DNA Cleavage System of Nanosized Graphene Oxide Sheets and Copper Ions

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ABSTRACT The exploration of efficient DNA intercalative agents (intercalators) is essential for understanding DNA scission, repair, and signal transduction. In this work, we explored systematically the graphene oxide (GO) interaction with DNA molecules using fluorescence spectroscopic (FL) and circular dichroism (CD) studies, gel electrophoresis, and DNA thermal denaturation. We demonstrated that the GO nanosheets could intercalate efficiently into DNA molecules. Significantly, we illustrated that the scission of DNA by GO sheets combining with copper ions could take place pronouncedly. The scission of DNA by the GO/Cu^{2+} system is critically dependent on the concentrations of GO and Cu^{2+} and their ratio. DNA cleavage ability exhibited by the GO with several other metal ions and the fact that GO/Cu^{2+}-cleaved DNA fragments can be partially relegated suggest that the mechanism of DNA cleavage by the GO/metal ion system is oxidative and hydrolytic. The result reveals that the GO/Cu^{2+} could be used as a DNA cleaving system that should find many practical applications in biotechnology and as therapeutic agents.

KEYWORDS: graphene oxide · DNA intercalation · DNA cleavage · copper ion · metal ions

DNA cleavage with certain chemical reagents is of importance for elucidating the mechanisms of DNA scission, repair, and signal transduction.[1,2] Cleaving agents also are promising candidates for therapeutic drugs.[3−6] It was found that the presence of a planar aromatic heterocyclic structure in DNA cleaving agents, which can insert and stack between the base pairs of double helical DNA (intercalation), is an essential requirement for their affinity to DNA and cleavage activity.[5,7,8] For example, the metal complexes containing planar ligands of phenanthroline and porphyrin were efficient DNA cleaving agents.[9,10] Increasing the surface area for the intercalative ligand of a metal complex, such as using an extended aromatic heterocyclic ligand, leads to a substantial increase in intercalative binding affinity, hence an increase in its DNA cleavage activity.[9] In addition, the dimension of the extended aromatic heterocyclic structure of DNA intercalators can change the intercalation site, which improves their specificity to DNA.[3,5,11] Although the number of DNA intercalators has been very high, it remains challenging to design and synthesize compounds with large dimension planar structure that allow a strong binding to DNA and have a high DNA cleavage activity.

Graphene oxide (GO), a single atomic layer of carbon arranged in a hexagonal lattice with oxygen containing functional groups,[12] emerged in recent years as a novel and important class of materials due not only to the new fundamental science that it has shown[13] but also to the prospect of a variety of applications.[14−23] The nanosized GO sheet (Figure 1), which closely resembles a small DNA intercalator with a planar aromatic cyclic structural component, should be a potential DNA intercalator. In comparison to those cleaving agents that have a small aromatic heterocyclic structure, the large planar structure of GO should be favorable to its intercalative binding to DNA and possibly affect the site of intercalation of DNA. The oxygen-containing groups on the surface of GO render more possibilities of chemical modification and reactions. This article presents an exploration of GO as a novel DNA cleaving system by taking advantage of both its structural and chemical properties. As will be shown, the planar single atomic GO sheets are ideal DNA intercalators. Interestingly, coupling to copper ions, the GO sheet can cause DNA cleavage.

GO Intercalation to DNA. The aqueous suspension of GO was prepared and characterized as described in the previous work.[20,24] Interaction between GO and DNA was first investigated by agarose gel electrophoresis using supercoiled PSICOR-GFP DNA incubated with different amount of GO at 37 °C. As shown in Figure 2, when the GO

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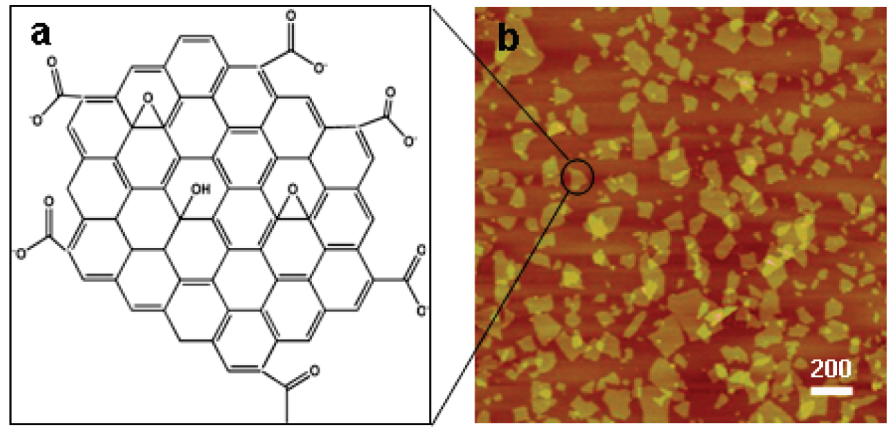


Figure 1. (a) Schematic representation of the chemical structure of the GO, and (b) atomic force microscopy (AFM) image of the GO nanosheets used in the work.

concentration increases, the percentage of the supercoiled DNA (Band I) decreases, while the nicked and linearized DNA (Band III) increase and other smeared bands are also observed. The band assignment was based on the DNA ladder and the enzymatic linearized DNA (Supporting Information Figure S1). The decrease in the intensity of Band I and the appearance of the smeared bands indicate that GO sheets interact with the DNA and very likely in a nonspecific manner. With the interaction time increase, the bands are more smeared (data not shown). Notably, as GO concentration was increased, about 10% of the DNA molecules dwelled in the sample wells. This may be attributed to the inhomogeneous sizes of the GO used, as shown in Figure 1. The large GO sheets may trap some DNA molecules, which affects the migration of DNA molecules in the gel.

Mode of the interaction between GO and DNA was studied by circular dichroism (CD). As shown in Figure 3a, the CD spectrum of DNA was dramatically changed upon addition of GO. The intensities of both the positive and negative ellipticity bands were decreased with the amount of GO increase, suggesting that the stacking mode and orientation of the base pairs of the DNA were disturbed. The decrease of the intensity for both positive and negative bands is a characteristic sign of intercalative interaction.[25] It is very likely the single atomic layered nanosized GO sheets intercalate into the planes between the base pairs of the DNA. To confirm the GO binding mode inferred from CD measurements, fluorescence spectroscopic (FL) studies were carried out with the DNA in the presence of GO. Ethidium bromide (EB), an indicator for DNA detection, was used to monitor the interaction between DNA and GO because a significant increase in fluorescence emission is generated when EB intercalates with DNA molecules.[26] Figure 3b depicts the changes of the FL intensity upon the addition of GO. The increase of the GO concentration induced a notable reduction of ~55% in emission intensity of the EB with DNA. Apparently, EB molecules were replaced by GO sheets and thus led to a decrease in FL intensity.[27] The FL quenching of the EB-bound DNA by GO is in agreement with linear Stern−Volmer equation,[28] and the quenching constant is about 0.45, indicating a strong interaction between the DNA and GO. To exclude the interaction between GO and EB (Supporting Information Figure S2a), the following experiment was performed. The DNA was first incubated with GO for 15 min, and EB was then added to the mixture. We found that FL intensity of the mixture was gradually increased and eventually reached a constant (Figure 4). For comparison, the FL intensities of the sample of DNA with EB in the absence of GO and the sample of GO with EB were also monitored, and their FL intensities remained unchanged during the same period of time as shown in Figure 4 and Supporting Information Figure S2b. The results clearly indicated that the intercalated GO was gradually replaced by EB, resulting in the increase of FL when EB was present in the system.

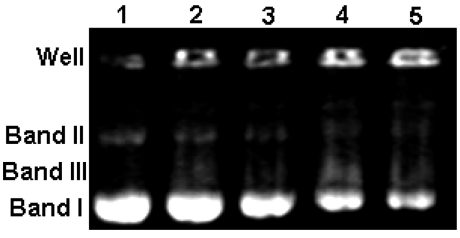


Figure 2. Agarose gel electrophoresis of PSICOR-GFP DNA (0.75 μg) incubated for 2 h with different amount of GO. Lane 1: control, GO concentrations were 50 (lane 2), 75 (lane 3), 125 (lane 4), 175 (lane 5), and 225 μg/mL (lane 6).

To further prove GO intercalation to DNA, the difference in helix−coil transition temperature (T_m) for the DNA with and without GO was measured (ΔT_m) (Supporting Information Figure S3). The ΔT_m of the DNA in the presence of 3 μg/mL GO was ~16 °C.[4,27] When GO concentration was further increased, ΔT_m values were too high to measure. Taking these results together, we conclude that GO sheets bind to the DNA in an intercalative manner.

DNA Cleavage. To explore the potential of the intercalated GO as a DNA cleaving agent, DNA cleavage activity of the GO was investigated in the presence of Cu^{2+}

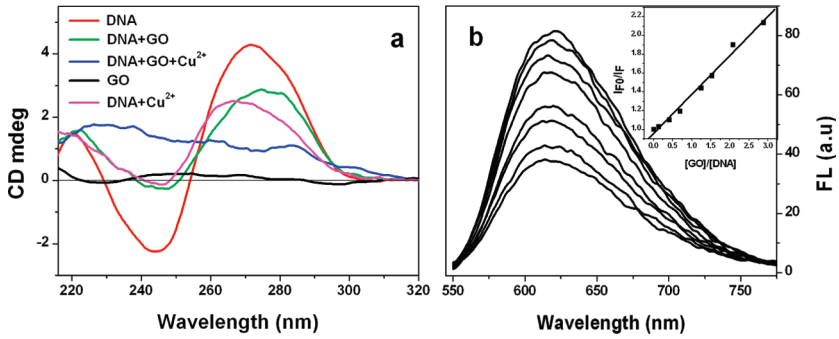


Figure 3. (a) CD spectra of the DNA (25 μg/mL) and the DNA in the presence of GO in 5 mM, pH 7.4, Tris-HCl buffer with 40 mM NaCl at room temperature. Blue line is GO alone. (b) FL spectra of the EB−DNA system (36 μg/mL EB and 18 μg/mL DNA) in the absence and presence of different amounts of GO (from 2.5 to 60 μg/mL). Inset is the Stern−Volmer plot.

ions. Cu^{2+} is chosen because it has been known that DNA has high affinity to Cu^{2+},[29] and copper complexes have been frequently used in the development of agents for the cleavage of DNA. Cu^{2+} ions alone have no effect on the DNA cleavage under the experimental condition (Supporting Information Figure S4). Figure 5 exhibited the agarose gel electrophoresis of the DNA and GO in the presence of 10 mM Cu^{2+}. In contrast to Figure 2, DNA bands in Figure 5 are well-separated, and the supercoiled DNA was converted to its relaxed form with the GO concentration increasing from lane 2 to 7 (from 2 to 50%, Table S1). Under the constant GO concentration, increasing Cu^{2+} concentration resulted in an increase of Band II from 20 to 80% (Figure 6a). Similarly, under the constant ratio of Cu^{2+} to GO, Band II was increased from 45 to 78% with the incubation time increasing from 2 to 8 h (Figure 6b). Under the same ionic strength, no obvious DNA cleavage activity was observed in the absence of Cu^{2+} ions, indicating that the cleavage activity of the GO/Cu^{2+} system is not attributed to the change of ionic strength caused by the presence of Cu^{2+} ions (Supporting Information Figure S5). These results indicate that the cleavage activity of the GO depends critically on the concentrations of Cu^{2+} and GO and their ratio. In addition, in the presence of Cu^{2+} ions, the intensities of both the positive and negative ellipticity bands were decreased in the CD spectra of DNA with GO (Figure 3a), and a significant decrease in fluorescence emission was generated in FL spectra of DNA with GO and EB (Supporting Information Figure S6).

We showed previously that the surface of the GO nanosheets is negatively charged from pH 4 to 10.[20] The GO nanosheet, therefore, is unlikely to interact with the phosphate of DNA molecules, leading to the ester bond scission. It has also been known that metal ions alone, other than lanthanides, are scarcely active in promoting DNA hydrolysis.[1] Hence we infer that the cleavage activity of GO/Cu^{2+} must be a result of the cooperation between GO and Cu^{2+}. It has been reported that the peripheral carboxylic acid groups and other surface groups of the GO sheets can chelate Mg^{2+} ions, forming a complex system.[17] Thus, it is likely in this case that the GO sheet chelates Cu^{2+} ions, forming a complex system, which is analogous to a copper compound with an expanded planar ligand. Compared to a small metal complex, the size of GO sheets ranges from a few to hundreds of nanometers (Figure 1), thus the GO/Cu^{2+} system possibly interacts with DNA at the major groove as schematically shown in Scheme 1 (left). In this system, some Cu^{2+} ions are chelated by GO sheets and delivered to the sites between base pairs of the DNA molecules, where Cu^{2+} can interact with the electron donor groups of bases that paired between two DNA strains. The interactions compete with hydrogen bonding between the paired bases that may be potentially transformed into a radical and eventually result in DNA unwinding.[6] On the other hand, some other Cu^{2+} ions

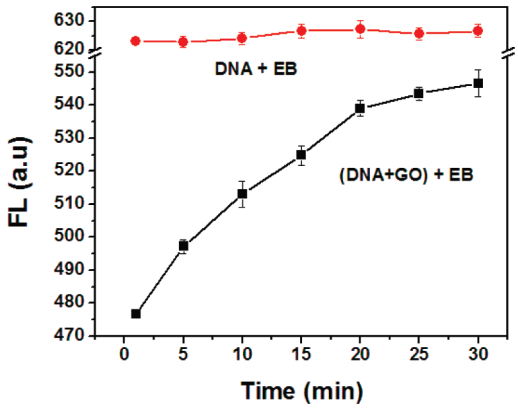


Figure 4. FL intensity variations of the samples of DNA with EB, and the DNA first incubated with GO (10 μg/mL), then with the same amount of EB (6 μg/mL DNA and 12 μg/mL EB).

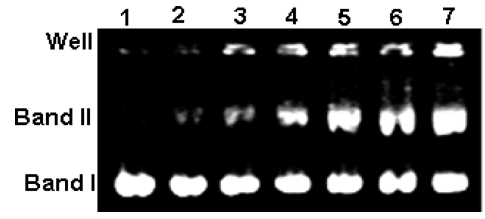


Figure 5. Cleavage of the DNA (1.25 μg) with different amounts of GO in the presence of 10 mM Cu^{2+}. Incubation time is 2 h (37 °C). Lane 1 is control. GO concentrations were 12.5 (lane 2), 25 (lane 3), 50 (lane 4), 75 (lane 5), 100 (lane 6), and 125 μg/mL (lane 7).

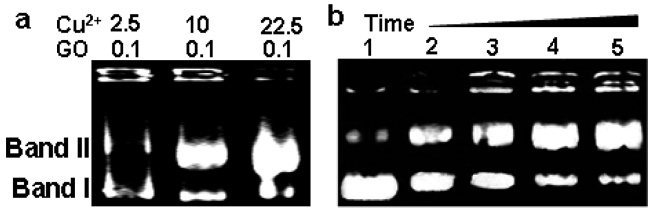


Figure 6. (a) Effect of the Cu^{2+} concentration on the DNA cleavage activity of the GO incubated at 37 °C for 2 h. DNA was 1 μg, Cu^{2+} was 2.5, 10, and 22.5 mM, GO was 100 μg/mL. (b) Agarose gel electrophoresis of the DNA (1.25 μg) incubated at 37 °C for different times in the presence of GO (150 μg/mL) and Cu^{2+} (10 mM). Lane 1 is control. Lane 2: 2 h. Lane 3: 4 h. Lane 4: 6 h. Lane 5: 8 h.

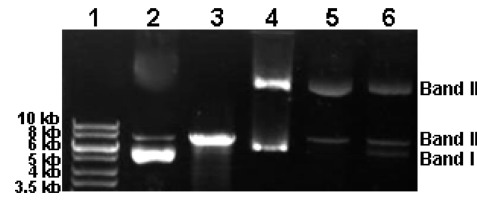
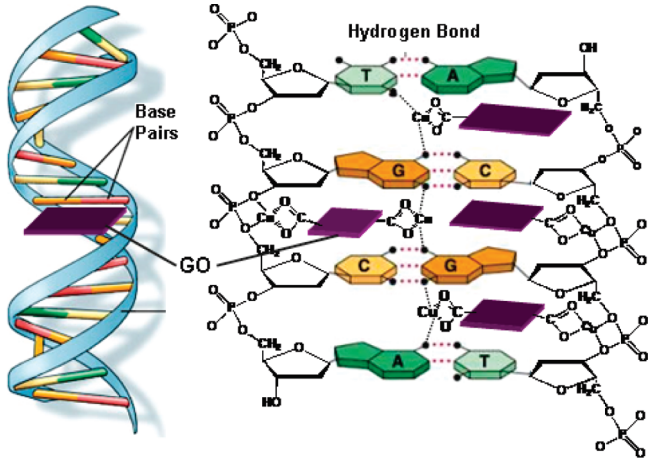


Figure 7. Agarose gel electrophoresis for ligation of the T4 ligase-treated DNA that was cleaved by the GO/Cu^{2+} system. Lane 1: DNA ladder. Lane 2: DNA control. Lane 3: DNA linearized by *Eco*RI. Lane 4: DNA cleaved by GO/Cu^{2+}. Lane 5: Bands II and III extracted from the sample of lane 4. Lane 6: sample of lane 5 relegated by T4 DNA ligase.

chelated by GO may be positioned close to the phosphate group of the DNA and thus can interact with the phosphate group just as free Cu^{2+} ions (Scheme 1, right).[29] According to this mechanism, in principle, any metal ions that can be chelated by GO and can interact with the nucleotide bases and phosphate groups could result in DNA cleavage. Therefore, several other divalent transition metal ions (Mn^{2+}, Mg^{2+}, Ni^{2+}, Zn^{2+}, Fe^{3+}, and Ca^{2+}) were tested. As shown in Supporting Information Figure S7, under the same reaction condition, redox inert Ca^{2+} and Mg^{2+} have no DNA cleavage activity. Mn^{2+} ion is less active than Cu^{2+} ion. Ni^{2+} and Zn^{2+} also exhibited some activity, and Fe^{3+} showed a stronger effect even with a relatively low concentration. GO coupled with redox-active metal ions is capable of cleaving DNA, suggesting that the mechanism of DNA cleavage by the GO/metal ion system is possibly oxidative as many metallointercalators. However, this cannot explain the behavior of the Ni^{2+} and Zn^{2+} metal ions and possible other untested metal ions. In addition, the DNA cleavage activity with GO/Cu^{2+} was not inhibited either by dimethyl sulfoxide (DMSO) or by *n*-butanol (BuOH), typical hydroxyl radical scavengers (Supporting Information Figure S8). To solve this puzzle, the experiment of relegation of the GO/Cu^{2+}-cleaved DNA fragment was performed. As shown in Figure 7, the cleaved products can partially be relegated by T4 DNA ligase, suggesting that the mechanism of the DNA cleavage by GO/Cu^{2+} is hydrolytic as some metal complexes.[1] However, the efficiency of the relegation was not very high, suggesting that some DNA was also cleaved in an oxidative manner.[4,30]



Scheme 1. Proposed DNA cleavage mechanism by the GO/Cu^{2+} system.

Taking these results together, we infer that the DNA cleavage ability of the GO/Cu^{2+} system is the combination of the planar structure of the GO sheets and its surface functional groups and the Cu^{2+} ions chelated on them. When GO/Cu^{2+} sheets intercalate to DNA, the Cu^{2+} ions were delivered between the base pairs and/or to the sugar−phosphate backbone of DNA. Cu^{2+} ions have high affinity to the electron donor groups of the bases and thus can indirectly promote DNA strand cleavage when they are located between base pairs; the Cu^{2+} ions that were delivered to the sugar−phosphate backbone could promote DNA hydrolytic cleavage by acting as Lewis acids. Cu^{2+} ions have higher affinity to the electron donor group of the bases, whereas the other metal ions can be displaced more easily from the base binding sites when conditions favor the formation of hydrogen bonds between strands;[31] that might be the reason for GO/Cu^{2+} system showing higher DNA cleavage activity. On the other hand, in terms of the preference for phosphate over base association, Ni^{2+} and Zn^{2+} show higher association ability than that of Cu^{2+}. In these cases, hydrolytic pathway is a major contribution. Therefore, in the GO/metal ion system, the DNA cleavage activity is determined by the metal ions and GO, in which GO sheets serve as a shuttle for metal ions, similar to the DNA binding domain in iron bleomycin.[6]

In conclusion, we demonstrate for the first time that GO combining with Cu^{2+} is capable of causing DNA scission. The cleaving activity of the GO/Cu^{2+} system was attributed to the combination of the unique structural and chemical features of the GO and high affinity of Cu^{2+} to DNA. The cleavage of the DNA strands by GO/metal ion systems is hydrolytic and oxidative. The cleavage reaction of the GO/Cu^{2+} described here is not efficient, especially in comparison to the oxidative cleavage reaction with many copper complexes, and the concentration of Cu^{2+} used is relatively high because the size of the GO sheets is not well-tailored. Yet, the findings open a new route to obtain DNA cleaving systems and may be useful in improving specificity

and efficiency of the DNA cleaving agent and thus may benefit the research of cancer pharmaceutics. Given that several metal ions could be coupled with GO, forming DNA cleavage systems, we expect that GO will be a crucial nanomaterial for discovery and delivery of cancer drugs. Detailed mechanistic investigation of the GO/Cu^{2+} system, cytotoxicity of GO, and the control of the size of GO sheets is currently in progress.

MATERIALS AND METHODS

Materials and General Methods. Supercoiled PSICOR-GFP DNA was purified from DHα5 cell using EndoFree Plasmid Kit (QIAGEN, USA). The stock solution (2 μg/μL) in TE buffer (10 mM Tris and 1 mM EDTA, pH = 8.0) was prepared. Copper salts and other metal salts and chemical reagents were commercially available (SinoPharm Chemical Reagent Co. Ltd.) in analytic grade and used as purchased. Solutions of the metal salts for strand scission experiments were prepared fresh daily and filtered with a 0.22 μm filter before use. GO was prepared using natural graphite powder through a modified Hummer's method. The aqueous suspension of GO was stored at room temperature on a lab bench and used for characterizations and DNA scission. Electronic absorption spectra of GO and DNA were recorded on a Cary 50 spectrophotometer (Varian, USA). IR spectra of GO were recorded on a Perkin-Nicolet FT-IR 200 in the range of 4000−400 cm^{-1}. Samples were run as KBr pellets. Atomic force microscopic images of graphene oxide were taken on a Nanoscope MultiMode V scanning probe microscopy (SPM) system (Veeco, USA). The scanning rate was set usually at 0.7−1 Hz. The samples for AFM were prepared by dropping aqueous suspension (~0.02 mg/mL) of the graphene oxide on freshly cleaved mica surface and dried under vacuum at 80 °C.

Agarose Gel Electrophoresis of DNA. The DNA cleavage activity of the GO/Cu^{2+} system was determined by monitoring the conversion of the supercoiled DNA (Band I) to open circular DNA (Band II) and/or linear DNA (Band III) using agarose gel electrophoresis. In general, PSICOR-GFP DNA in 50 mM Tris buffer (pH 7.2) was treated with different concentrations of GO and Cu^{2+} (10 mM) for 2 h at 37 °C. Each reaction was quenched by adding 2 μL of a loading buffer solution (0.05% bromophenol blue, 1% SDS, and 50% glycerol, pH 8.0) and then subjected to electrophoresis on a 0.9% agarose gel containing 50 μg of ethidium bromide (EB) in 40 mL of TBE buffer (89 mM Tris, 89 mM boric acid, and 2 mM EDTA, pH 8.3) at 90 V for approximately 1.5 h. To study the involvement of hydroxyl radicals in the mechanism of the DNA cleavage performed by the GO/Cu^{2+} system, 5% DMSO and BuOH were added to the reaction mixtures. Agarose gel electrophoresis was carried out with DYY-6C electrophoresis apparatus (Liuyi Instrumental Co., China). The agarose gels were visualized and digitized with the FR-200A gel image analysis system and analyzed by the SmartView software.

Fluorescence and Circular Dichroism Measurements. Fluorescence measurements were carried out with a Hitachi F4600. In the fluorescence measurement, EB solution was gradually added to 36 μg/mL DNA, and at each time, the fluorescence patterns were scanned from 550 to 800 nm at an excitation wavelength of 526 nm. The fluorescence intensity of DNA-bound EB reached the maximum when EB concentration was 55 μg/mL. At this point, GO was added gradually, up to 60 μg/mL, until the fluorescence pattern decreased and reached the saturation. A JASCO J815 spectropolarimeter (Jasco International Co. Ltd., Japan) equipped with a Jasco temperature controller (model PTC-423S) and controlled by a PC was used for all circular dichroism measurements at 22 °C. A 1 mL quartz cell of 1 cm path length was used. Each spectrum was averaged from five successive accumulations at a scan rate of 50 nm/min.

Thermal Denaturation Measurement. The thermal denaturation experiments were carried out according to the literature with a HITACHI U-2910 spectrometer connected to a temperature controller. The DNA solution (10 μg/mL in 5.0 mM Tris-HCl buffer, pH 7.55) was prepared, and the increase of the absorbance at 260 nm with increase of temperature was recorded as a function of temperature. For the sample in the presence of GO, the DNA was mixed with GO (3 μg/mL) and measured with the same method as that for DNA alone. To compare the changes of the helix−coil transition temperature (T_m) for the DNA with and without GO, the $(A - A_0)/(A_f - A_0)$ values for both samples were plotted *versus* the temperature, where A_0 and A_f are the absorption intensities at 55 and 97 °C, respectively; A is the intensity at a given temperature between 55 and 97 °C. Because the intensity at the total denaturation point for the sample of DNA with GO (A_f(DNA + GO)) was more than 100 °C, and practically not possible to measure, A_f(DNA + GO) was calculated as A_f(DNA) + (A_0(GO) − A_0(DNA)).

DNA Relegation Experiment. The supercoiled DNA was treated with the GO/Cu^{2+} system at 37 °C and pH 7.2 for 2 h. Next, the cleavage products were extracted with DNA extraction kit (TIANGEN Midi Purification Kit, China). Ligation experiment was performed in 10 μL with 5 μL of ligation buffer and 5 units of T4 DNA ligase (Fermentas). The ligation reaction was performed for 17 h at 16 °C.

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Supporting Information Available: DNA thermal denaturation plots, the effects of Cu^{2+} concentration, ionic strength, radical scavengers, and different metal ions on the DNA cleavage activity of GO/Cu^{2+} system. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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