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Direct Visualization of Enzymatic Cleavage and Oxidative Damage by Hydroxyl Radicals of Single-Stranded DNA with a Cationic Polythiophene Derivative

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Abstract: A new method has been developed for the label-free, convenient, and real-time monitoring of the cleavage of single-stranded DNA by single-strand-specific S1 nuclease and hydroxyl radical based on cationic water-soluble poly[3-(3′-*N*,*N*,*N*-triethylamino-1′-propyloxy)-4-methyl-2,5-thiophene hydrochloride] (PMNT). The PMNT can form an interpolyelectrolyte complex with ssDNA (duplex) through electrostatic interactions, in which PMNT takes a highly conjugated and planar conformation, and thus PMNT exhibits a relatively red-shifted absorption wavelength. When ssDNA is hydrolyzed by S1 nuclease or hydroxyl radical into small fragments, the PMNT/ssDNA duplex cannot form. In this case, the PMNT remains in random-coil conformation and exhibits a relatively short absorption wavelength. The nuclease digestion or oxidative damage by hydroxyl radical of DNA can be monitored by absorption spectra or just visualized by the "naked-eye" in view of the observed PMNT color changes in aqueous solutions. This assay is simple and rapid, and there is no need to label DNA substrates. The most important characteristic of the assay is direct visualization of the DNA cleavage by the "naked-eye", which makes it more convenient than other methods that rely on instrumentation. The assay also provides a promising application in drug screening based on the inhibition of oxidative damage of DNA.

Introduction

The cleavage of DNA by nucleases such as restriction nucleases and nonspecific nucleases has been shown to be involved in many important biological processes, such as DNA replication, recombination, and repair.[1−3] So far, only a few techniques for the nuclease assay are performed, such as gel electrophoresis, high performance liquid chromatography (HPLC), sedimentation, and enzyme-linked immunosorbent assay (ELISA).[4−7] These methods are time-consuming, laborious, and require substrate radiolabeling. Convenient assays based on fluorescence resonance energy transfer (FRET) have been developed; however, most of them only work under restriction endonucleases for doubled-stranded DNA substrates.[8,9] The single-strand-specific nucleases have been widely used as a tool in molecular biology and biotechnology, such as for removal of nonannealed polynucleotide tails and hairpin loops in RNA and DNA duplexes, molecular cloning, and gene analysis.[10−14] Although several intriguing strategies based on FRET[15] and electrochemistry techniques[16] have also been developed to assay the cleavage efficiency of these nucleases, they require the expensive DNA strands labeled at the terminus with dual dyes or electro-active chromophores. It still remains a challenge to find simple, rapid, and more convenient approaches to assay single-strand-specific nucleases and probe the process of ssDNA cleavage.

In recent years, oxidative damage of DNA by reactive oxygen species (ROS), such as hydroxyl, alkoxyl, and peroxyl radicals and singlet oxygen, has received much attention due to its involvment in mutagenesis, carcinogenesis, and aging.[17−20] DNA damage by hydroxyl radicals (•OH) generates characteristic mutagenic base lesions, such as 8-oxoguanine, 8-oxoadenine, thymine glycol, and 8-hydroxycytosine, and the strand breaks

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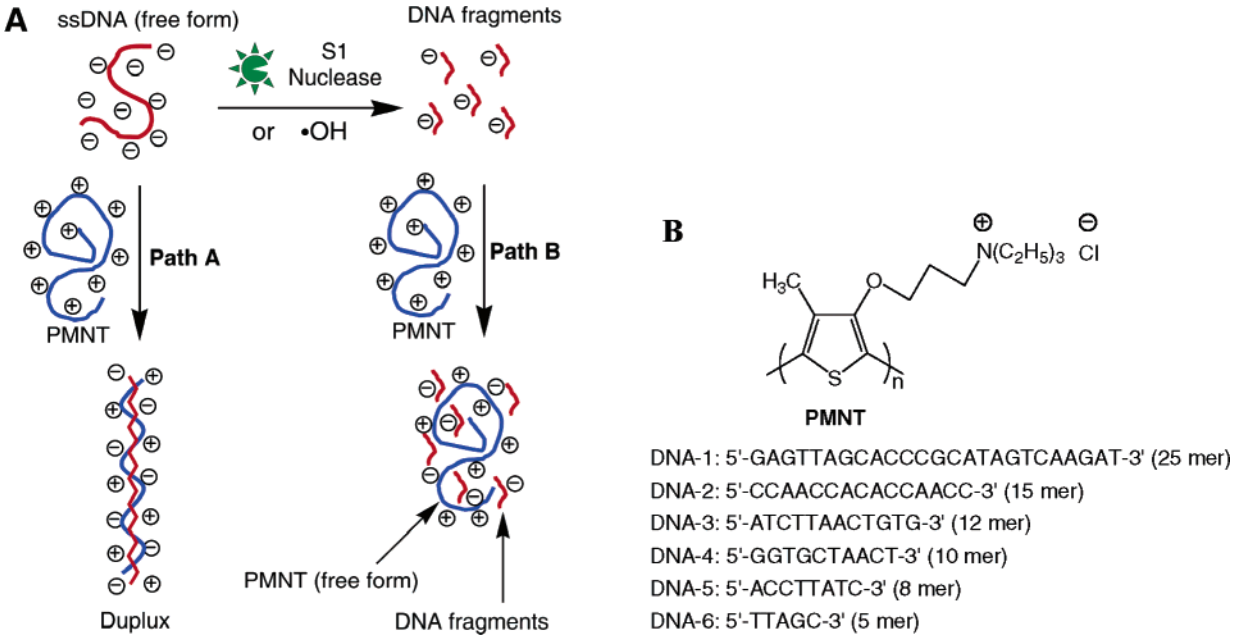
Scheme 1. (A) Schematic Representation of the Assay for Nuclease; (B) Chemical Structures of PMNT and ssDNAs with Different Base Lengths



into fragments.[21] To assay the damage of DNA by •OH, the same methods have been employed as for enzymatic cleavage, such as gel electrophoresis, high performance liquid chromatography (HPLC), and FRET technique based on doubly labeled DNA probes.[22−24] Thus, there is also a need for more sensitive and convenient methods to assay the cleavage of DNA by •OH.

Recently, conjugated polymers (CPs) provide a unique platform for chemical and biological sensors in view of their optical signal amplification effect.[25−27] We and others have utilized this property of conjugated polymers to detect DNA, RNA, protein, and metal ions.[28−37] Leclerc[30] and Inganäs groups[31] have developed new water-soluble polythiophene derivatives, which show conformational or aggregation changes upon forming interpolyelectrolyte complexes with single-stranded DNA. The induced conformational or aggregation changes can be conveniently monitored by using absorption or fluorescence spectroscopies.[38] As demonstrated here, it is possible to take advantage of the conformational change of polythiophene to design a simple, homogeneous, rapid, and label-free method to assay nucleases, oxidatively damage DNA by •OH, and probe the process of DNA cleavage. Rather than rely on the complexities of other DNA cleavage assay methods, our method provides visual detection by the "naked-eye", without requiring additional instrumentation.

Results and Discussion

Design of the Optical Probe for DNA Cleavage. Our new assay for enzymatic cleavage or oxidative damage of ssDNA is illustrated in Scheme 1. Cationic water-soluble poly[3-(3′-*N*,*N*,*N*-triethylamino-1′-propyloxy)-4-methyl-2,5-thiophene hydrochloride] (PMNT) was selected as the optical probe for DNA cleavage, which was prepared from an oxidative polymerization in chloroform with $FeCl_3$ as the oxidizing agent according to the procedure in the literature.[30a,39,40] The PMNT itself takes a

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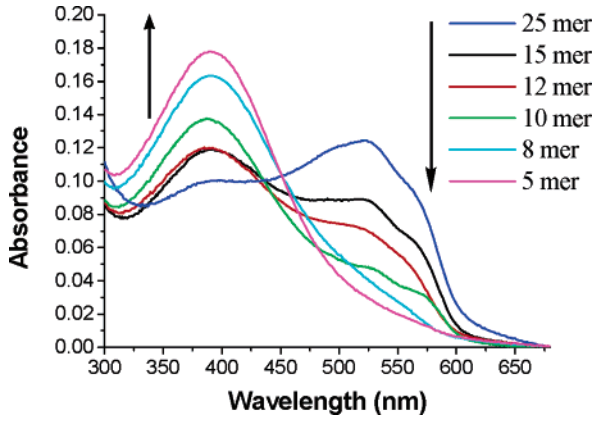

Figure 1. The absorption spectra of PMNT in the presence of ssDNAs with different base lengths. [PMNT] = 5.6×10^{-5} M, [DNAs] = 1.2×10^{-6} M. The measurements were performed in buffer solution (2 mM CH_3COONa, 15 mM NaCl, 0.1 mM $ZnSO_4$, pH = 4.6).

random-coil conformation and exhibits relatively short absorption wavelength. It can form an interpolyelectrolyte complex with ssDNA (duplex) through electrostatic interactions, in which PMNT takes a highly conjugated and planar conformation, and thus PMNT exhibits a relatively red-shifted absorption wavelength (Scheme 1, path A).[30] When ssDNA is cleaved by S1 nuclease or •OH into small fragments, the PMNT/ssDNA duplex cannot form. In this case, the PMNT remains in random-coil conformation and exhibits a relatively short absorption wavelength (Scheme 1, path B). The cleavage of DNA can be monitored by absorption spectra or just visualized by the "naked-eye" in view of the observed PMNT color changes in aqueous solutions.

To check the critical lengths of DNA fragments that can form the color changing adduct with PMNT, the absorption spectra of PMNT in the presence of ssDNAs with different base lengths (see sequences in Scheme 1B) were studied (Figure 1). The results show that the minimum length of ssDNA to form strong and well-defined complexes with PMNT contains 10 bases at the same DNA concentration ([DNAs] = 1.2×10^{-6} M, [PMNT] = 5.6×10^{-5} M). Because dsDNA cannot form the obvious color changing adduct with PMNT, this approach does not work with ds-DNA.[41]

The ssDNA-1 is used as the nuclease substrate, and S1 nuclease is chosen as the model enzyme. The S1 nuclease is a ssDNA-specific nuclease, which exhibits endo- and exolytic hydrolytic activity for the phosphodiester bonds of ssDNA or RNA and produces mono- or oligonucleotide fragments.[42]

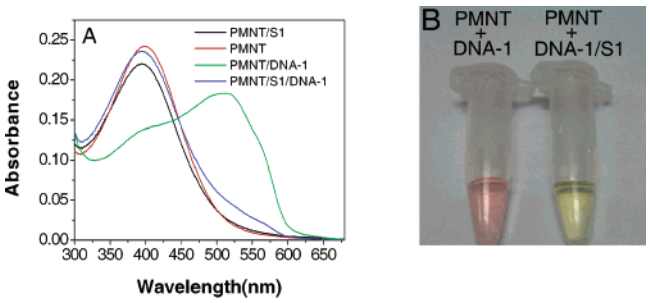

Figure 2. (A) UV−vis absorption spectra of PMNT, PMNT/S1 nuclease, and PMNT in the presence of ssDNA-1 before and after digestion with S1 nuclease. (B) Photographs of PMNT in the presence of ssDNA-1 before and after digestion with S1 nuclease. The solution involved 12.0 nmol of PMNT, 0.25 nmol of ssDNA, and 0.33 units S1. All measurements were performed in buffer solution (2 mM CH_3COONa, 15 mM NaCl, 0.1 mM $ZnSO_4$, pH = 4.6).

Assay for the DNA Cleavage by S1 Nuclease. Figure 2A compares the absorption spectra of PMNT ([PMNT] = 5.6×10^{-5} M) in the presence of DNA-1 ([DNA-1] = 1.2×10^{-6} M) before and after digestion with S1 nuclease ([S1 nuclease] = 0.33 units). The absorption maximum of PMNT in buffer solution (2 mM CH_3COONa, 15 mM NaCl, 0.1 mM $ZnSO_4$) at pH 4.6 appears at around 394 nm, which is related to its random-coil conformation. The control experiment shows that the S1 nuclease has little effect on the absorption spectra of PMNT. Upon adding DNA-1, the absorption maximum of PMNT is red-shifted to 520 nm with a dramatic color change from yellow to pink-red that is noticeable to the naked eye. In constrast, when DNA-1 is hydrolyzed by S1 nuclease at 37 °C for 30 min, the solution is still yellow upon adding PMNT, and the absorption maximum of PMNT appears at around 394 nm (Figure 2B). From the changes of solution colors and absorption spectra of PMNT in buffer, it is convenient and visible to probe the ssDNA cleavage by S1 nuclease.

Figure 3A shows the absorption spectra of PMNT as a function of the S1 nuclease digestion time. In these experiments, the S1 nuclease (0.33 units) was added to the solution of DNA-1 (1.17×10^{-6} M) in buffer (2 mM CH_3COONa, 15 mM NaCl, 0.1 mM $ZnSO_4$) at pH 4.6 at 37 °C, and PMNT was added after a specific incubating period, and then absorption spectra were measured. The absorption maximum of PMNT at 520 nm was gradually decreased and that at 394 nm was gradually increased with the incubating time from 0 to 30 min (Figure 3C). The gradual color changes of the solutions from pink-red to yellow were observed along with the DNA-1 digestion by S1 nuclease from 0 to 30 min (Figure 3B). In fact, the absorbance ratio of 394 nm to 520 nm did not increase any more after 30 min, which indicated that the digestion of DNA-1 was nearly completed. In contrast, the control experiment showed that the ratio of 394 nm to 520 nm was kept almost unchanged in the presence of S1 nuclease inhibitor, ATP.[43] It was observed that ATP could inhibit S1 nuclease rapidly and almost completely (Figure 3C). The PMNT-based assay thus makes it possible to probe the process of ssDNA digestion by its specific nuclease.

To investigate the specific property of S1 nuclease, another phosphodiesterase enzyme (E2)[44] was chosen to cleave DNA-1 under the same condition as S1 nuclease. As shown in Figure

(40) As reported for water-soluble polythiophene with a free amino acid side chain (see ref 31c) or anionic poly(3-alkoxy-4-methyl-thiophene)s (see ref 39) prepared by the same method as that of PMNT, size exclusion chromatography (SEC) or matrix-assisted laser-desorption ionization time-of-flight spectroscopy (MALDI-TOF-MS) showed that they have number-average molecular weights (M_n) in the 6000−10 000 range. These molecular weights show that the polymers contain 20−40 thiophene repeat units in the backbone. However, Leclerc group's (see ref 30a) and our attempts to determine the molecular weight of PMNT were all not successful by SEC and MALDI-TOF-MS techniques. Lukkari et al. also obtained the same result for a similar cationic polythiophene (see ref 39).

(41) The thermal property of PMNT was investigated by thermal gravimetric analysis (TGA), which showed good thermal stability up to 210 °C, and only 5% weight loss occurred below this temperature. Because the dehybridization temperature for most double-stranded oligonucleotides is below 70 °C, the PMNT would not be degraded during the thermal denature of double helixes.

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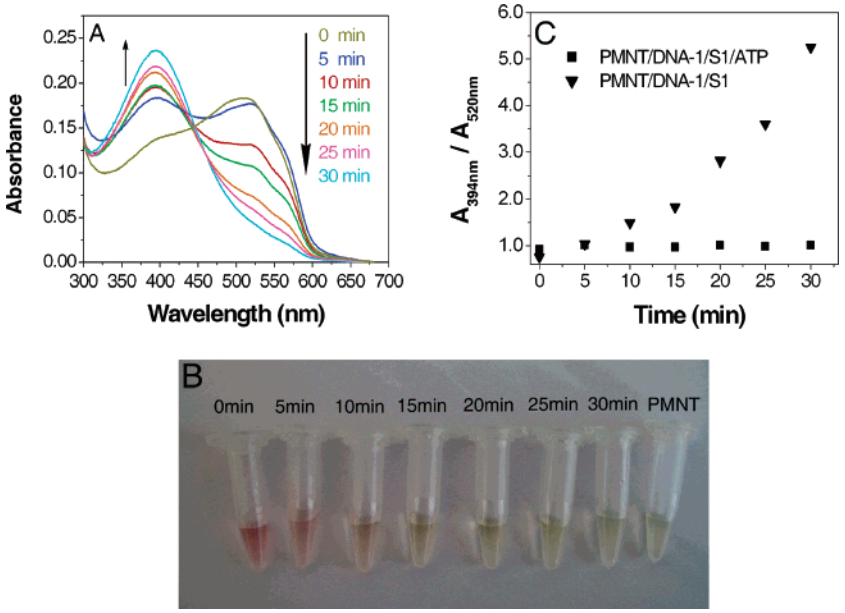

Figure 3. (A) Absorption spectra of PMNT as a function of the S1 nuclease digestion time. (B) Photographs of solutions corresponding to the absorption spectra. (C) The PMNT absorption ratio of 394 nm to 520 nm (A_{394nm}/A_{520nm}) in the absence or presence of ATP. The solution involved 12.0 nmol of PMNT, 0.25 nmol of ssDNA, 0.33 units S1, and 1.0 mM ATP. All measurements were performed in buffer solution (2 mM CH_3COONa, 15 mM NaCl, 0.1 mM $ZnSO_4$, pH = 4.6).

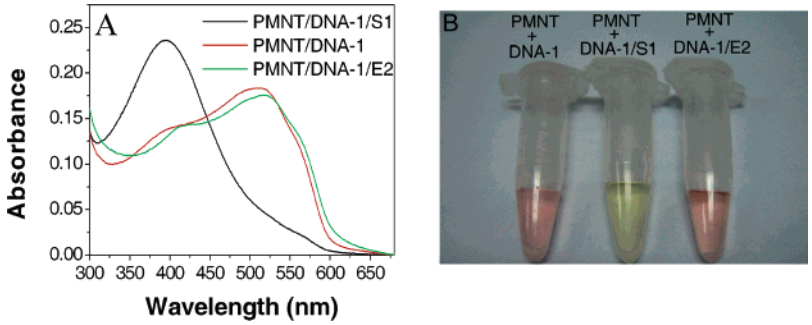

Figure 4. (A) UV−vis absorption spectra of PMNT in the presence of DNA-1 after respective digestion with S1 nuclease and E2. (B) Photographs of solutions corresponding to the absorption spectra. The solution involved 12.0 nmol of PMNT, 0.25 nmol of ssDNA, and 0.33 units S1 or 1.7×10^{-3} units E2. All measurements were performed in buffer solution (2 mM CH_3COONa, 15 mM NaCl, 0.1 mM $ZnSO_4$, pH = 4.6).

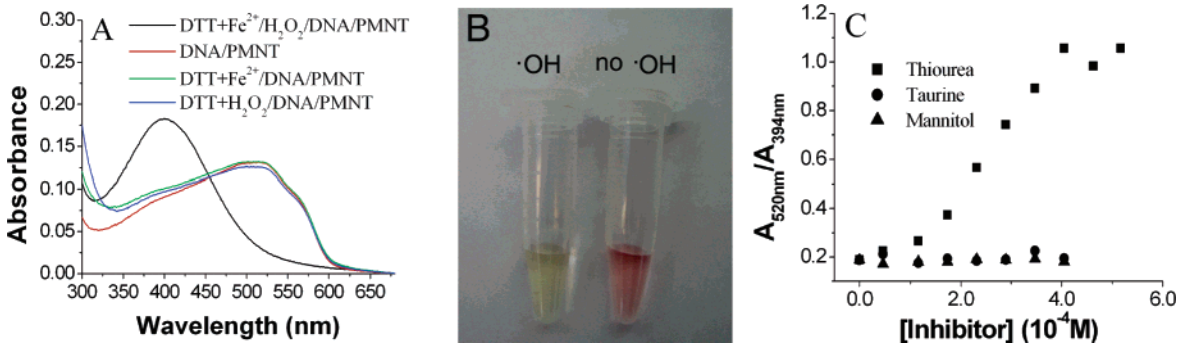

Figure 5. (A) UV−vis absorption spectra of PMNT/DNA-1 in the presence and absence of Fenton's reagent (H_2O_2 + Fe(II)). (B) Photographs of solutions corresponding to the absorption spectra. (C) The dependence of absorbance ratio of 520 nm to 394 nm in the presence of various antioxidants. [PMNT] = 5.6×10^{-5} M, [DNA-1] = 1.2×10^{-6} M, [Fe^{2+}] = 4.6×10^{-5} M, [H_2O_2] = 6.9×10^{-3} M, [antioxidants] = 0−(5.2×10^{-4}) M. All measurements were performed in water including 0.46 mM DTT.

4, the absorption spectra of PMNT/DNA-1 were not affected in the presence of E2, and the color change of the solution from pink-red to yellow was not observed. These observations indicate that S1 nuclease is a specific enzyme for ssDNA digestion, although both S1 nuclease and E2 belong to phosphodiesterase.[44] These results also validate the possibility of using the PMNT/ssDNA system as a platform for rapidly screening other ssDNA-specific nucleases using visual detection by the "naked-eye".

Assay for the DNA Cleavage by Hydroxyl Radicals. Figure 5A compares the absorption spectra of PMNT/DNA-1 ([PMNT] = 5.6×10^{-5} M, [DNA-1] = 1.2×10^{-6} M) in the presence and absence of Fenton's reagent (H_2O_2 + Fe^{2+}). Fenton's

reagent can generate •OH from Fe^{2+} and H_2O_2, and •OH can cut the DNA into different sequence fragments and even single bases.[8a] Upon adding DNA-1, the absorption maximum of PMNT is red-shifted to 520 nm with a dramatic color change from yellow to pink-red that is noticeable to the naked eye. In constrast, when DNA-1 is cut by •OH at 25 °C for 2 min, the solution is still yellow upon adding PMNT, and the absorption maximum of PMNT appears at around 394 nm (Figure 5B). The control experiments showed that H_2O_2 or Fe^{2+} itself had nearly no effect on the absorption spectra of PMNT. From the changes of solution colors and absorption spectra of PMNT in buffer, it is convenient and visible to probe the cleavage of DNA by •OH.

Some antioxidants have capabilities to scavenge •OH; therefore, they are able to inhibit the cleavage of DNA by •OH.[45] These •OH scavengers exhibit different abilities in cleaning •OH in the concentration range from 4.6×10^{-5} M to 4.0×10^{-4} M, and so the methodology described here provides a rapid and convenient method to distinguish their inhibiting ability for screening anti-oxidation drugs. Figure 5C shows the absorbance ratio of 520 nm to 394 nm in the presence of various antioxidants, taurine, mannitol, and thiourea. High inhibiting efficiency for thiourea over mannitol and taurine is observed.

Conclusions

In summary, we demonstrate practical usefulness of cationic water-soluble polythiophenes for monitoring the cleavage of single-stranded DNA by single-strand-specific nuclease and hydroxyl radical. This assay is simple and rapid, and there is no need to label DNA substrates. The most important characteristic of the assay is direct visualization of the DNA cleavage by the "naked-eye", which makes it more convenient than other methods that rely on instrumentation. The technique shows the potential to screen new ssDNA-specific nucleases and to monitor their cleavage reactions. It also provides a promising application in drug screening based on the inhibition of oxidative damage of DNA.

Experimental Section

Materials and Measurements. Cationic water-soluble poly[3-(3′-*N*,*N*,*N*-triethylamino-1′-propyloxy)-4-methyl-2,5-thiophene hydrochloride] (PMNT) was prepared according to the procedure in the literature.[30a] S1 nuclease and phosphodiesterase (E2) were obtained from Sigma. ATP and single-stranded oligonucleotides were purchased from Shanghai Sangon Biological Engineering Technology & Service Co., Ltd. The concentration of DNA was determined by measuring the absorbance at 260 nm in a 200 μL quartz cuvette. UV−vis absorption spectra were acquired on a JASCO V-550 spectrophotometer. The cleavage experiments by S1 nuclease were measured in buffer (2 mM CH_3COONa, 15 mM NaCl, 0.1 mM $ZnSO_4$, pH 4.6), and those by hydroxyl radical were measured in water. The water was purified using a Millipore filtration system.

Assay for DNA-1 Cleavage by S1 Nuclease (Figures 2 and 4). To a 0.5 mL eppendorf cup were added 3.0 μL of DNA-1 (8.4×10^{-5} M), 5.0 μL of S1 nuclease (0.066 units/μL), and 200 μL of buffer, and then the mixed solution was incubated for 30 min at 37 °C. After incubation, 12.0 μL of PMNT (1.0×10^{-3} M) was added. The UV−vis absorption spectra were measured immediately in a 200 μL quartz cuvette at room temperature. The DNA cleavage experiments for E2 enzyme are the same as those for S1 nuclease, except for addition of E2 enzyme (8.5×10^{-6} units/μL) instead of S1 nuclease. The photographs were obtained in 100 μL of buffer with the same added substances.

Assay for DNA Cleavage by S1 Nuclease as a Function of Incubating Time (Figure 3). To seven 0.5 mL eppendorf cups were added 3.0 μL of DNA-1 (8.4×10^{-5} M), 5.0 μL of S1 nuclease (0.066 units/μL), and 200 μL of buffer (samples 1−7). Samples 1−7 were incubated at 37 °C for 0, 5, 10, 15, 20, 25, and 30 min, respectively. After incubation, 12.0 μL of PMNT (1.0×10^{-3} M) was added into every cup. The UV−vis absorption spectra were measured immediately in a 200 μL quartz cuvette at room temperature. The inhibition experiments were the same as the above procedure, except for addition of 15.0 μL of ATP (15 mM) into every eppendorf cup before incubation. The photographs were obtained in 100 μL of buffer with the same added substances.

Assay for DNA Cleavage by Hydroxyl Radicals (Figure 5A). To a 0.5 mL eppendorf cup were respectively added DNA-1 (1.2×10^{-6} M), DTT (4.6×10^{-4} M), iron(II) (4.6×10^{-5} M), and hydrogen peroxide (6.9×10^{-3} M), and then the cup was incubated for 2 min at 25 °C. After incubation, 20 μL of the solution in eppendorf cup was added into 185 μL of water, and then PMNT (5.6×10^{-5} M) was added. The absorption spectra were measured in a 200 μL quartz cuvette.

Assay for DNA Cleavage under Inhibitors (Figure 5C). Taurine, mannitol, and thiourea were added into three 0.5 mL eppendorf cups, respectively; next, DTT (4.6×10^{-4} M), iron(II) (4.6×10^{-5} M), and hydrogen peroxide (6.9×10^{-3} M) were added into every cup, respectively (samples 1−3). After 2 min, DNA-1 (1.2×10^{-6}M) was added, and the cups were incubated for 2 min at 25 °C. After incubation, 24 μL of the solution in eppendorf cup was taken out and added into 180 μL of water, and then PMNT (5.6×10^{-5} M) was added. The absorption spectra were measured in 200 μL quartz cuvettes.

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