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Detection of DNA damage by using hairpin molecular beacon probes and graphene oxide

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ABSTRACT

A hairpin molecular beacon tagged with carboxyfluorescein in combination with graphene oxide as a quencher reagent was used to detect the DNA damage by chemical reagents. The fluorescence of molecular beacon was quenched sharply by graphene oxide; while in the presence of its complementary DNA the quenching efficiency decreased because their hybridization prevented the strong adsorbability of molecular beacon on graphene oxide. If the complementary DNA was damaged by a chemical reagent and could not form intact duplex structure with molecular beacon, more molecular beacon would adsorb on graphene oxide increasing the quenching efficiency. Thus, damaged DNA could be detected based on different quenching efficiencies afforded by damaged and intact complementary DNA. The damage effects of chlorpyrifos-methyl and three metabolites of styrene such as mandelieaeids, phenylglyoxylieaeids and epoxystyrene on DNA were studied as models. The method for detection of DNA damage was reliable, rapid and simple compared to the biological methods.

1. Introduction

Vast amounts of new chemicals were produced in our world every year, playing an important role in our modern society. The safety of the majority of these chemicals cannot be fully proved. Exposure to the dangerous compounds would increase the chance of getting cancer. So, it is necessary to determine the carcinogenic toxicity of a new chemical before it is produced on a large scale. The mechanism of the cancer brought on by a chemical reagent is complicated. However, the main pathway is that DNA was firstly damaged by chemical reagent or its metabolic products. Then, DNA mutation occurred if the damage cannot be repaired timely, which can cause the introduction of DNA replication errors during cell division, which may then be propagated to additional generations of cells. Depending on the nature of those errors, the result may be the development of cancer [1]. So, detection of DNA damage could predict the carcinogenic toxicity of a new chemical reagent beforehand, thus could serve as a basis for in vitro carcinogenic toxicity screening.

The single cell gel electrophoresis, called "comet assay" is the most frequently used biologic method, which was firstly developed by Ostling and Johanson in 1984 for detecting DNA damage at the level of the single cell [2]. However, it reflects only the level of overall DNA damage in single cell. Most important, in the comet assay 10–800 kb fragments are analyzed and fragments smaller than 10 kb might get lost in agarose gel. Combination of fluorescence in situ hybridization with the comet assay was proved to be a promising technique for evaluating the distribution of DNA and chromosome damage in the entire genome of individual cells [3]. In addition, unscheduled DNA synthesis test [4], sister chromatid exchange test [5] and SOS (a regulatory signal initiated by damage to DNA or the physiological consequences of such damage in prokaryotes)-chromotest [6] were also used to detect the carcinogenic toxicity. However, these methods usually need to culture cells, which make them complex, time consuming, of low efficiency and low sensitivity. In addition, the parameters to demonstrate the damage of DNA in this assay cannot be standardized, which limit the application of these methods.

A considerable battery of simple, quick, highly sensitive and high accuracy analytical methods were developed to detect the damaged DNA. High performance liquid chromatography–mass spectra (HPLC–MS) were often used to detect nucleobases adducts or oxidized nucleobases [7–9]. These methods are very sensitive and could provide specific and detailed molecular information, while it may be limited for screening by throughput, analysis time, and cost. Damaged nucleobases lead DNA to partly unwind, which allows better access of the catalyst to guanines compared to intact DNA. On the other hand, the partial unwinding of DNA may lead to the increase or decrease of the amount of exposed electroactive substances in different systems. Many methods based on these differences were set up for detection of damaged DNA. For example, Rusling et al. developed several electrochemical and

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electrogenerated chemiluminescence method to detect DNA damage [10–13]; Photoelectrochemical sensors were developed for the rapid detection of DNA damage by Guo et al. [14,15]. Most electrochemical methods for detection of DNA could be improved to detect gene mutation [16–18]. The amount of exposed negative charge for intact and unwinding duplex strand DNA is different, which led to the precipitation of negative charged nanoparticles such as single wall carbon nanotube (SWCN) and graphene oxide (GO) in different degrees, which was used to detect single nucleotide polymorphism by simple colorimetric methods [19,20]. Fluorescence probes were also extensively used to detect the DNA damage based on its different fluorescence in the presence of intact or mismatched target DNA [21–24].

Hairpin Molecular beacons (MB), dual-labeled with a reporter fluorophore at one end and a quencher at the opposite end, are one class of important fluorescence probes. They are designed to have a specific probe sequence positioned centrally between two short self-complementary segments that, in the absence of target, near to form a hairpin structure. In this configuration, the fluorophore is in close proximity with the quencher and the MB is in the dark state. After hybridization with a complementary DNA the hairpin opens, separating the fluorophore and quencher, restoring fluorescence, and resulting in the bright state [25]. Hairpin MB can be used to differentiate the intact and damaged DNA due to their different ability to recovery its fluorescence [26,27]. Nano materials such as GO [28–32], gold nanoparticles [33], SWCN [34] and multiple wall carbon nanotube (MWCN) [35] were introduced into the MB to substitute the traditional fluorescent quenching reagent, such as dabcyl, to acquire high quenching efficiency, low background signal and high detection sensitivity.

Here, the MB probes tagged with carboxyfluorescein (FAM) on the 3′ end that is quenched by GO was used to detect the DNA damage induced by chemical reagents. The damaging effect of chlorpyrifos-methyl (CM) and three metabolites of styrene such as mandelieaeids (MA), phenylglyoxylieaeids (PGA) and epoxystyrene (SO) were studied as models. The MB fluorescence was efficiently quenched by GO and the quenching efficiency decreased in the presence of the complementary target DNA. After treating the complementary target DNA with the above mentioned chemical reagents, the quenching efficiency of GO increased in different degrees in presence of these treated target DNA, indicating that DNA was damaged at various levels by these reagents.

2. Experimental section

2.1. Chemicals and materials

Oligonucleotides were purchased from Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China). Their sequences were listed in Table 1. T1, T2 and T3 were complementary to the loop, the loop and one of the stems and the whole sequence of the MB respectively.

The graphene oxide used herein was synthesized by our group following the Hummers method [36].

The primitive sample was centrifuged at 5000 rpm for 5 min and then dissolved to 1×10^{-4} mol L^{-1} with 20 mM pH 7.4 Tris–HCl buffer containing 100 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$ and 0.5 mM EDTA for storage. This solution was protected from light and deposited at 0–4 °C and shaken gently if needed. The hairpin structure of the FAM modified molecular beacon was formed by thermal denaturation at 93 °C in water bath for 5 min and then deposition to room temperature. The stock solution of graphene oxide was obtained by sonicating the samples for 6 h in aqueous solution (pH 7.0). A 0.2 mol/L of SO, PGA and MA stock solution was prepared by diluting the original solution ($\geq$97%, $\geq$98%, and $\geq$99% respectively, Sigma) with double distilled water. 0.2 mol/L CM was also prepared with double distilled water. All other chemicals and solvents were of analytical grade.

Safety note: CM, SO, MA and PGA are suspected human carcinogen and somewhat volatile. Gloves were worn, weighing and manipulations were done under a closed hood. All reactions were done in closed vessels.

2.2. Apparatus

Fluoromax 4 spectrofluorometer (Horiba, Japan) was used in the experiment. The fluorescence anisotropy was measured on a circular dichroism spectrometer (Chirascan, Applied Photophysics, UK). The CS/FP accessory was used for Fluorescence Anisotropy. The TEM image of GO was obtained on transmission electron microscope (JEM2100, Japan). The electrochemical impedance spectroscopy was measured on the Versa STAT 3 (Riceton Applied Research, UK).

2.3. Procedures

The fluorescence intensities of MB, mixed MB/GO and MB-Target/GO solution were measured on Fluoromax 4 spectrofluorometer. The excitation wavelength (λ_{ex}) was 480 nm. The excitation and emission slit width was 1.0 nm. The incubation of the MB/GO solution was just 1 min, and 30 min was needed for the hybridization of the MB with the target DNA.

Glassy Carbon Electrodes (GCE, diameter 3 mm) were polished with 0.3, 0.05 μm alumina powder in order, after rinsing with double-distilled water, the electrodes were sonicated in ethanol and double-distilled water in sequence. Then, the electrodes were dried under a low speed stream of nitrogen. GO modified electrode was prepared by dropping 10 μL 0.35 mg/mL GO on the surface of electrode for 3 h at room temperature. Then, 10 μL MB was dropped on the above prepared electrode for preparation of GO–MB modified electrode. GO–MB-target DNA modified electrode was obtained by dipping GO–MB modified electrode in target DNA solution for 2 h. Then, their electrochemical impedance spectroscopy (EIS) was measured in 0.1 M $[Fe(CN)_6]^{3-}$/$[Fe(CN)_6]^{4-}$ solution.

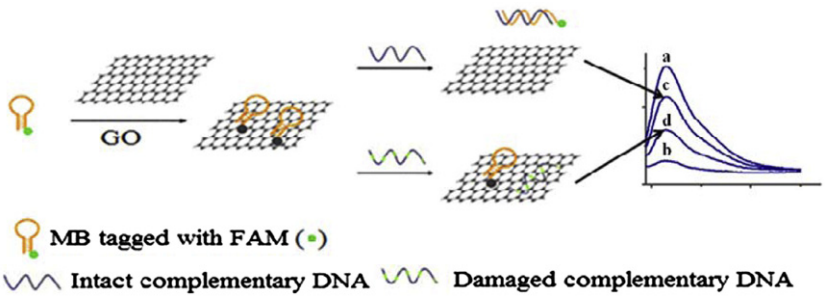
3. Results and discussion

3.1. Design strategy for detection of DNA damage by chemical reagents

The MB used in this study was the hairpin-structured oligonucleotide with 3′ end labeled with FAM, which emitted high fluorescence intensity (Scheme 1a). Graphene oxide (GO) sheet

Table 1
Sequences of oligonucleotide used.

Oligonucleotide	Sequences
Molecular beacon (stem bases were underlined)	5′-CGACGGAGAAAGGGCTGCCACGTCG-FAM-3′
T1: complementary to the loop bases	5′-TGGCAGCCCTTTCTC-3′
T2: complementary to the loop bases and 3′ end of the stem bases	5′-TGGCAGCCCTTTCTC CGTCG-3′
T3: complementary to the loop bases and two end of the stem bases	5′-CGACGTGGCAGCCCTTTCTC CGTCG-3′



Scheme 1. Detect damaged DNA based on the different quenching efficiency of GO on MB in the presence of it or intact DNA: (a) the spectra of free MB, (b) the spectra of MB in the presence of GO, (c) the spectra of MB in the presence of intact target DNA and GO,and (d) the spectra of MB in the presence of damaged target DNA and GO.

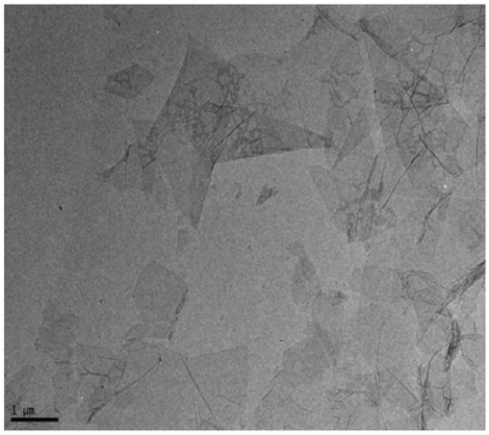


Fig. 1. TEM images of GO used as the quencher.

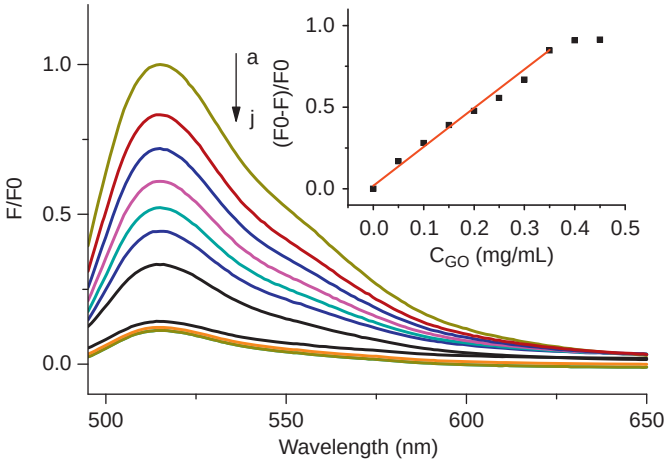


Fig. 2. Fluorescence of 25 nM MB in the presence of 0, 0.05, 0.1, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45 mg/mL GO (a–j). Inset: The effect of GO concentration on the quenching efficiency.

was used as both "nanoscaffold" for the oligonucleotide and "nanoquencher" for the FAM. The fluorescence of MB was quenched sharply due to the high fluorescence resonance energy transfer (FRET) efficiency between GO and FAM because of the strong π-stacking interaction between the ring structures in the nucleobases and the hexagonal cells of GO (Scheme 1b) [37]. In the presence of a complementary target DNA, hybridization of DNA strands, led to the partial release of adsorbed MB from GO, as a result, the quenching efficiency decreased and the released MB emitted strong fluorescence (Scheme 1c); If the complementary DNA were damaged by the chemical reagents, they cannot effectively hybridized with MB to form intact duplex DNA, as a result, the quenching efficiency of GO on MB increased and the MB emitted weaker fluorescence (Scheme 1d) compared to that in the presence of intact complementary target DNA. Thus, the damaged DNA could be detected based on this strategy.

Fig. 1 shows the TEM image of single layer graphene oxide. The cross-sectional view of the image shows that the GO sheet was wrinkled with occasional folds, and rolled edges.

3.2. *The quenching efficiency of GO on the MB fluorescence*

In order to minimize the background fluorescence, GO concentration was optimized to achieve the highest quenching efficiency. The fluorescence change of the MB in the presence of different concentrations of GO was shown in Fig. 2. It shows that 25 nM MB emitted strong fluorescence at 517 nm with the excitation wavelength at 480 nm in the absence of GO (F0). After it was mixed with GO for 1 minute, the fluorescence of MB decreased with increasing the amount of GO from 0 to 0.45 mg/mL (F) (a–j), and reached a maximum quenching efficiency of 94% ((F0 − F)/F0) at 0.35 mg/mL GO (inset in Fig. 2). The high quenching effect of GO on FAM was due to the strong noncovalent binding of GO with nucleobases and aromatic compounds [27,38], which is similar to the mechanism that the carbon nanotubes quench the fluorescence of organic dyes [39,40]. Thus, 0.35 mg/mL GO was used to quench the fluorescence of MB and 1 min was chosen for incubation of MB and GO in the following studies.

The fluorescence anisotropy of MB and MB–GO complex was measured to prove the interaction of ssDNA and GO. The fluorescence anisotropy of free MB is 0.1081 whereas that for the MB–GO complex is 0.8513. The increase of the fluorescence anisotropy indicated that the ssDNA is adsorbed on GO and the free rotation of FAM was further restricted, which was in accordance with previous reported results [41].

Fig. 3 shows the Nyquist plots obtained in a whole impedimetric experiment of the GO system. The equivalent circuit $R_1(C_1R_2)(QR_3)(C_2R_4)$ was used to fit the experimental data. In the circuit, R_1, R_2, R_3 and R_4 represent to the resistance of the solution, electrolyte resistance, charge transfer and Warburg resistance due to the contribution of diffusion respectively. Q means the constant phase element which was associated to the capacitance of the double layer. In fact, the charge transfer process, due to the redox reaction of the couple $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ at the applied potential, is strongly influenced by any electrode surface modification. The Rct of the GO-modified electrode (1) significantly increased from 2178.0 Ω/cm^2 to 2721.0 Ω/cm^2 after MB immobilization (2) onto it. This is due to hindrance of the electron transfer process of $[Fe(CN)_6]^{3-/4-}$ at the electrode surface after immobilization. After hybridization with the complementary target, the Rct (3) decreased to 2430.0 Ω/cm^2 due to the partial release of the MB probes from the electrode surface, which decreased the total

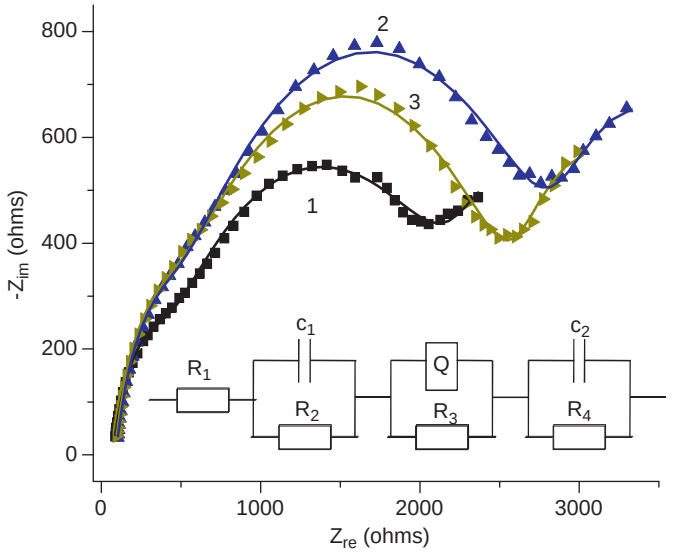


Fig. 3. Nyquist plots for the (1) GCE-GO, (2) GCE-GO-MB and (3) GCE-GO-MB-Target modified electrode at 0.35 mg/mL GO, 25 nM MB and 50 nM T3. The symbols represent the experimental data, while solid lines were fitted curves using equivalent circuits shown in the inset. All measurements were performed in 0.1 M tris solution containing 0.1 M $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$.

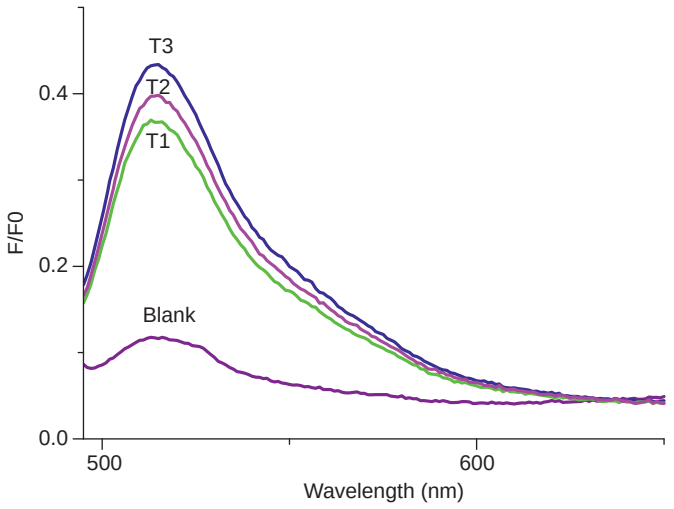


Fig. 4. Influence of different lengths of complementary target DNA on the quenching efficiency of GO on FAM. 0.35 mg/mL GO, 25 nM MB, 50 nM T1, T2 and T3 respectively.

charge present onto the electrode surface, thus reducing the resistance to charge transfer [42].

3.3. Influence of different length of target DNA on the quenching efficiency of GO on MB

The target DNA of T1, T2 and T3 with different lengths was chosen to study their restoration ability on the quenched MB (Fig. 4). After hybridization of target DNA with MB, the fluorescence quenching of MB was also observed during the addition of GO into the hybridization solution. Compared to the MB-GO system without target DNA, however, the presence of each one of the target DNA decreased the quenching efficiency of GO on MB in the following order T1 < T2 < T3. T3 that has longest sequence, complementary to the loop bases and two end of the stem bases, decreased the quenching efficiency of GO on MB with the largest scale. The suggested reason was that the longest complementary sequences produced the strongest interaction with the MB, as a result, most MB were released from GO. Thus, T3 was chosen as the complementary target DNA due to the lower quenching efficiency could leave wider range for the damaged DNA to increase the quenching efficiency, which is beneficial to improve the detection sensitivity.

The T3 concentration also influenced the quenching efficiency of GO on MB. A series of different concentrations of T3 from 0 to 60 nM were used to hybridize with MB and then 0.35 mg/mL GO were added to quench the fluorescence. As shown in Fig. 5, the fluorescence intensity increased with the increasing concentration of T3 in the linear range from 0.8 to 50 nM, with the detection limit of 0.2 nM at 3 times the standard deviation of the control, which indicated that the higher T3 concentration prevented more MB to interact with GO leading to the decreased quenching efficiency of GO [27,38,41]. The linear regression equation was $y = 0.842 - 0.0127x$ (x denotes the concentration of T3 (nM)), with the regression coefficient $R = 0.993$. However, the presence of more T3 could not restore the fluorescence of MB to the values that in the absence of GO because other unknown types of interaction still existed between MB and GO. Considering the detection sensitivity, 50 nM T3 was chosen as the complementary target DNA.

3.4. Detection the DNA damage by chemical reagents

CM is a broad-spectrum organophosphate insecticide used to control the pests in grain and leafy crops storage, nevertheless, its residue is harmful to the the human body. Its damage effect on DNA was studied by choosing 50 nM T3 as a model DNA. First, T3 was treated with CM for different times. Then, 25 nM MB hybridized with this treated T3 followed by adding 0.35 mg/mL GO. Compared to the quenching efficiency in the presence of intact T3, they increased in the presence of these treated T3. This indicated that T3 had been damaged by CM, as a result, it could not form the intact duplex structure with MB to prevent its strong interaction with GO effectively (Fig. 6). The longer incubation time for T3 and CM led to increased quenching efficiency, indicating CM induced more damage on T3 with increasing incubation time. After 2.5 h, the damage reached maximum. The result was in agreement with the reported results that CM led to DNA damage, which was proved by comet assay [43,44].

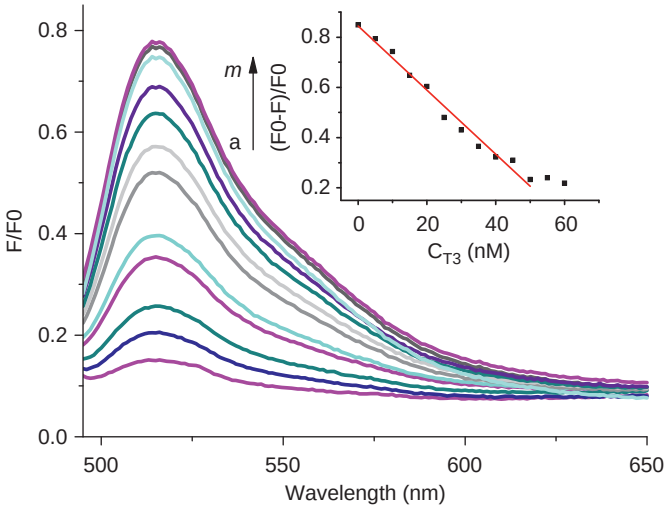


Fig. 5. Influence of different concentrations of T3 on the fluorescence of FAM: 25 nM MB; 0.35 mg/mL GO; 0, 5, 10, 15, 20, 25, 30, 35, 40, 50, 55, 60 nM T3 from a to m. Inset: Influence of different concentration of T3 on the quenching efficiency of GO on FAM.

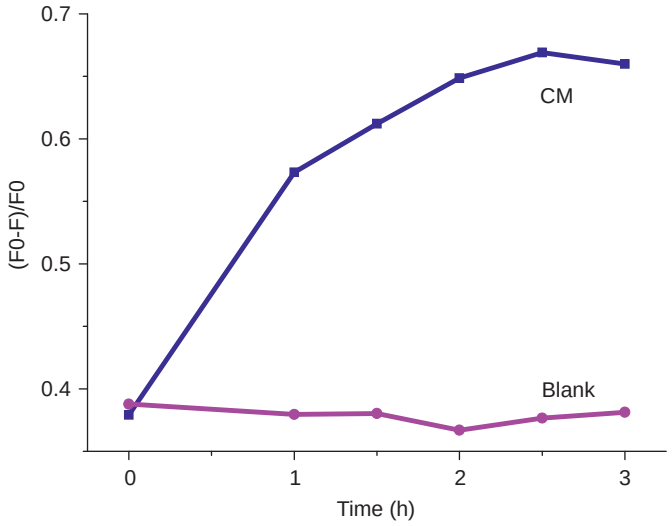


Fig. 6. Comparison of quenching efficiency of GO on MB in the presence of intact T3 and T3 that was damaged by CM at different times. Fluorescence was excited at 480 nm and emitted at 517 nm. *Other conditions*: MB, 25 nM; GO, 0.35 mg/mL; T3, 50 nM; CM, 0.2 mol/L.

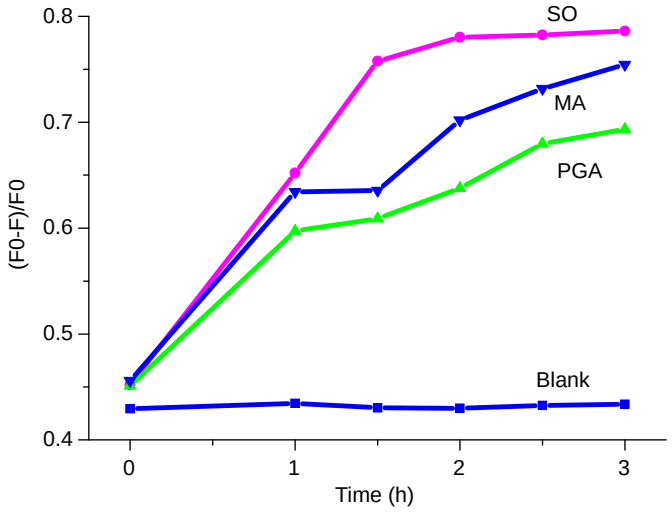


Fig. 7. Comparison of quenching efficiency of GO on MB in the presence of intact T3 and damaged T3 treated by MA, PGA and SO at different times. MA, PGA and SO, 0.2 mol/L. Other conditions are the same as that in Fig. 6.

Styrene is one of the aromatic classes. It is widely used in the chemical industry and is also suspected to be carcinogenic. In vivo, styrene is metabolized into mandelieaeids (MA), phenylglyoxylieaeids (PGA) and epoxystyrene (SO). Their damaged effects on DNA were also studied by this method. 0.2 mol/L of each metabolite was prepared as the working solution. 1 μL metabolite was mixed with 50 nM T3for some time. Experiment results showed that treated T3 increased the quenching efficiency of GO on MB sharply compared with the intact T3. In addition, the quenching efficiency increased with increase in the incubation time between styrene metabolites and T3, which indicated that MA, PGA and SO exert the damage on T3 and these damage increased with increase in exposure time (Fig. 7). The quenching efficiency of GO was higher in the presence of T3 treated with SO than that treated with MA and PGA, indicating that among these metabolites, SO exerted the most serious damage on DNA. These were in accordance with the literature which reported the results that genotoxicity of styrene is from its epoxide, SO, which damage DNA by forming DNA adducts and causing DNA strand breaks [45].

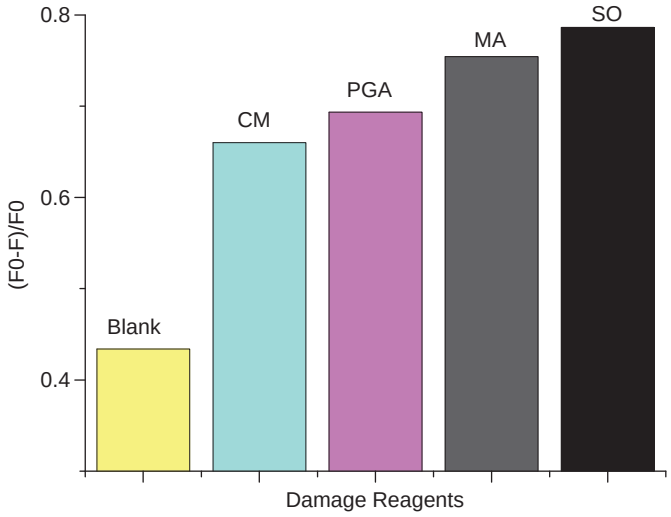


Fig. 8. Comparison of quenching efficiency of GO on MB in the presence of intact T3 and damaged T3 treated by CM, PGA, MA and SO for 3 h. Other conditions are the same as that in Figs. 6 and 7.

However, this method for detection of DNA damage for CM, MA, PGA and SO was simple and sensitive (25 nM 25 b oligonucleotides were used) compared to biological methods [43–48] which are complex, time consuming and low efficiency because most of them need to culture cells. In addition, biological methods are low in sensitivity and usually need high content and long chain DNA (comet assay can be used only for 10–800 Kb base containing DNA). Compared to HPLC-MS method, the method is simple and inexpensive, however, it cannot supply the detailed damage information such as oxidized base or adduct base and so on [7–9].

Fig. 8 shows the quenching efficiency of GO on MB in the presence of T3 that damaged by CM, MA, PGA and SO for 3 h, respectively. It indicated that all of them decreased the quenching efficiency compared to the intact T3. Different quenching effect resulted from the different damage degree of T3.

4. Conclusions

A new method for detection of DNA damage by chemical reagents was proposed in this paper. The main theory was that the intact and the damaged target DNA have different effect on the quenching efficiency of GO on MB. Four chemical reagents CM, PGA, MA and SO were incubated with intact target DNA. Results showed that the treated DNA increased the quenching efficiency of GO on MB compared to intact target DNA, indicating that all of them exert damage effect on DNA. The present work was provided a promising analytical method for detection of DNA damage by chemical reagents with the advantages of being rapid, simple and reliable.

Acknowledgments

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