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Detection of DNA damage by thiazole orange fluorescence probe assisted with exonuclease III

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ABSTRACT

This work reports a fluorescent dye insertion approach for detection of DNA damage. The capture DNA with overhanging 3'-terminus was immobilized on silicon surface to hybridize with target DNA. The intercalation of cyanine dye of thiazole orange (TO) to the double helix structure of DNA (dsDNA) allowed intense enhancement of fluorescence signal. The DNA damage with chemicals led to poor intercalation of TO into double helix structure, resulting in the decrease of the fluorescence signal. This signal decrease could be further enhanced by exonuclease III (Exo III). With this approach, the target DNA could be detected down to 47 fM. Seven chemicals were chosen as models to monitor DNA damage. The results suggested that the present strategy could be developed to detect DNA damage, to classify the damaging mechanism with chemicals and to estimate the toxic effect of chemicals.

1. Introduction

Nowadays millions of chemicals are making an important impact on all aspects of human life. Unfortunately, the vast majority of chemicals are not safe and have health effects, thus causing some great potential risks to human health and the ecosystem. So, it is important to evaluate the mutagenic and carcinogenic action of certain chemicals. Many chemicals produce their carcinogenic toxic effect by causing DNA damage leading to gene mutation. The traditional methods of detecting DNA damage mainly focus on the toxicology and pathology, which are related to the alkaline comet assay, the sister chromatid exchange test and the cytokinesis-block micronucleus test. However, these methods are relatively complicated, time-consuming, inefficient and need expensive instruments.

Recently, a lot of research work had been carried out to simplify the analysis steps and improve the detection sensitivity of the monitoring of DNA damage. For example, the distance between the fluorophore and quencher or nanomaterials could be varied by "open" or "closed" loops due to the molecular beacon-labeled fluorophore [1]. So the method could be used to detect the single nucleotide polymorphisms (SNP) [2,3], target DNA [1,4–7], small biomolecules [8,9], and protein on the basis of the fluorescence quenching and recovery [7,10]. In a similar way, the change of the electrochemical signal could also be used for DNA detection when some electron transfer mediators are modified onto the molecular beacon [11–13]. The rolling circle amplification (RCA) or the circular strand-displacement polymerization could lower the detection limit efficiently through replacement of the signal probe [7,14–16]. Other materials were also reported in the detection of DNA. Copper-nanoparticles (CuNPs), whose size was controlled by the length of dsDNA, could lead to preparation of dsDNA–CuNPs with different fluorescence signals and were used to detect DNA [17]. Gold-nanoparticles (AuNPs) were used to detect DNA by colorimetry based on their various aggregations in the presence of different concentrations of DNA [18,19]. Silver nanoclusters (AgNCs) in hybridized DNA duplex scaffolds were highly sequence-dependent and could be used to identify a typical single-nucleotide mutation [20–25]. Some small molecules could be labeled or inserted to DNA as fluorescence probe to distinguish DNA [26–29]. Electrogenerated chemiluminescence substances, e.g., $[Ru(bpy)_2PVP_{10}]^{2+}$ and $Ru(phen)_3Cl_2 \cdot H_2O$, were also used to recognize DNA damage [30–32]. These methods could shorten the detection time effectively and improve the detection sensitivity.

Thiazole orange (TO), which possesses unique fluorescence property, attracted intense interest of researchers. It was reported that the fluorescence quantum yield increased 3000 times upon inserting to the double helix structure of DNA compared to the direct binding with single-stranded DNA (ssDNA) [33,34]. Exonuclease III (Exo III) could degrade blunt or recessed 3′-terminus in

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dsDNA to produce 5'-mononucleotide, while the degradation of Exo III to dsDNA with overhanging 3'-terminus was negligible. Combined with the characteristics of TO and Exo III, a label-free fluorescence detection method for chemicals damage to DNA was developed. As shown in Scheme 1, the TO was facilitated to insert in the completely double helix structure of dsDNA and produced a strong fluorescence signal, while the fluorescence intensity decreased significantly due to the weakened interaction between damaged DNA and TO. When the DNA was damaged and formed incision, Exo III could catalyze the stepwise removal of mononucleotides from 3'-hydroxyl terminus of dsDNA and the double helix structure of dsDNA was destroyed more seriously. As a result, the fluorescence signal of TO reduced sharply.

2. Materials and methods

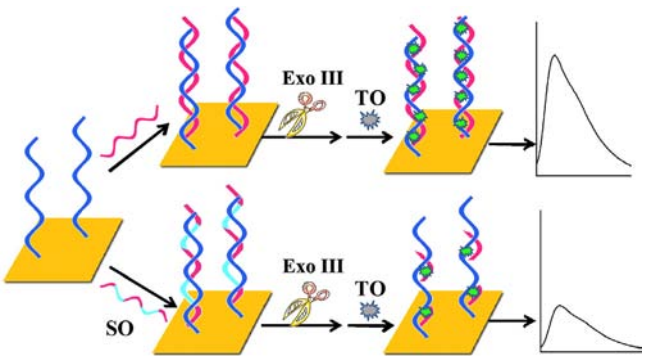
2.1. Reagents and apparatus

The oligonucleotides were synthesized and purified using high-performance liquid chromatography (HPLC) by Sangon Biological Engineering Technology & Co. Ltd. (Shanghai, China). The sequences were as follows.

capture DNA: 5'-HOOC-T GTG AAA TTG TTA TCC GCT AAAAA-3';
target DNA: 5'-AGC GGA TAA CAA TTT CAC A CAGGA-3';
single-base mismatch DNA: 5'-AGC GGA TAA GAA TTT CAC A CAGGA-3'.

The oligonucleotides were stored in phosphate buffer solution (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2.0 mM KH_2PO_4, concentrated hydrochloric acid to adjust pH 7.4). Prior to use, the oligonucleotides were centrifuged at 5000 rpm for 5 min. PBS buffer containing 1 mM Ca^{2+} and 0.5 mM Mg^{2+} was used for hybridization of DNA.

Exonuclease III (Exo III, EC 3.1.11.2, 100 U/μL), glucose oxidase (GOD, EC 1.1.3.4, 158.9 U/mg), horse radish peroxidase (HRP, EC l.11. l.7, 300 U/mg), laccase (EC 1.10.3.2, 50 U/mg), thiazole orange (TO), styrene-7,8-oxide (SO), phenylglyoxylic acids (PGA), mandelic acids (MA), sudan III and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were obtained from Sigma-Aldrich. The stock solution of 1.68 μM TO was prepared in dimethylsulfoxide and diluted with deionized water to a desired concentration when used. 200 μM SO, PGA and MA solution in ethanol, 200 μM sudan III solution in acetone and 200 μM sodium arsenite solution in deionized water were prepared freshly before each measurement. All other chemicals and solvents were of analytical grade.



Scheme 1. Schematic representation of TO intercalation to the double helix structure of DNA, DNA damaging by chemicals, DNA degrading by Exo III and biosensing mechanism.

Fluorescence spectra were recorded at room temperature with a FluoroMax-4 spectrofluorometer (Horiba, Japan).

Safety note: SO, MA, PGA and sudan III are considered as human carcinogens and somewhat volatile. Gloves should be worn. Weighing and manipulations should be done under a fume hood.

2.2. Coupling capture DNA to silicon surface

The monocrystalline silicon wafers (0.8×1.2 cm^2) were cleaned by sonication successively in ethanol, acetone and deionized water each for 10 min and dried in nitrogen. The cleaned monocrystalline silicon wafers were dipped into freshly prepared "Piranha" solution (30% H_2O_2/98% H_2SO_4 (V/V) of 3/7) for 5 h to further remove contaminant and derived –OH from the surface of monocrystalline silicon wafers (Si–OH). After drying in nitrogen, the pretreated Si–OH wafers were immersed in a 2% APTES/toluene solution for 24 h. Then they were washed with toluene and ethanol successively and dried in nitrogen to obtain Si–NH_2 wafers [35,36].

The coupling of capture DNA to silicon surface was performed by dipping of Si–NH_2 wafers into 100 nM capture DNA solution for 3 h after the capture DNA was activated by freshly prepared 50 μM EDC for 0.5 h. After rinsing with PBS and water, the Si–ssDNA was formed and stored at 4 °C for future use.

2.3. Detection of DNA damage

DNA damage was determined by the intense fluorescence signal when the intercalating of thiazole orange into the double helix structure of dsDNA occurred. In detail, the Si–ssDNA wafer was incubated with target ssDNA solution at 60 °C for 10 min to form a double helix structure of dsDNA through hybridization. After rinsing thoroughly with PBS, 10 μL 0.42 nM TO solution in dimethylsulfoxide was dropped on the Si-dsDNA and allowed to react for 1 h. After washing with deionized water and drying in air, the fluorescence spectra of the resultant TO inserted Si–dsDNA was recorded upon 480 nm excitation with a 0.1 s integration time and 5 nm integration step at room temperature. For measurement of DNA damage, target ssDNA was incubated with damaging agents, such as SO, PGA, MA, ethanol, sodium arsenite, sudan III, and methanal, for 4 h. Excessive damaging agents were removed from target ssDNA solution by using an ultrafiltration centrifuge tube (~3000 NMWL, centrifuge 14,000 rcf, 20 min). The ultraviolet induced DNA damage was performed by irradiating the target ssDNA sample with an ultraviolet source (150 W, height 5 cm) at 254 nm for 6 h.

The Si–ssDNA wafer was incubated with damaged target ssDNA solution at 60 °C for 10 min. Damaged target ssDNA was hybridized with capture ssDNA to form defective Si–dsDNA. Then the defective Si–dsDNA was dipped into 1 mL 2.5 U/mL Exo III-PBS solution at 40 °C for 30 min. After rinsing thoroughly with deionized water and drying in air, 10 μL 0.42 nM TO was dropped on Si–dsDNA and allowed to react for 1 h. The fluorescence spectra were then recorded.

3. Results and discussion

3.1. Interaction of TO with DNA

Previous studies demonstrated that TO displayed high affinity to double-stranded DNA [33,34,37]. TO itself in an aqueous solution gave a very weak fluorescence signal as shown in Fig. 1A, curve a. The interaction of TO with ssDNA (Fig. 1A, curve b) led to the fluorescence signal increase of about 193% compared

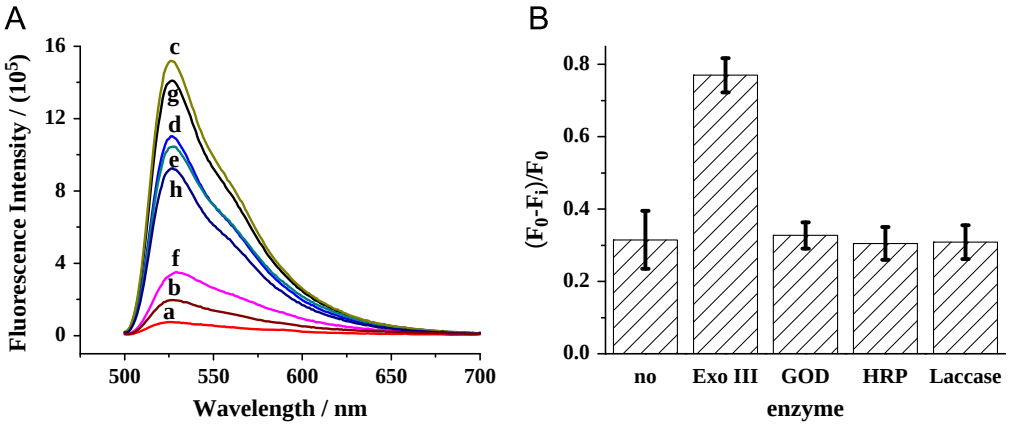
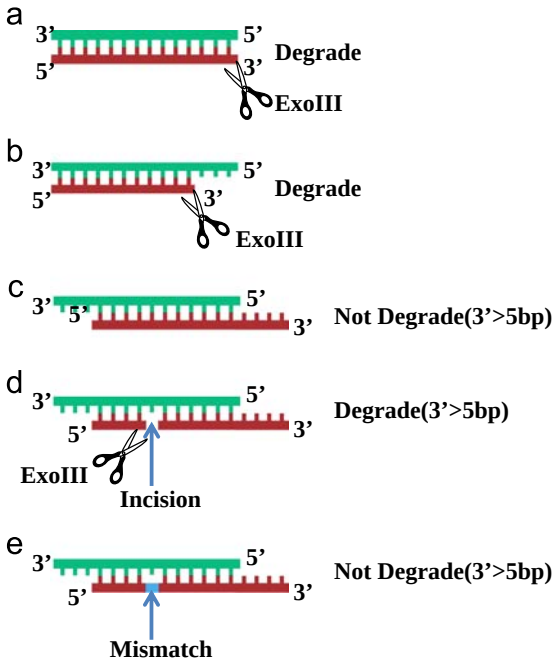


Fig. 1. (A) Fluorescence intensity of TO (a), intercalation to ssDNA (b), dsDNA (c), single-base mismatch hybridized dsDNA (d), dsDNA when target DNA was damaged with SO before (e) and after Exo III degrading (f), Exo III degrading of intact dsDNA without chemical's damaging (g) and single-base mismatch hybridized dsDNA (h). (B) Fluorescence intensity ratio $(F_0-F_i)/F_0$ of dsDNA when target DNA was damaged with SO in the presence of different enzymes.

to TO free solution. When TO bound to dsDNA, the intercalation of TO to the double helix structure of dsDNA allowed intense enhancement of fluorescence signal (Fig. 1A, curve c). The fluorescence intensity of dsDNA/TO increased to 660% of that of ssDNA/TO, while it was 2120% increase compared to TO free solution. As expected, the result is consistent with previous reports [33,34,37]; there was good binding affinity between TO and dsDNA. When target ssDNA was base mismatched, it could not hybridize completely with the capture ssDNA to form a fully double helix structure. This led to a decrease in the amount of TO bound to DNA double helix structure that resulted in a weakened fluorescence signal. For example, a single base mismatch (Fig. 1A, curve d) caused the decrease of 28% fluorescence signal compared with complete double-stranded DNA (Fig. 1A, curve c).

In addition, the damaged nucleobases led DNA to partly unwind or alter in DNA sequence. Compared with intact DNA, the target ssDNA damaged by chemicals could not hybridize completely with the capture ssDNA to form a full double helix structure; thus the weak intercalation of TO with double helix structure decreased the fluorescence signal. We defined the fluorescence intensity of completely hybridized dsDNA/TO as F_0, while the fluorescence intensity of base mismatched or chemical damaged dsDNA/TO as F_i. As shown in Fig. 1A, when target ssDNA was incubated with SO for 3 h, followed by hybridization with Si–ssDNA and exposure to TO, the fluorescence intensity decreased by 31% ($(F_0-F_i)/F_0$) of its original value without incubation with SO (Fig. 1A, curve c and e). The decrease of fluorescence intensity of damaged DNA was further enhanced by Exo III. Exo III can catalyze the stepwise removal of mononucleotides from 3'-hydroxyl terminus of dsDNA. Therefore, the activity of Exo III on ssDNA was limited, and thus, Exo III preferred substrates were blunt or recessed 3'-terminus.[38] Exo III (Scheme 2) can degrade blunt (a) or recessed (b) 3'-terminus of dsDNA to produce 5'-mononucleotide, while this degradation action is negligible for overhanging 3'-terminus of dsDNA (> 5 bp) (c). In this experiment, both target DNA and capture DNA possess overhanging 3'-terminus, which cannot be degraded by Exo III. However, if there are some incisions in the dsDNA produced by chemical reagents and formed recessed 3'-terminus, they could be degraded by Exo III from the 3'-terminus of incisions (d). DNA strand break always happened if they were damaged seriously by chemicals reagents. These fractured target ssDNA hybridized with capture ssDNA to form dsDNA with incisions, which made it more prone to be degraded by Exo III and the double helix structure was damaged seriously. On the other hand, if chemical reagents induced only base mutation but not incision, so it could not form recessed 3'-terminus, the degrade efficiency decreased sharply (e). As a result, fewer TO



Scheme 2. Schematic mechanism of DNA degrading by Exo III. Exo III can degrade blunt (a) or recessed (b) 3'-terminus of dsDNA to produce 5'-mononucleotide, and the degradation action is negligible for overhanging 3'-terminus of dsDNA (overhanging 3' > 5 bp) (c). Some incisions in the dsDNA were produced by chemical reagents and formed recessed 3'-terminus, and they could be degraded by Exo III from the 3'-terminus of incisions (d). The base mutation could not form recessed 3'-terminus, the degrade efficiency decreased sharply (e).

could be inserted in the dsDNA and its fluorescence signal reduced to 67% compared with no Exo III (Fig. 1A, curve e and f), while it was only 7% decrease of fluorescence signal for impact DNA without damage (Fig. 1A, curve c and g). Thus, the DNA damage induced by various chemical reagents could be studied based on these facts. Exo III also increases the signal in the case of base mismatch. After added Exo III, the signal of fluorescence decreased 16% comparing with no Exo III (Fig. 1A, curve d and h). Control experiment was performed by using other enzymes, including GOD, HRP and laccase, which were added to prove the signal amplification of Exo III. The results showed that only Exo III increased the signal readout, while the other three enzymes showed almost no remarkable effect (Fig. 1B). All these results demonstrated that the damaging of ssDNA could be detected by monitoring the decrease

of the fluorescence intensity, and the signal could be further enhanced by Exo III. This provided a novel approach to detect DNA damage.

3.2. Optimum conditions

The fluorescence signal of Si–dsDNA depended on TO concentration. As shown in Fig. 2A, the fluorescence intensity increased with the TO concentrations and up to its maxmum value at 0.42 nM. After that the fluorescence intensity decreased with increasing TO concentrations due to the formation of the dimer or polymers. Therefore, 0.42 nM was chosed as optimum TO concentration. The fluorescence intensity was also affected by the period for DNA hybridization. It had been found that the hybridization was basically completed in about 10 min (data not shown). Therefore, 10 min hybridization time was used in all measurements.

For the DNA damage by chemicals, the dosage and reaction time had a significant impact. The fluorescence signal decreased considerably with the increase of chemicals contained in the incubation solution at the SO concentrations of 0–50 nM, and then the fluorescence signal decrease began to flatten out at the SO concentrations of 50–100 nM (Fig. 2B). The fluorescence signal decrease was attributed to the damage of target ssDNA with SO, which could not hybridize completely with the capture ssDNA to form a fully double helix structure and decrease the insertion of TO. The concentration of chemicals was thus chosen as 50 nM. Similarly, at the SO concentration of 50 nM, the reaction time of SO with DNA displayed significant impact on the detection signals (Fig. 2C). The fluorescence signal was reduced by 32% after 4 h incubation of target DNA with SO. Therefore 4 h was chosen as the reaction time for target DNA with chemicals.

Exo III could cut the incision of dsDNA, which led to decrease in insertion of TO and weakened the fluorescence signal. The effect of Exo III quantity as well as reaction temperature and duration on the cutting of defective dsDNA was investigated. In 1 mL PBS, the fluorescence intensity of dsDNA/TO decreased considerably after addition of Exo III with the quantity in the range 0–2.5 U/mL. Further increase of Exo III quantity led to small increase of the detection signal (difference of the fluorescence intensity between dsDNA and the damaged dsDNA in the presence of TO and Exo III) in the quantity range of 2.5–7.5 U/mL. Thus 2.5 U/mL Exo III was chosen as the optimal quantity for all measurements (Fig. 3A).

The enzymic activity of Exo III was influenced by the reaction temperature. The target DNA was damaged with SO, hybridized with Si–ssDNA, the resultant Si–dsDNA was allowed to react with Exo III at various tempratures and then its fluorescence spectrum was detected using TO insertion approach. The detection signal increased from 0 to 25 °C due to the enhancement of enzymic activity with the increase of reaction temperature (Fig. 3B). With further increase of the reaction temperature up to 45 °C, the detection signal remained at a constant value. Increasing the reaction temperature from 45 °C to 70 °C led to the decrease of the detection signals, which may be ascribed to the decrease of the enzymic activity at high temperature. In our case, 40 °C was chosen for all measurements.

The duration for the cutting of defective dsDNA also affected the fluorescence signal. The fluorescence signal decrease considerably increased with reaction time in the first 30 min (Fig. 3C). After that the increasing of signal decrease slowed down and it tended to reach a constant value. Thus 30 min was used for Exo III cutting time.

3.3. Detection of target DNA and its damaging by chemicals

By using TO insertion approach, the target DNA could be detected with fluorescence spectrum. It was found that the fluorescence emission intensity increased with the increasing concentration of target DNA in the hybridization solution (Fig. 4). The plots of the difference of fluorescence intensity from Si–dsDNA and Si–ssDNA vs. the logarithm value of target concentration showed a linear calibration in the range from 100 fM to 100 nM. The detection limit for target DNA was 47 fM at signal-to-noise of 3. The detection limit was much lower than that of 12 nM by using the fluorescence resonance energy transfer method from quantum dots to graphene oxide [4], suggesting the high sensitivity of the present TO insertion approach.

Previous studies demonstrated that the metabolites of styrene in vivo, such as SO, PGA and MA, could injure DNA [39] and 150 W UV irradiation at 245 nm led to the DNA strand break [40]. Herein, the DNA damage by these chemicals and UV irradiation were investigated correspondingly. The results are summarized in Table 1. All the chemicals showed damaging effects on DNA. Ethanol and methanal could cause cross-link of DNA and formed DNA adducts. The cross-link of DNA could destroy the double helix structure of DNA and led to the decrease of amount of TO bound to DNA double helix structure, resulting in weakened fluorescence signal. However, it did not produce incision and the degrading property of ExIII was not reflected. The result demonstrated that this strategy could be applied for detection of the damage in DNA cross-linking; it did not need Exo III and had high sensitivity.150 W UV irradiation at 245 nm showed no serious injury for DNA so that only a slight increase of signals was observed in the presence of Exo III. The chemicals SO, PGA, MA, sodium arsenite and sudan III had almost the same damaging effect. However, obvious signal amplification by Exo III was observed after DNA damage by SO and

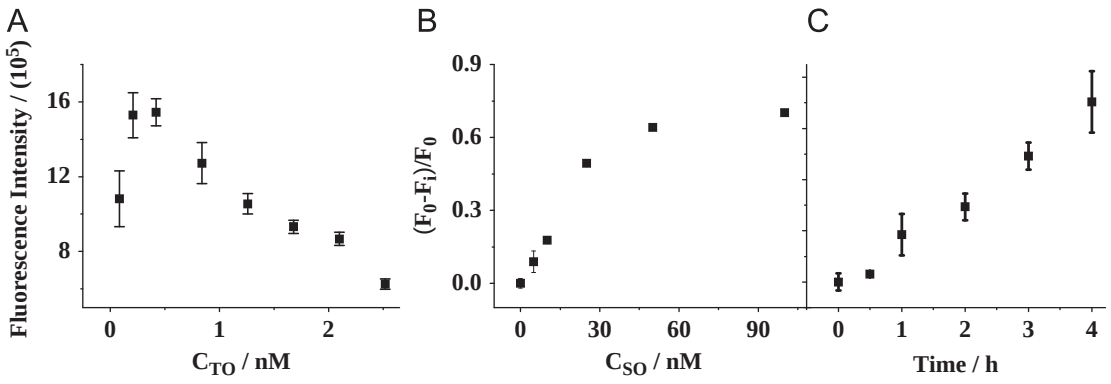


Fig. 2. (A) Plots of fluorescence intensity vs. TO concentration. (B) Plots of fluorescence intensity ratio $(F_0-F_i)/F_0$ vs. concentration of SO. (C) Plots of fluorescence intensity ratio $(F_0-F_i)/F_0$ vs. SO damaging time. In all cases, the Si–ssDNA was incubated with 100 nM target DNA.

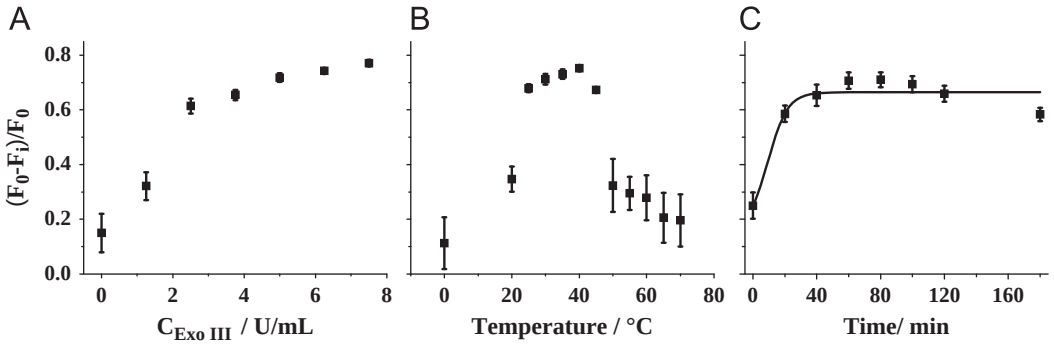


Fig. 3. Plots of fluorescence intensity ratio $(F_0-F_i)/F_0$ vs. (A) amount of Exo III, (B) reaction temperature and (C) reaction time of Si–dsDNA with Exo III.

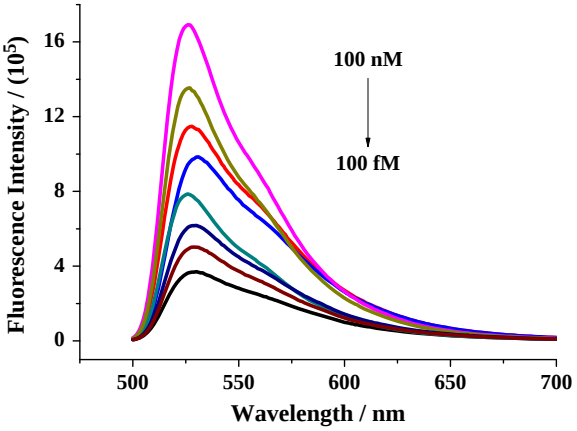


Fig. 4. Fluorescence spectra of TO intercalation to Si-ssDNA incubation with 100 nM, 10 nM, 1 nM, 100 pM, 10 pM, 1 pM, and 100 fM target DNA.

Table 1
A variety of damaging effects on DNA by different chemicals and the signal amplification of Exo III.

No	Chemical	$(F_0-F_i)/F_0$			
		Without Exo III		With Exo III	
		Mean value	Standard deviation	Mean value	Standard deviation
1	Not added	0	0.0027	0.07	0.0081
2	SO	0.2839	0.0474	0.8357	0.0045
3	PGA	0.2383	0.0302	0.4070	0.0270
4	MA	0.2192	0.0080	0.3296	0.0183
5	Ethanol	0.5010	0.0372	0.7734	0.0188
6	Sodium arsenite	0.3065	0.0337	0.5387	0.0283
7	Sudan III	0.3581	0.0281	0.8740	0.0131
8	Methanal	0.8889	0.0069	0.9284	0.0042
9	150 W UV, 6 h	0.6809	0.0068	0.8319	0.0192

sudan III, while only a little increase of the detected signals after addition of Exo III to the DNA damaged with PGA, MA and sodium arsenite was obtained. Exo III could incise the dsDNA and amplify the signal only for the specific damages of DNA as shown in Scheme 2. This indicated that the difference in the signal amplification was possibly due to the different damaging effects or damaging mechanisms with various chemicals. Therefore, the present strategy could be developed to detect DNA damage, to classify the damaging mechanism with chemicals and to estimate the toxic effect of chemicals.

4. Conclusion

In this paper, we developed a novel strategy for monitoring DNA damage by chemicals. The insertion of TO in dsDNA is closely related to the double helix structure of dsDNA. Chemicals, e.g., SO, PGA, MA, ethanol, sodium arsenite, sudan III, and methanal, could damage the target DNA. When target DNA was damaged by various chemicals and hybridized with capture ssDNA to form an incomplete double helix structure of dsDNA, the insertion of TO in dsDNA decreased and the fluorescence signal was thus decreased. Exo III degraded the dsDNA with incisions when the double helix structure was damaged seriously, and it enhanced detection signal considerably. This suggested that the present strategy could be developed to detect DNA damage, to classify the damaging mechanism with chemicals and to estimate the toxic effect of chemicals.

Acknowledgments

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