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# Effect of pH on the Iron Autoxidation Induced DNA Cleavage

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Fenton reaction and iron autoxidation have been debated for the major process in ROS mediated DNA cleavage. We compared both processes on iron oxidation, DNA cleavage, and cyclic voltammetric experiment at different pHs. Both oxidation reactions were preferred at basic pH condition, unlike DNA cleavage. This indicates that iron oxidation and the following steps probably occur separately. The ROS generated from autoxidation seems to be superoxide radical since sod exerted the best inhibition on DNA cleavage when $H_2O_2$ was absent. In comparison of cyclic voltammograms of $Fe^{2+}$ in NaCl solution and phosphate buffer, DNA addition to phosphate buffer induced significant change in the redox cycle of iron, indicating that iron may bind DNA as a complex with phosphate. Different pulse voltammogram in the presence of ctDNA suggest that iron ions are recyclable at acidic pH, whereas they may form an electrically stable complex with DNA at high pH condition.

**Key Words :** Iron autoxidation, DNA cleavage, Fenton reaction, Reactive oxygen species, Hydroxyl radical

## Introduction

Oxidative DNA damage may be an important process in mutagenesis, carcinogenesis, and aging process. Reactive oxygen species (ROS) for oxidative DNA damage include singlet oxygen, hydroxyl radical, superoxide radical, and hydrogen peroxide. Reduced iron ions have been known to produce such ROS upon their oxidation process. It has been known that the concentration of the cellular iron ions is kept as low as 5% of the total cell iron (50-100 μM) in a quiescent conditions, although that fraction can increase dramatically under stress,[1] thus inflicting oxidative damage on various biomolecules, including proteins, DNA, lipids and tissue cells.[2,3] Two major mechanisms for iron oxidation reaction have been proposed. $Fe^{2+}$ catalyzes the conversion of less reactive $H_2O_2$ into highly reactive hydroxyl radicals as shown in Eq. (1) through the well-known "Fenton reaction".[4,5] Consequently the hydroxyl radicals generate multiple damage products from all four DNA bases.[6-9] The reactive species in this process may be either ferryl or perferryl compounds.[10-15] The autoxidation of $Fe^{2+}$ with dissolved oxygen molecules is another process that can produce ROS. The mechanism of ROS production by $Fe^{2+}$ autoxidation is still under debate. Reinke *et al.* proposed that the biologically active radical is either the ferryl or perferryl complexes upon the observation of the inconsistency of the radical products between the Fenton reaction and iron autoxidation as probed by EPR experiment.[16] Urbanski and Beresewicz also reported that ferryl ion is capable of promoting hydroxyl radical generation and the efficiency is comparable with $Fe^{2+}$-driven Fenton reaction.[17] However, there is no observed evidence for the existence of those Fe-O complexes. One of the alternative mechanisms is superoxide radical-intermediated hydroxyl radical generation. Many papers reported the involvement of superoxide radicals in not only $Fe^{2+}$-driven Fenton reaction but also iron autoxidation, which is proved by the action of superoxide dismutase (SOD) and EPR spectroscopy.[18-21] Eq. (2) shows the generation of superoxide radicals from the reaction between $Fe^{2+}$ ion and molecular oxygen.[18,22] Subsequently the superoxide radicals are dismutated to $H_2O_2$, which can be served as the precursor of hydroxyl radical in the Fenton reaction. The involvement of $H_2O_2$ is supported by the observation that the attenuation of the hydroxyl radical generation as well as DNA cleavage efficiency by the addition of catalase.[18,20,23,24]

$$H_2O_2 + Fe^{2+} \xrightarrow{H^+} Fe^{3+} + {}^{\bullet}OH + H_2O \quad (1)$$

$$Fe^{2+} + O_2 \rightleftharpoons [Fe^{2+}\text{–}O_2 \rightleftharpoons Fe^{3+}\text{–}O_2^{\bullet-}] \rightleftharpoons Fe^{3+} + O_2^{\bullet-} \quad (2)$$

$$2O_2^{\bullet-} + 2H^+ \rightarrow O_2 + H_2O_2 \quad (3)$$

There has been alternative hypothesis that the reactive species is not a free hydroxyl radical but a 'crypto hydroxyl radical', which may originate from the ·OH-Fe-chelator complex.[25,26] Saran *et al.* observed that the hydroxylation of terephthalic acid by hydroxyl radicals, which was generated by iron autoxidation, exhibited a large dependency on pH while that by gamma radiation was markedly less affected by the pH change.[27] Welch *et al.* also observed the different EPR products when comparing the Fenton reaction and iron autoxidation reaction.[24] Other studies reported that chelators and ligands affect the efficiencies of iron autoxidation and the hydroxyl radical generation.[24,28,29] However, these proposals are still unclear and under debate.

The prior studies usually focused on a specific step and did not relate these steps to overall autoxidative DNA cleavage reaction. Therefore, in this study, we investigated $Fe^{2+}$

oxidation, hydroxyl radical generation, and DNA cleavage reaction under various conditions and tried to relate the all processes to elucidate the overall mechanism of iron autoxidative DNA cleavage reaction.

## Experimental

**Materials.** The pBR322 plasmid DNA stock solution (1.0 mg/mL) containing 10 mM Tris-HCl and 1.0 mM EDTA at pH 8.0 was purchased from New England Biolabs (Massachusetts, USA). Calf-thymus DNA (hereafter referred to as ctDNA) was purchased from Worthington Biochemicals (New Jersey, USA). All DNAs were dissolved in the required buffers containing 100 mM NaCl and 1.0 mM EDTA by exhaustive stirring at 4 ºC. All DNAs were dialyzed several times at 4 ºC against 5 mM sodium phosphate buffer at required pH or 5 mM NaCl for the reactions in $H_2O$. Both the ctDNA and pBR322 super coiled DNA (hereafter referred to as scDNA) concentrations were determined spectrophotometrically using the absorption coefficient, $\varepsilon_{260nm}$ = 6700 $M^{-1}$ $cm^{-1}$. $FeSO_4$, glycerol, $H_2O_2$, SOD from *E. coli*, bovine liver catalase, and terephthalic acid (TPA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Stock solution of $FeSO_4$ was prepared in 5 mM $H_2SO_4$ to prevent oxidation. The $H_2O_2$ concentration was determined using a spectrophotometer at $\varepsilon_{230nm}$=74 $M^{-1}$ $cm^{-1}$.[30] Buffer solutions with different pHs were prepared by mixing the same concentrated acidic and basic forms until the required pH was obtained. The pH range investigated in this work was 5-9 in which DNA is stable. Although the pH range exceed the buffering range of sodium phosphate buffer (pH 6-8), the same buffer was used because the nature of buffer may affect the DNA cleavage.

**Oxidation of $Fe^{2+}$ and Hydroxyl Radical Generation Measurement.** The decrease of $Fe^{2+}$ was detected using 1,10-phenanthroline that forms a colored complex with $Fe^{2+}$. 1,10-phenanthroline was added after different incubation times of $Fe^{2+}$ in buffer solution in order to determine the remained relative amount of ferrous ions. Absorption intensities of $Fe^{2+}$-phenanthroline complex were measured at 512 nm using 1 cm quartz cell. All measurements were recorded on a Cary 100 spectrophotometer (Victoria, Australia). Hydroxylation of non-fluorescent TPA yields 2-hydroxyterephthalate which emits strong fluorescence around 430 nm when excited at 323 nm. TPA was dissolved in the required buffer to make a 10 mM stock solution and was diluted to a final concentration of 400 mM. All spectral measurements were performed after completion of the reaction at 37 ºC. A Jasco FP-777 fluorimeter was used for all measurements with 5/5 nm of excitation and emission slit widths.

**Electrochemical Measurements.** Voltammetry was collected using a CH instrument potentiostat (CHI630C) with a single-compartment voltammetric cell equipped with an indium tin oxide (ITO) working electrode (area = 0.32 $cm^2$), a Pt wire counter electrode, and an Ag/AgCl reference electrode. In a typical experiment of cyclic voltammetry, a sample containing 0.1 mM $Fe^{2+}$ with or without 51 μM calf-thymus DNA dissolved in buffered aqueous solution containing 50 mM Na/phosphate buffer (at various pH) was scanned at 100 mV/s at room temperature. Cyclic voltammograms of the samples were collected at different time interval when needed. Differential pulse voltammograms (DPV) were collected using the same apparatus and conditions described for cyclic voltammetry. The pulse height was 50 mV, scan rate = 3 mV/s, interval time 0.2 s, modulation time = 0.05 s, step potential = 6 mV, and quiet time = 2 s.

**DNA Cleavage.** The reaction mixture (12 μL) consisted of 200 ng of scDNA and 0.1 mM $FeSO_4$, unless otherwise indicated. Since preparation order is crucial, the metal ion was always added last. After incubation at the required temperature, a 4 μL solution comprised of 50% glycerol (v/v), 40 mM EDTA, 0.025% bromophenol blue and 0.025% Xylene cyanol FF was added to stop the reaction. The reaction mixtures were then subjected to 1% agarose gel electrophoresis. The samples were placed in a horizontal slab gel apparatus in TAE buffer (40 mM Tris, 20 mM sodium acetate and 2 mM EDTA) set at 20 V for 12 hours. The gel was stained with ethidium bromide for 15 minutes followed by destaining in TAE buffer for 2-3 hours. Finally, the gels were photographed using a UV transilluminator.

## Results

**pH Dependent $Fe^{2+}$ Oxidation: The Fenton *vs.* Autoxidation Mechanism.** $Fe^{2+}$ ion has been known to be stable at acidic pH, which can be readily oxidized to form a complex with hydroxide. Welch *et al.* measured the autoxidation rate of $Fe^{2+}$ ion using ferrozine, $Fe^{2+}$ ion specific chelator, in phosphate buffer at two different pHs, 6.5 and 7.0.[24] The autoxidation rate is faster at lower pH, although the consumption of $Fe^{2+}$ ion was rapid at both pHs. Similar measurements were performed in this work. The conditions for the $Fe^{2+}$ oxidation rate were slightly different being pH 5.4, 7.0, and 9.2 in the absence and presence of $H_2O_2$. Oxidation rates were recorded by decrease in absorbance at 512 nm due to the formation of a complex with 1,10-phenanthroline (Figures 1 and 2). In the absence of phosphate buffer and $H_2O_2$, the oxidation of $Fe^{2+}$ did not occur at any pHs as it is shown in Figure 1(a) (no further absorption change detected up to 2 hours). The presence of phosphate ion stimulated the oxidation of $Fe^{2+}$ in a pH dependent manner (Figure 1(b)). When the reaction was performed in the 5 mM buffer solution, a rapid pH-dependent chelation of 1,10-phenanthroline was observed even in the absence of $H_2O_2$: at pH 5.4, no evidence for the $Fe^{2+}$oxidation was observed, while at pH 9.1, oxidation was so fast that the reaction completed in about 2 minutes. In the presence of phosphate ions but absence of $H_2O_2$, the mechanism of $Fe^{2+}$ oxidation has been believed to be an autoxidation. Contrarily, the presence of $H_2O_2$ induces the $Fe^{2+}$ oxidation even in the absence of phosphate ions (Figure 2(a)). This Fenton oxidation seems to be independent of pH in the absence of phosphate ion, and are generally fast compared to that

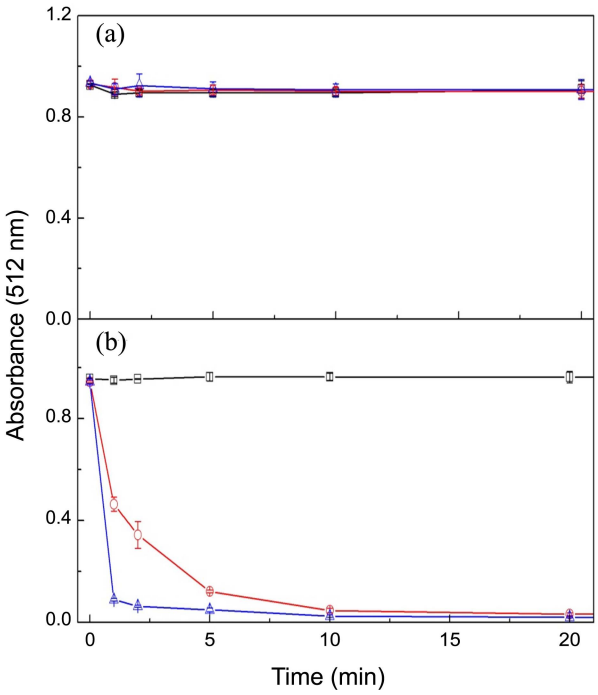


**Figure 1.** Oxidation of $Fe^{2+}$ in 5 mM NaCl aqueous solution in the absence (panel (a)) and presence of $H_2O_2$ (panel (b)) detected by changes in an absorbance at 512 nm. The symbols denote different pHs. Black square: pH 5.4; red circle: pH 7.0; blue triangle pH 9.1. Reaction was initiated by addition of $FeSO_4$ and stopped by addition of 1,10-phenanthroline. [$FeSO_4$] = 0.1 mM, [phenanthroline] = 0.5 mM, and [$H_2O_2$] = 1.0 mM. Error bars denotes the standard deviation of the 3 measurements.

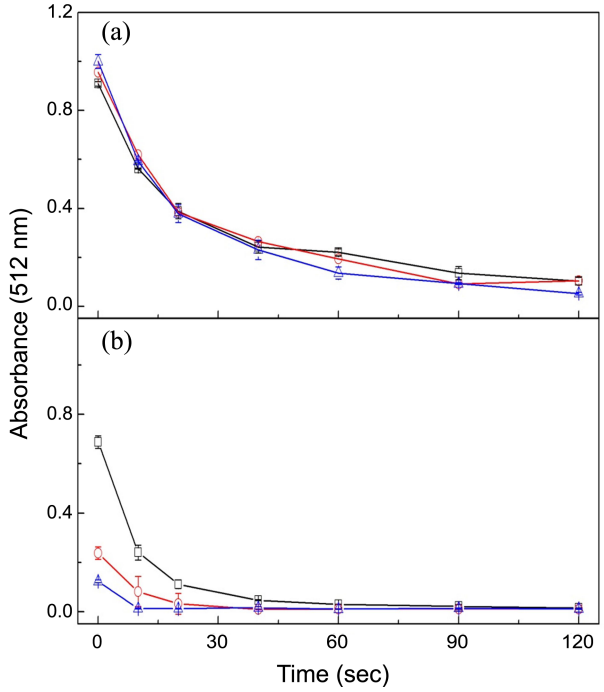


**Figure 2.** Oxidation of $Fe^{2+}$ in 5 mM phosphate buffer in the absence (panel (a))and presence of $H_2O_2$ (panel (b)) detected by changes in an absorbance at 512 nm at various pHs. Symbol assignments and the concentrations are the same as in Figure 1.

occurred by autoxidation. As it was observed in the autoxidation case, the presence of phosphate ion also stimulated Fenton-type oxidation (Figure 2(b)) and the pH dependence. In the latter case, the absorbance already decreased at the time of $Fe^{2+}$ addition, and what was recorded is probably the tail of the reaction. In both autoxidation (Figure 1(b)) and Fenton-oxidation (Figure 2(b)) cases, the reactions appeared to be strongly pH-dependent. In summary, phosphate ion was required for autoxidation of $Fe^{2+}$, and the reaction rate was enhanced at higher pH. In the Fenton reaction, the presence of phosphate ion is not a necessary condition but it also enhances the reaction rate in a pH-dependent manner.

**Electrochemical Behaviors of Iron Ions.** It is clear that the oxidation of $Fe^{2+}$ ions is required to initiate the DNA cleavage reaction both through the Fenton mechanism and by the autoxidative reaction. It has been reported that $Fe^{2+}$ oxidation is greatly favored at high pH, which is in agreement with the result presented in Figures 1 and 2. A cyclic voltammetric experiment was carried out at various pHs in the absence and presence of phosphate and DNA in order to determine the effects of pH, buffer and DNA on the oxidation of $Fe^{2+}$ ions, and the results are presented in Figure 3. In 5 mM NaCl solution (but in the absence of phosphate), the voltammogram was not significantly altered in pH 5.4 and pH 7.0, while it shrunk at pH 9.1 (in comparison of curves a in each panel). In contrast, the presence of phosphate ion significantly decreased the reduction potentials

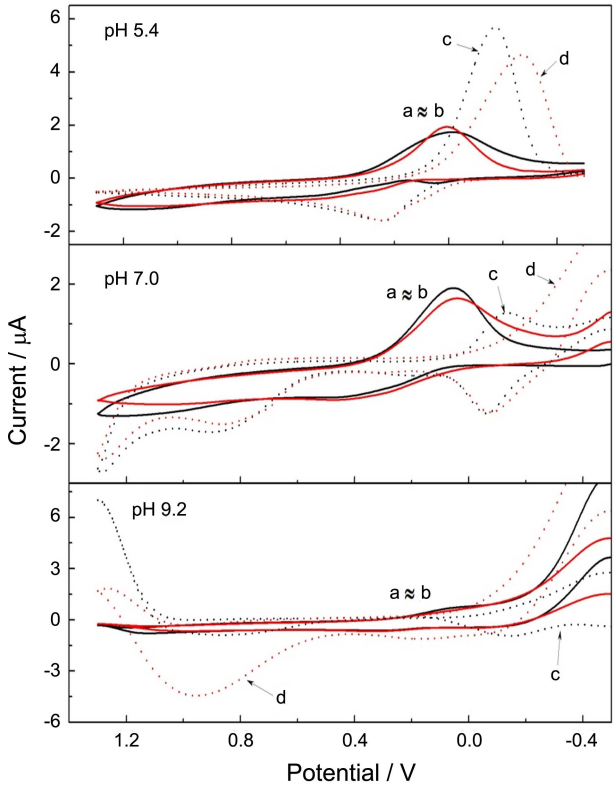


**Figure 3.** Cyclic voltammograms of $FeSO_4$ at various pHs in 5 mM NaCl (solid curves) and in 5 mM phosphate buffer (dotted curves) in the presence (black) or absence of DNA (red). The voltammograms in phosphate buffer at pH 7.0 and pH 9.1 were multiplied by the factor of 25 for easier comparison. [$FeSO_4$] = 0.1 mM, [DNA] = 0.051 mM.

from −0.014 V to −0.171 V at pH 5.4 (curves a and c, in panel pH 5.4). The current at this pH was also increased by more than two fold. This indicates that reduction became much difficult and the relative number of $Fe^{3+}$ ions increased in a large extent. At pH 7.0, the reduction potential also shifted to negative value from 0.054 to −0.136 V by the presence of phosphate ion, whereas the current decreased about 25 times (curves a and c, in panel pH 7.0), indicating that $Fe^{3+}$ may form electrically stable complex with phosphate ions at this pH. At pH 9.1, the redox cycle was largely disturbed, and an extreme decrease in the current was observed. In an aqueous environment, appearance of voltammogram was not largely altered by the presence of DNA (in comparison of curves a and b in each panel). On the other hand, the presence of DNA affects both the reduction potential and the current in the phosphate buffer solution (curves d in each panel): reduction potential as well as the reduction current was notably deceased, suggesting the interaction of the Fe-phosphate complex with DNA.

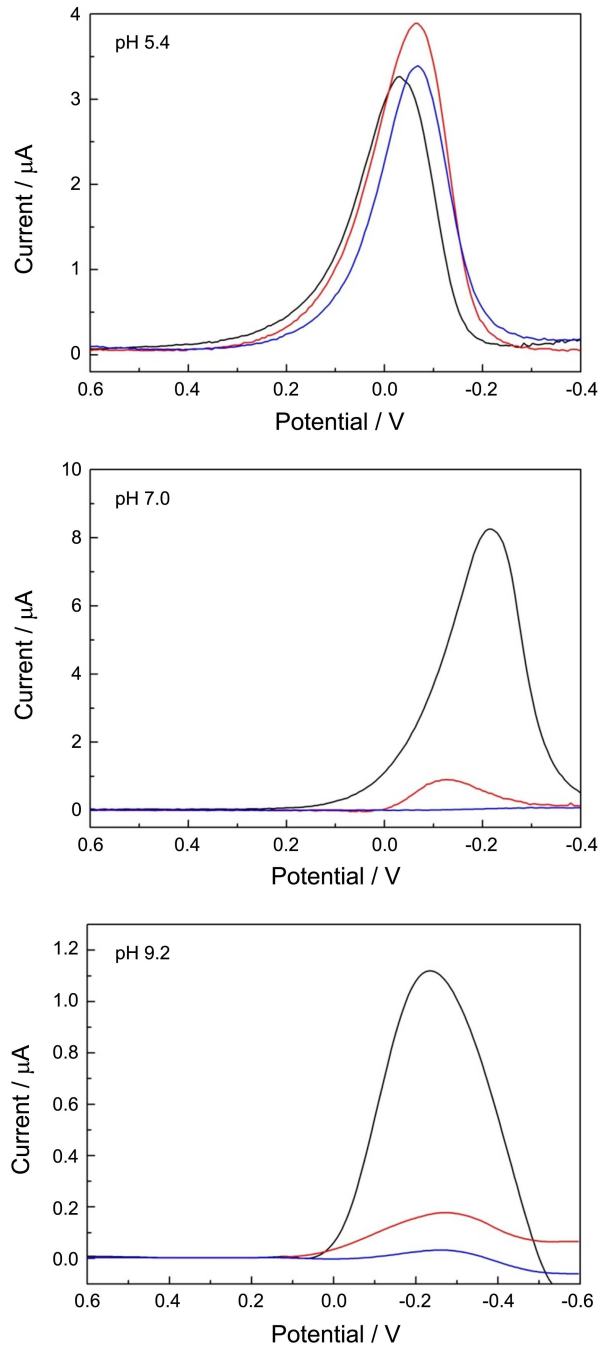


**Figure 4.** Different pulse voltammograms in phosphate buffer at various pHs in the absence of DNA (black), and immediately (red) and 10 minutes (blue) after the addition of DNA. Concentrations were same as in Figure 3.

Figure 4 depicts different pulse voltammogram of iron ions in phosphate buffer in the absence of DNA, at the time of mixing and 10 minutes after addition of DNA. $Fe^{3+}$ was formed at all pHs in the absence of DNA. However, the reduction was most preferred at pH 5.4: the reduction potential shift to the positive direction as pH decrease. They were −0.032, −0.218, and −0.236 V for pH 5.4, pH 7.0, and pH 9.1, respectively. The presence of DNA affects little to different pulse voltammogram at pH 5, even 10 minutes after DNA addition. However, a large decrease of reduction current at higher pHs were observed. 10 minutes after mixing of DNA, the current was almost zero at both pH 7.0 and 9.1. This observation indicated that the relative number of $Fe^{3+}$ ions decreased rapidly after DNA mixing at higher pHs.

**pH Dependent Hydroxyl Radical Generation.** Hydroxyl radicals generated from Fenton reaction are thought to be the reactive speciesthat is responsible for the damage of biomolecules. Formation of the hydroxyl radical can be detected by observing the fluorescence of terephthalic acid (TPA), which becomes highly fluorescent 2-hydroxy-terephthate (TPA-OH) upon binding with hydroxyl radicals. TPA-OH emits strong fluorescence around 430 nm when excited at 323 nm. Changes in the fluorescence intensity in 5 mM NaCl solution and 5 mM phosphate buffer are shown in Figure 5(a) and (b), respectively. In the absence of $H_2O_2$, no fluorescence was found in an aqueous solution (Figure 4(a)). Upon increasing the $H_2O_2$ concentration, the fluorescence intensity increased gradually, suggesting the generation of the hydroxyl radical, and thus suggesting the gradual increase in $Fe^{2+}$ oxidation. Although the extent of the fluorescence increases was significant at all pHs, it is most pronounced at pH 5.4 and least at pH 9.1. In the phosphate buffer, hydroxyl radical generation upon increasing $H_2O_2$ concentration was also noticed (Figure 5(b)). However, the manner of increasing was in contrast with that in the absence of phosphate ions. The fluorescence intensities at their maxima were lower, and the pH dependent was more significant in the presence of phosphate. At pH 9.1, the generation of the hydroxyl radical was significantly inefficient compared to that at pH 5.4. Effect of DNA on the hydroxyl radical generation in both NaCl solution and phosphate buffer was tested (Figure 6). In the absence of ctDNA, hydroxyl radicals were readily generated in phosphate buffer, which is in contrast with that no hydroxyl radical formation was observed in the absence of phosphate ions. Strong interaction between $Fe^{2+}$ and phosphate may be the reason for this observation. Addition of ctDNA induced slight increase of hydroxyl

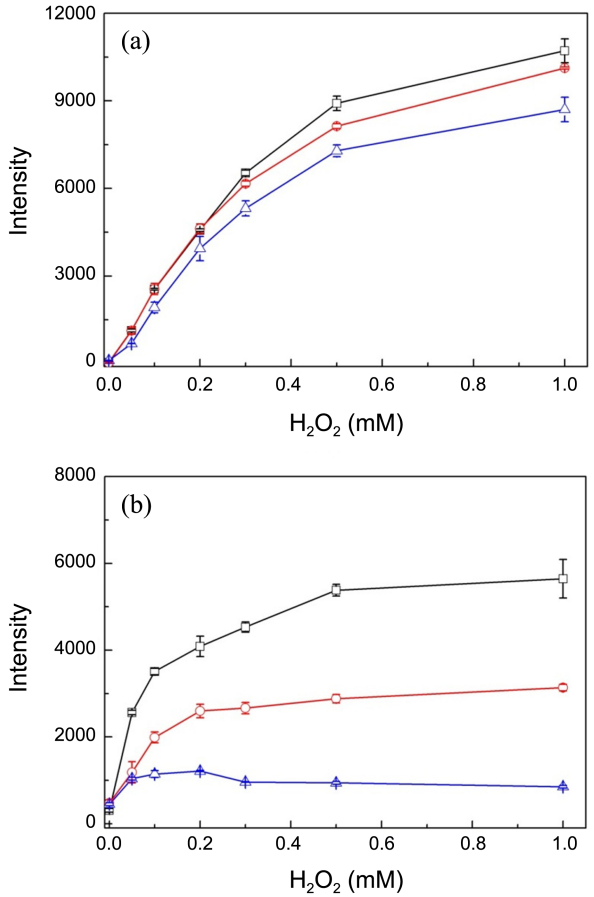


**Figure 5.** $H_2O_2$concentration dependent hydroxyl radical generation at various pHs in 5 mM NaCl (panel (a)) and 5 mM phosphate buffer panel (b)). Black square: pH 5.4; red circle: pH 7.0; and blue triangle: pH 9.1. The fluorescence of TPA-OH was detected through excitation at 323 nm and emission at 430 nm. All samples were incubated at 25 °C for six hours to allow the complete reaction. $[FeSO_4]$ = 0.1 mM.

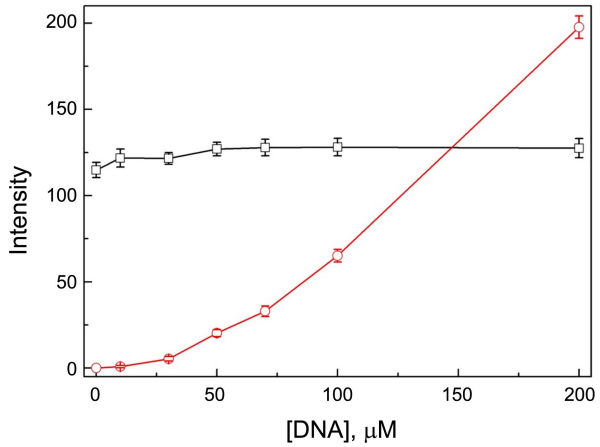


**Figure 6.** Dependence of the hydroxyl radical generation on the DNA concentrations in 5 mM NaCl (red circle) and 5 mM phosphate buffer (black square). All samples were incubated at 25 °C for six hours. DNA was dialyzed several times against 5 mM NaCl solution for the measurement in NaCl (none-buffer) solution. $[FeSO_4]$ = 0.1 mM.

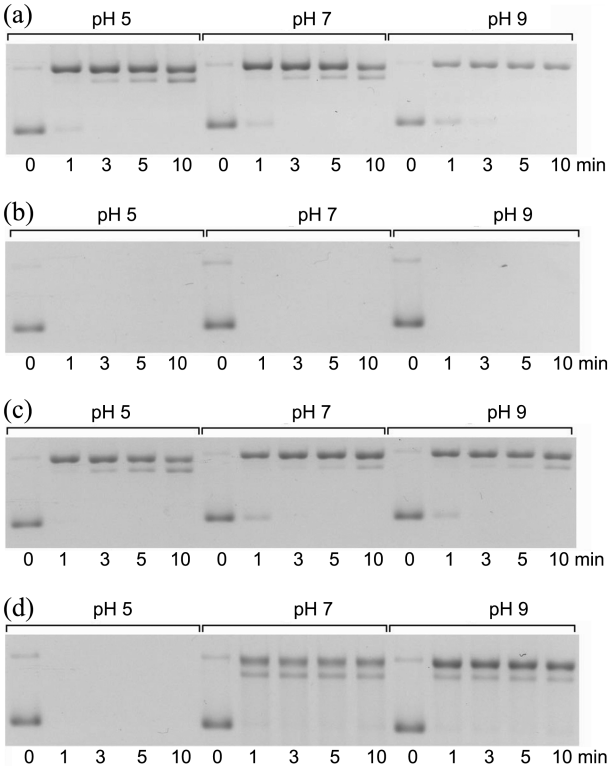


**Figure 7.** Time dependent cleavage of scDNA in 5 mM NaCl solution (panels (a) and (b)), and in 5 mM phosphate buffer (panels (c) and (d)) at various pHs. Panels (b) and (d) contained $H_2O_2$ while panels (a) and (c) do not. $[FeSO_4]$ = 0.1 mM, [scDNA] = 200 ng, $[H_2O_2]$ = 1.0 mM.

radical in the phosphate buffer. Contrarily, increase of the ctDNA concentration induced gradual increase of hydroxyl radical in NaCl solution, although the effect is very low at the low DNA concentrations. The amount of the generated hydroxyl radical by ctDNA in the NaCl solution surpass at the ctDNA concentration between 100 and 200 μM.

**pH Dependent Supercoiled DNA Cleavage; Autoxidation *vs.* Fenton Reaction.** Figure 7 shows the time dependent scDNA cleavage at three different pHs both in 5 mM NaCl solution and in 5 mM phosphate buffer in the absence and presence of $H_2O_2$. In the absence of $H_2O_2$, the cleavage was the fastest at pH 5.4 both in NaCl solution and in phosphate buffer (Figure 7, panels a and c). As the pH elevated, the cleavage rates became slower. Both in NaCl and phosphate solutions, the reaction rate seemed to be similar at pHs 5.4 and 7.0. At pH 9.1, linear DNA was observed in phosphate buffer but not in NaCl solution, suggesting that the ligation of the phosphate ion to $Fe^{2+}$ seems to stimulate the cleavage reaction at pH 9.1. In the presence of $H_2O_2$, the cleavage reactions were extremely stimulated. The stimulation was more pronounced in aqueous NaCl solution compared to that in phosphate buffer (in comparison of Figure 7(b) with 7(d)): all DNAs were fragmented to small pieces at all pHs in aqueous solution, while the fragmentation was still in progress at pH 7.0 and 9.1 in phosphate buffer. From these results, it is obvious that the reaction rates of both $Fe^{2+}$ autoxidative- and Fenton reaction driven DNA cleavages are

favored at acidic pH condition. In addition, phosphate may hamper the Fenton reaction-driven DNA cleavage.

**Scavengers Effect on Supercoiled DNA Cleavage Reaction.** The major reactive species responsible for the DNA cleavage induced either by the Fenton reaction or by $Fe^{2+}$ autoxidation is still under debate. The candidates proposed are $Fe^{2+}$-O complexes and hydroxyl radical, whether it is free- or crypto-forms. In order to clarify this point, the effects of various scavengers on DNA cleavage reaction under various conditions including pHs, buffer conditions, and existence of $H_2O_2$ were tested. The scavengers used in this work are ethanol, sodium azide, catalase, and SOD which are known to suppress the action of hydroxyl radicals, singlet oxygen, $H_2O_2$, and superoxide anion radical, respectively. The inhibitory efficiencies of various scavengers cannot be directly compared at different pHs since the activities of the scavengers would be different upon pH change. As it is shown in Figure 8, all scavengers inhibited the scDNA cleavage at all tested conditions. The most remarkable actions of scavengers are noticed for catalase in the presence of $H_2O_2$ and SOD in the absence of $H_2O_2$. The largest effect found for SOD in the absence of $H_2O_2$ was irrespective of the buffer condition (whether phosphate ions are present or not), and so was pHs (Figures 8(b) and 8(d)). In the presence of $H_2O_2$, catalase exhibited the best inhibition effect at lower pHs. As the pH increased, its inhibitory activity diminished: at pH 9.2, in phosphate buffer, almost no inhibitory effect was observed (Figure 8(d)). Ethanol and $NaN_3$ also displayed some inhibition effect and their actions are prominent at higher pHs in phosphate buffer (pHs 7.0 and 9.2 in Figures 8(c) and pH 9.2 in Figure 8(d)). In summary, in the absence of $H_2O_2$, SOD was the best inhibitor, suggesting the predominant species may be superoxide anion radical. In contrast, the largest inhibitory effect was observed for catalase, which suggests the hydroxyl radical is the main species responsible for Fenton-type DNA cleavage.

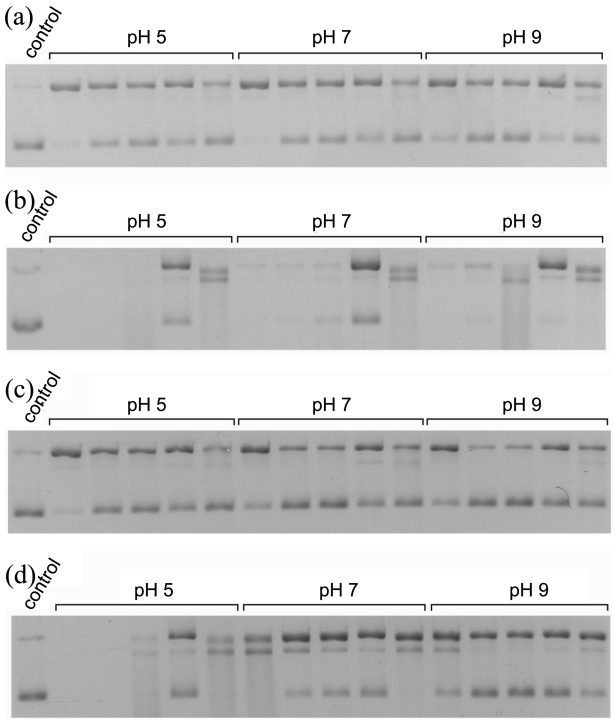


**Figure 8.** Effect of various scavengers on the cleavage of scDNA in 5 mM NaCl solution (panels (a) and (b)), and in 5 mM phosphate buffer (panels (c) and (d)) at various pHs. Panels (b) and (d) contained $H_2O_2$ while panels (a) and (c) do not. Lane 1: no scavenger, lane 2: 10 mM ethanol, lane 3: 10 mM $NaN_3$, lane 4: 10 units catalase, lane 5: 10 units SOD. [$FeSO_4$] = 0.1 mM, [scDNA] = 200 ng, [$H_2O_2$] = 0.5 mM.

## Discussion

**Low pH Dependency of DNA Cleavage.** $Fe^{2+}$ autoxidative DNA cleavage seems to proceed through several consecutive steps. First, $Fe^{2+}$ must be oxidized by the interaction with dissolved oxygen molecules to produce ROSs. It has been known that singlet oxygen and hydroxyl radical can directly attack biomolecules but superoxide anion and $H_2O_2$ cannot.[6,20,31,32] Produced ROS from $Fe^{2+}$ autoxidation may not attack DNA directly since interaction between $Fe^{2+}$ and molecular oxygen produces superoxide anion as it is shown in Eq. (2). Hence, in the second step the produced superoxide anion should be converted to another ROS form which can attack DNA (Eq. (2) and (3)) from which DNA cleavage reaction finally occurs. Several studies reported that pH markedly influences the autoxidation of $Fe^{2+}$ ions as well as the hydroxyl radical generation. Welch and Svoboda observed the same high pH dependency of $Fe^{2+}$ autoxidation in phosphate buffer at pH range 6.8-8.1.[23,24] Although the same result was observed in phosphate buffer in this study (Figure 1(c)), opposite results was reported in our previous work[33] that both hydroxyl radical generation and DNA cleavage were preferable at low pH. The latter result was reconfirmed in this work (Figures 5, 6 and 7). These pH dependencies seem to be in conflict: autoxidation of $Fe^{2+}$ is preferred at basic conditions while hydroxyl radical generation and DNA cleavage are preferred at acidic conditions. High pH dependency of $Fe^{2+}$ autoxidation can be attributed to the higher binding constant of $Fe^{2+}$ ion to $H_2PO_4^-$ (501 $M^{-1}$) than to $HPO_4^{2-}$ (3981 $M^{-1}$).[34] Cyclic voltammetric data explain the lower efficiency of $Fe^{2+}$ autoxidation at acidic environment. At pH 5.4 in phosphate buffer (Figure 3), reduction became much more difficult than in NaCl solution. On the other hand, the redox cycle seemed smoother at pH 5.4 while it was hampered at high pHs. This indicates that iron ions may form chemically stable complex with phosphate and it's hardly reduced to $Fe^{2+}$ state at high pH, whereas the redox cycle might be reversible at low pH.

Oxidation of $Fe^{2+}$ probably converts molecular oxygen to superoxide radical since SOD exhibited remarkable inhibition effect on DNA cleavage both in NaCl solution and in phosphate buffer when $H_2O_2$ is absent. In contrast, catalase exhibited the best inhibition effect when $H_2O_2$ is present. It seems reasonable that hydroxyl radical generation is preferred at acidic condition because the conversion of superoxide anion to $H_2O_2$ requires two moles of protons as it is shown in Eqs. (2) and (3). Accordingly, DNA cleavage

reaction can be efficient at acidic conditions due to the high yield of hydroxyl radicals. Moreover, time dependent DPV data suggest that redox cycle is conserved after addition of DNA at low pH: the DPV current was not largely altered by the addition of DNA (Figure 4(a)). On the other hand, iron ions probably forms stable complex with DNA and cannot be recycled at high pH (Figure 4(b) and 4(c)). Finally, it was conclusive that the produced $H_2O_2$can serve as a reactant for the consecutive Fenton reaction since the same type of ROS were detected in the scavenger experiment on DNA cleavage reaction irrespective of $H_2O_2$ addition (Figure 8).

**Phosphate Inhibits DNA Cleavage in Fenton Reaction.** It is obvious that phosphate is required for autoxidation of $Fe^{2+}$ ions (in comparison of Figures 1(a) and 2(a)). Fenton-type oxidation was also stimulated in large extent by the phosphate ions (in comparison of Figures 1(b) and 2(b)). These results are consistent with the previous studies reporting the buffers or chelators effects on the $Fe^{2+}$ oxidation.[18,19,24] Our previous work also reported that Fenton reaction-driven hydroxyl radical generation was more stimulated in 20 mM phosphate buffer than in aqueous solution (in the absence of phosphate ion),[33] and the DNA cleavage efficiency was also proportional to the phosphate concentration (data not shown). Several studies suggested that excess amount of $H_2O_2$ inhibit the action of hydroxyl radicals: the proper concentration of $H_2O_2$ was reported as about 0.05 mM from the DNA nicking experiment of phage DNA. However, the Fenton-type DNA cleavage was shown to be almost entirely quenched in the 5 mM of phosphate buffer at all tested pHs (Figure 8(d)) compared to that in NaCl solution (inhibition at pH 5.4 was confirmed at lower concentration of $Fe^{2+}$, data not shown). This decrease in the cleavage activity is probably due to the lower hydroxyl radical generation in phosphate buffer (Figure 5(b)). When $H_2O_2$ is present in the NaCl solution, the efficiency of hydroxyl radical generation by ctDNA surpassed that in phosphate buffer. The difference may be elucidated by the difference in the efficiency of $Fe^{2+}$ oxidation. Previous study reported that DNA phosphate acts as better oxidizing agent than inorganic phosphate.[23] Our data on hydroxyl radical generation presented similar result (Figure 6(c)). The observed efficiency in the generation of hydroxyl radical by the presence of ctDNA indicated that the $Fe^{2+}$ ions remained to be tethered to phosphate. The effect of phosphate and $H_2O_2$ concentration on DNA cleavage efficiency needs further study.

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