombination of electron–hole pairs which are localized within the domain of sp^2 carbon embedded in an sp^3 matrix (Eda et al., 2010). The fluorescence characteristics of GO have been demonstrated by showing that GO, containing porphyrin modifications, has excellent optical limiting properties to act as electron acceptors (Xu et al., 2009).

In this study, we have utilized the properties of GO outlined above to design a novel biosensor by employing GO as an efficient fluorescent label for detecting DNA–DNA interactions. In the GO-based DNA biosensor, GO sheets which are covalently linked with probe DNAs are deposited on a positively charged glass slide in an array format. The complementary target DNA, which is labeled

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with Au NP, is specifically hybridized with the probe DNA on the GO. We observed DNA hybridization event by measuring the fluorescence intensities of GO arrays, and characterized the Au NP labeled duplex DNA on the GO surface to investigate the effect of fluorescence resonance energy transfer between the GO and Au NPs.

2. Materials and methods

2.1. Chemicals and instruments

Sodium citrate ($C_6H_5Na_3O_7$), hydrogen tetrachloroaurate ($HAuCl_4$), dithiothreitol (DTT), *N*,*N*-dimethylformamide (DMF), 2-[morpholino]ethanesulfonic acid (MES) and graphite flakes were purchased from Sigma–Aldrich (USA). 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC) and *N*-hydroxy sulfosuccinimide (Sulfo-NHS) were obtained from Pierce (USA). All the DNA oligonucleotides were ordered from Bioneer Corporation (Korea), and a PD-10 column was purchased from GE Healthcare. UV–vis absorption spectra and fluorescence emission spectra of GO, single stranded DNA linked GO (ssDNA-GO) and Au NP labeled duplex DNA on GO (Au NP-dsDNA-GO) were recorded by using a UV–vis spectrophotometer (UV-2450, SHIMADZU), and a spectrofluorophotometer (RF-5301PC, SHINMADZU), respectively. The fluorescence emission signals of GO and Au NP-dsDNA-GO deposited on glass slides were analyzed with an Axon GenePix 4000A fluorescence reader (Axon, Union City, CA). The fluorescence median intensities of each spot were calculated with GenePix Pro 3.0, and the average and standard deviation were obtained from quadruplicate experiments. High-resolution transmission electron microscope (HRTEM, Tecnai F30, FEI) and energy dispersive X-ray (EDX) analyses were performed by dropping 1 μL of GO and Au NP-dsDNA-GO solutions onto a lacey carbon-coated grid. The morphology and height profile of GO and Au NP-dsDNA-GO were measured by atomic force microscope (AFM, Veeco D3100, USA) under the conditions of relative humidity of 43% at 20 °C with a tapping mode at a 1–3 Hz scan rate and a 512 × 512 pixel resolution.

2.2. Synthesis of Au NP-dsDNA-GO in a solution phase

The Au NP labeled single stranded DNA (18mer: 5′-SH TTT TTT GTG CGC GTG TGC-3′, T_m: 56.3 °C) was synthesized according to Hill et al. and a water-soluble GO was prepared by using a modified Hummers method (see Supplementary Information). To immobilize a probe DNA on the GO surface, GO (0.06 mg) was dissolved in a 300 μL of DMF and water mixture (2:1, v/v ratio) and then sonicated for 30 min. Subsequently, EDC (2.1 μmol) and Sulfo-NHS (4.2 μmol) were added to activate the GO carboxylic acid functional groups in order to convert them into an amine-reactive NHS-ester form. A 3 nmol of an amino-modified ssDNA (5′ H_2N-TTT TTT GCA CAC GCG CAC-3′) was reacted with the activated GO carboxylic acid groups. Excess EDC, Sulfo-NHS, NH_2-ssDNA and by-products were removed by repeated centrifugation at 14,000 rpm and the precipitated ssDNA-GO was recovered and washed with DI water. A 4 nmol of Au NP labeled target DNA was mixed with 3 nmol of probe DNA immobilized GO, and incubated for 2 h to proceed hybridization reaction. The hybridized product, Au NP-dsDNA-GO, was purified by centrifugation at 10,000 rpm for 30 min and washed with DI water three times.

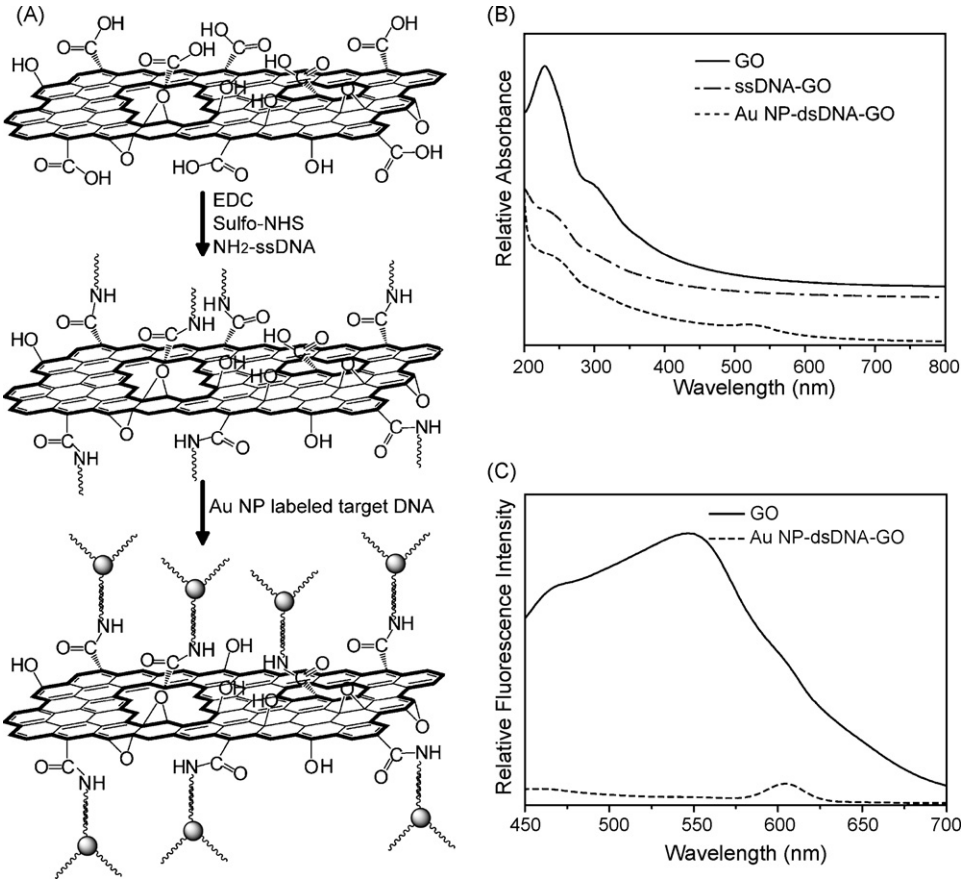

Fig. 1. (A) Schematics illustrating the GO-based DNA biosensor. (B) UV–vis absorption spectra of GO, ssDNA-GO and Au NP-dsDNA-GO. (C) Relative fluorescence intensities of GO and Au NP-dsDNA-GO in a solution phase.

2.3. GO arrays for specific DNA hybridization

One microlitre GO solution (0.5 mg/mL) was deposited in an array format on an amine-modified glass slide by using a micropipette. The slide was dried at room temperature, washed with copious DI water to remove the excess GO sheets and then dried in N_2 gas. One microlitre of EDC (0.5 nmol) and Sulfo-NHS (5 nmol) in a 0.1 M MES buffer was dropped on the GO spots in order to activate the carboxylic acid groups of the GO. After incubating in a humidity chamber for 1 h, 0.5 μL of an amino-modified DNA (40 pmol) was added and the array was kept in a humidity chamber for 10 h to allow the covalent amide bond forming process to take place. After thorough washing with DI water and drying with N_2 gas, the Au NP labeled target DNA solution (4, 2, 1, 0.1, and 0.01 pmol in 0.5 μL) was dropped on the deposited GO array, and then incubated for 10 h. Finally, the slide was extensively washed with DI water, and subjected to fluorescence analysis by using a fluorescence scanner.

3. Results and discussion

3.1. Principle of DNA sensing on a GO sheet

The scheme for DNA hybridization on a GO sheet is illustrated in Fig. 1A. Graphite is chemically oxidized to form graphite oxide which disperses in water to generate a colloidal suspension of GO sheets. The presence of oxygen functionality, including carboxylic acids and hydroxyl moieties disrupts the π system present in the parent graphite, so that it reduces the van der Waals forces between the graphene sheets and allows facile exfoliation upon sonication in water (William and Offeman, 1958; Xu et al., 2009). As demonstrated in the CNT researches, carboxylic acids serve as key functional groups for carbodiimide based covalent linkage with other organic and biomolecular species (Banerjee et al., 2005; Gao and Kyratzis, 2008). However, we have observed that aggregation of GO sheets occurs when DNA is immobilized on the surface owing to charges present in EDC and Sulfo-NHS molecules. It has been reported that the GO sheets likely aggregate in the salt or protein solutions as a result of electrostatic and nonspecific binding. To circumvent GO aggregation, surface functionalization with hydrophilic molecules such as PEG (Liu et al., 2008), and 7,7,8,8-tetracyanoquino-dimethane (TCNQ) anion (Hao et al., 2008) has been used. In our efforts, we found that a polar aprotic solvent, *N*,*N*-dimethylformamide (DMF), helps to disperse the non-surface modified GO sheets. Therefore, we used a DMF/water mixture (2:1, v/v ratio) as a medium, which preserves the homogeneity of the GO solution and is biocompatible with DNA molecules.

The GO sheets are covalently modified with DNA by using carbodiimide assisted amidation, yielding a highly water-soluble adduct that does not aggregate in the aqueous DMF solution. The micron-sized 2D areas of GO sheets afford a sufficient number of covalent binding sites for the probe DNA to increase the sensitivity of DNA detection. To prove a potential application of GO as a DNA biosensor, Au NP labeled complementary DNAs are hybridized with probe DNAs on GO in a solution phase. Hybridization of the probe DNA on GO with Au NP labeled target DNA would take place on both sides of the sheets, resulting in the formation of heavier entities, Au NP-dsDNA-GO, which quickly precipitate during centrifugation. The uniform distribution of negatively charged functional groups and the immobilized probe DNA prevents nonspecific binding of Au NP labeled DNA on the GO surface, allowing specific DNA hybridization reaction. Au NPs have been widely employed as excellent building blocks for biosensing based on FRET, SERS, and SPR analysis (Ray et al., 2007; Wang and Zhou, 2008). In particular, when Au NPs are in close proximity to organic fluorophores or nanocrystals, they act as an efficient quencher through the Förster resonance energy transfer (FRET) mechanism. Thus, specific DNA hybridization causes the Au NPs to be bound on the GO surface which brings about quenching of the GO fluorescence.

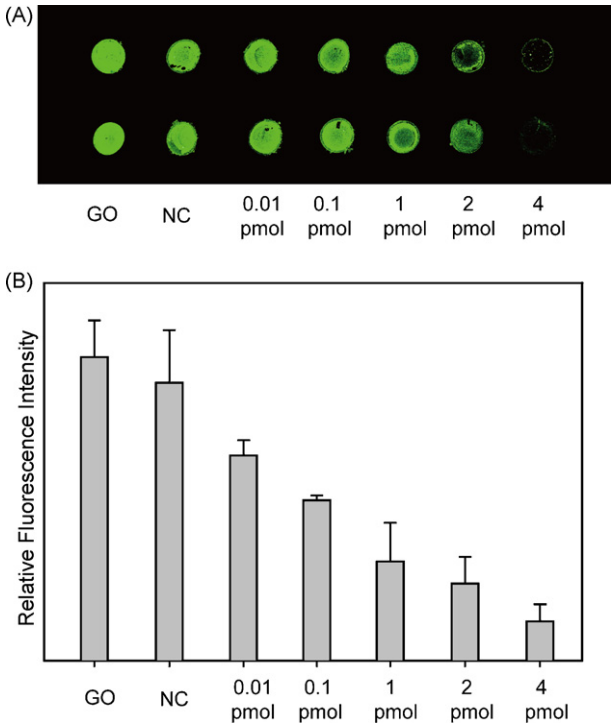

Fig. 2. (A) The scanned fluorescence images of the GO array. The amount of Au NP labeled target DNAs used for DNA hybridization was 4, 2, 1, 0.1, 0.01 pmol, and the quenching efficiency of fluorescence signal was proportional to the target DNA concentration. (B) As the target concentration varied from 4 to 0.01 pmol, the quenching efficiency was changed from 87 to 32% compared with that of the pristine GO. The fluorescence intensity difference between the GO and the negative control (NC) is less than 8.3%.

3.2. Optical characterization of GO and its derivatives

Fig. 1B shows UV–vis absorption spectra of GO, ssDNA-GO and Au NP-dsDNA-GO solutions. The spectrum of GO contains a strong absorption band at 230 nm and a weak band around 290 nm, which correspond to C=C $\pi \rightarrow \pi^*$ and C=O $n \rightarrow \pi^*$ transitions, respectively (Sun et al., 2008). The UV–vis spectrum of ssDNA-GO contains a distinct absorption at *ca.* 235 nm, a 5 nm red shift relative to GO owing to the change of the GO electronic ground state induced by DNA conjugation. This observation shows that DNA probes are successfully linked to the graphene via carbodiimide promoted coupling. In the case of the Au NP-dsDNA-GO complex, absorption band is observed at 519 nm, which is associated with 15 nm diameter. Au NP in addition to the DNA peak at *ca.* 240 nm, confirms that Au NP-DNA is hybridized with probe DNA on the GO surface.

In order to demonstrate the quenching effect of Au NPs on GO in the Au NP-dsDNA-GO complex, the fluorescence emission intensities of GO and the Au NP-dsDNA-GO complex were compared. The fluorescence emission spectrum of the pristine GO (Fig. 1C, solid line) displays a strong fluorescence emission band centered at 547 nm with excitation of 400 nm. The disruption of the fully delocalized π electron system in graphene crystals caused by the introduction of oxygen-containing groups leads to localization of the excitation energy at defect sites, resulting in fluorescence emission. After the probe DNAs on the GO surface are hybridized with Au NP labeled complementary DNAs, the GO fluorescence emission is reduced by more than 95% owing to the effective quenching by the

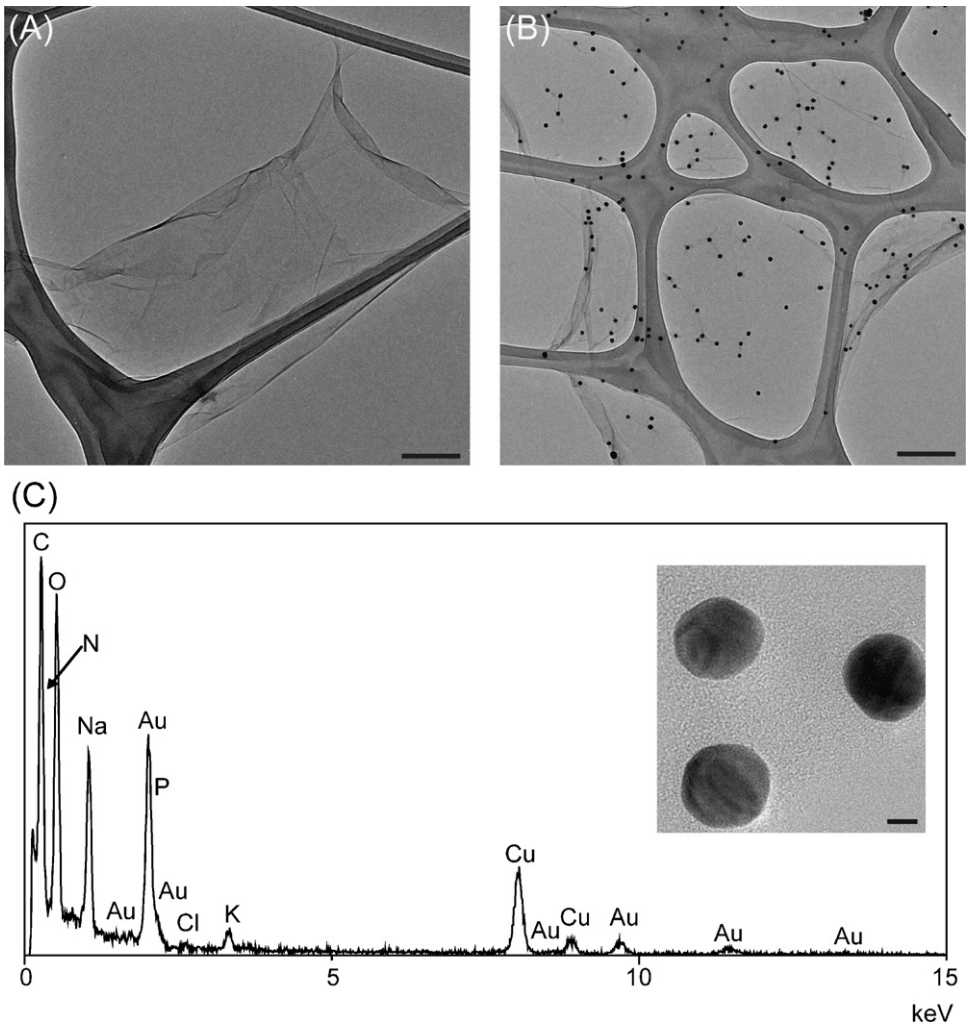

Fig. 3. HRTEM images of (A) the pristine GO sheet and (B) the Au NP-dsDNA-GO complexes. Scale bar: 200 nm. (C) EDX data of Au NP-dsDNA-GO complexes and the enlarged picture of Au NP on the GO sheet (inset). Scale bar: 5 nm.

Au NPs (Fig. 1C, dotted line). In the Au NP-dsDNA-GO complex, the closely located GO and Au NPs act as an energy donor and acceptor pair, which leads to quenching of GO fluorescence through the FRET phenomenon. This observation suggests that GO has a potential of a novel fluorescence label for selective detection of DNA-DNA hybridization and DNA biosensing applications.

3.3. GO arrays for DNA biosensing

Having demonstrated the fluorescence properties of GO and its efficient quenching effect upon hybridization with Au NP labeled DNA in a solution phase, we explored the use of GO sheets in an array format to selectively recognize complementary DNAs. Negatively charged GO sheets were found to self-assemble on a positively charged amine-glass slide, and the strong electrostatic attractive force between the negative GO sheets and the glass surface which is coated with ammonium ions prevents detachment of the GO sheets during a washing step. Thus, a regular array of single graphene sheets can be generated at a defined location with desired patterns on a large substrate, which is ideal for the rational design of GO arrays for biosensing applications. Following the probe DNA conjugation and hybridization reaction as described above, the fluorescence of the GO and Au NP-dsDNA-GO was determined by a microarray scanner. After loading 0.5 μg of GO on each spot, we applied serially diluted target DNAs ranging from 4 pmol (8 μM) to 10 amol (20 nM) to investigate the effect of the concentration of target DNAs on the quenching efficiency. As the concentration of Au NP labeled target DNAs increased, the quenching effect became more dominant, resulting in the gradually reduced green fluorescence signals as shown in Fig. 2A. The average relative fluorescence intensities were measured according to the target DNA concentration of 4, 2, 1, 0.1, and 0.01 pmol, and the corresponding quenching efficiency was calculated as *ca.* 87 ± 6%, 74 ± 8%, 67 ± 11%, 47 ± 2%, and 32 ± 4%, respectively in comparison to that of the pristine GO array (Fig. 2B). Since the quenching phenomenon correlates with the area of graphene oxide sheet, the number of the hybridized target DNA, which contains Au NPs as an energy acceptor, is an important factor to quench the fluorescence of the GO array. Therefore, these results are reasonable in a sense that the attomole level of target DNAs generated relatively low quenching effect, while higher amount of target DNAs causes dramatic change in the fluorescence intensity. If we assume that the threshold of quenching efficiency should be more than 50% to recognize the target DNA, ~0.1 pmol (200 nM) of Au NP labeled target DNAs is a limit of detection (LOD) under our experimental conditions which is comparable to the amount commonly used in a conventional DNA microarray technology. If the threshold is set over 90%, at least 4 pmol of the target DNA should be required. However, we anticipate that the LOD can be improved by reducing the size of graphene sheets into a nanometer scale and employing a multiple and larger Au NP labeled target

DNA to enhance the quenching effect. To prove that the GO quenching is a consequence of a selective DNA hybridization, a negative control experiment was conducted in parallel, in which 2 pmol of a noncomplementary target DNA (5′-HS TTT TTT TTT TTT TTT TTT-3′) conjugated with Au NPs was incubated with the probe labeled GO array. The difference of fluorescence intensities between the GO and the negative control was measured less than 8.3% after a vigorous washing step, thereby demonstrating that the probe DNA on the GO arrays is hybridized specifically by complementary DNA strands with minimal nonspecific interactions.

3.4. Fluorescence quenching of GO with Au NPs

The morphology of the GO sheets and Au NP-dsDNA-GO complex was investigated by HRTEM in order to clarify the interactions that take place between the GO and Au NPs. Fig. 3A shows that a typical GO sheet has lateral dimensions ranging from 500 nm to 3 μm and it contains many wrinkles on the surface center and folds at the edges with a monolayer thickness (Fig. SI-1A). The wrinkle and folded structures are commonly found in 2D graphene in order to maintain thermodynamic stability. Although a previous study showed that the negative carboxylic acid functional groups are uniformly distributed on the GO surface (Park and Ruoff, 2009), the crumpling areas generated during oxidation process seem to have more carboxylic acid groups as crystal defects. This hypothesis is supported by the HRTEM image of the Au NP-dsDNA-GO complex in Fig. 3B. The Au NPs are positioned over the entire surface, particularly at the folded edges and wrinkle sites. The number of Au NP per μm^2 ranged from 70 to 120 with an average *ca.* 80, which lead to 87% fluorescence quenching of GO sheets. The EDX spectrum shows the authenticate composition of the Au NP-dsDNA-GO complex (Fig. 3C). Although it is difficult to detect clearly low level of nitrogen (*ca.* 0.392 keV) and phosphorous (*ca.* 2.013 keV) atom peaks of DNA due to the high intensities of the carbon (*ca.* 0.277 keV) and gold (*ca.* 2.120 keV), the automatic assignments of C, O, Au, N, and P elements suggest that the Au NPs are linked to the GO through DNA hybridization interactions.

4. Conclusion

In summary, we demonstrated the use of GO sheets as a novel fluorescent label for DNA biosensor. The combination of photoluminescence of GO and the fluorescence quencher of Au NPs can be utilized as a basis for selective and sensitive detection of DNA–DNA hybridization interaction. Our results serve as a concrete proof that the fluorescent GO array based biosensor can be applied for a variety of bioanalytical fields such as nanomedicine, nanobiotechnology and immunoassay.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.02.022.

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