Potential-modulated DNA Cleavage by (*N*-Salicylidene-glycinato)-copper(II) Complex

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The interaction of aqua (*N*-salicylidene-glycinato)copper(II) ($\text{Cu}(\text{salgly})^{2+}$) complex with calf thymus DNA has been investigated by cyclic voltammetry. Potential-modulated DNA cleavage in the presence of $\text{Cu}(\text{salgly})^{2+}$ complex was performed at a gold electrode in a thin layer cell. DNA can be efficiently cleaved by electrochemically reducing $\text{Cu}(\text{salgly})^{2+}$ complex to $\text{Cu}(\text{salgly})^+$ complex at -0.7 V (vs. Ag/AgCl). When the solution was aerated with a small flow of O_2 during electrolysis, the extent of DNA cleavage was dramatically enhanced, and hydroxyl radical scavengers inhibited DNA cleavage. These results suggested that O_2 and hydroxyl radical were involved in potential-modulated DNA cleavage reaction. The percentage of DNA cleavage was enhanced as the working potential was shifted to more negative values and the electrolysis time was increased. It was also dependent on the ratio of $\text{Cu}(\text{salgly})^{2+}$ complex to DNA concentration. The cleaved DNA fragments were separated by high performance liquid chromatography (HPLC). The experimental results indicated that the method for potential-modulated DNA cleavage by $\text{Cu}(\text{salgly})^{2+}$ complex was simple and efficient.

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The preparation of new DNA cleavage reagents and the establishment of a new method for DNA cleavage have important biomedical and biotechnological implications, since they can potentially be used for cancer therapy or as restriction nucleases. For many years, metal complexes have been used for DNA cleavage;¹⁻⁹ in fact, bleomycin, a mononuclear Fe(III) complex has been used as an antitumor therapy, and $\text{Fe}(\text{EDTA})^{3+}$ and $\text{Cu}(\text{phen})^{2+}$ have been used extensively for DNA footprinting.¹⁰⁻¹² The mechanism of action of these complexes was a radical-based, oxidative cleavage reaction using the classical Fenton reaction.^{13,14} In the process of cleavage reaction, the reduced transition metal complexes [such as Fe(II), Cu(I), Cr(II)] react with hydrogen peroxide in a one-electron redox (Fenton) reaction, producing hydroxyl radicals and hydroxide anions:

However, the reduction of the metal complex required some strong reductive agents to be added into the reaction system, such as thiols,^{15,16} ascorbate¹⁷ or NADH¹⁸. Karlin and coworkers¹⁹ have reported the use of multinuclear complexes for cleavage of DNA without any addition of extra reductive agents. But these complexes were not stable in aqueous solutions. For this reason, the studies of the reactivity of these complexes were performed in a nonaqueous solvent such as acetonitrile.^{20,21} If these complexes were to be used as antitumor therapies, it was imperative to find that these complexes were soluble and to establish a new method where DNA cleavage can be performed under physiological conditions.

The electrochemical method can modulate redox reaction under physiological condition. It can perform the reduced reaction on the electrode without addition of extra reductive

agents. Rodriguez *et al.*²² studied nicking of supercoiled (sc) DNA by oxygen reduction at an electrode in the presence of transition metals, observing potential-modulated DNA cleavage during electrolysis of aerobic DNA solution. Johnston *et al.*²³ studied *trans*-dioxorhenium-mediated electrocatalytic oxidation of DNA at indium and tin-oxide electrodes. Fojta *et al.*²⁴ studied DNA cleavage by reactive oxygen species generated by a modulated electrochemically reaction of copper ions on a hanging mercury drop electrode in the presence of hydrogen peroxide and/or oxygen. According to height of peak 3, which is the redox signal of base in the DNA molecules on the electrode ($E_p = -1.43$ V) increasing, the cleavage of DNA was ascertained. With those values in mind, we chose potential-modulated DNA cleavage in the presence of $\text{Cu}(\text{salgly})^{2+}$ complex. This method can cleave DNA efficiently without addition of extra reductive agents. And $\text{Cu}(\text{salgly})^{2+}$ complex was chosen because it possesses good aqueous soluble properties and the extent of DNA cleavage is high in the presence of it. The fragments of DNA cleavage were separated and detected by high performance liquid chromatography. The influences of several experimental conditions including working potential, electrolysis time and the concentration of $\text{Cu}(\text{salgly})^{2+}$ complex on DNA cleavage were studied in detail. The experimental results revealed that the proposed method for DNA cleavage is highly efficient.

Experimental

Apparatus and reagents

CHI660A electrochemical analyzer (Shanghai Chenhua Apparatus Corporation, China) and Model 363 potentiostat/galvanostat (EG & G Princeton Applied Research) were used. The gold disk electrodes (diameter 1 mm and 5 mm) were used as working electrodes. A platinum auxiliary electrode and Ag/AgCl reference electrode were used for the measurement. A L-7100 high performance liquid chromatography

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Fig. 1 Cyclic voltammograms of 2.5×10^{-4} mol/L Cu(salgly)^{2+} complex in the absence of (a) and presence of 3.0×10^{-4} mol/L DNA (b). Supporting electrolyte, 50 mM phosphate buffer (pH 6.5); scan rate, 100 mV/s.

(Hitachi High-Technologies Corporation, Tokyo Japan) was equipped with a L-7420 UV-Vis spectrophotometric detector. The Shim-pack Diol-300 chromatographic column (7.9 mm ϕ $\times$ 25 cm) packed with 5 μm porous silica microspheres (pore diameter 300 $\text{\AA}$) which have a diol-bonded phase, was employed to separate the cleaved DNA fragments.

All chemicals were of reagent grade and were used without further purification. Calf thymus DNA was purchased from Sigma. DNA stock solution was prepared in double-distilled deionized water. It was stored at 4°C and used for not more than 5 days. The purity of DNA was checked by monitoring the absorption spectrum, and the ratio of the absorbance at 260 nm to 280 nm. The ratio being 1.87 indicated that the DNA was fully free of protein.²⁵ The concentration of DNA in nucleotide phosphate was determined spectrometrically at 260 nm using the absorption coefficient = 6600 $\text{M}^{-1} \text{cm}^{-1}$.²⁶ The Cu(salgly)^{2+} complex was prepared according to the literature.²⁷ Doubly distilled deionized water was used throughout. The experiments were carried out at room temperature.

Procedure

The interaction of Cu(salgly)^{2+} complex with DNA. The working electrode surfaces were freshly polished with 0.25 μm Al_2O_3 and then cleaned ultrasonically prior to each experiment. The cyclic voltammetric (CV) experiment was performed in a single compartment cell with a three-electrode configuration. The volume of the cell was 5 mL. A gold disk electrode (diameter 1 mm) was used as a working electrode. The supporting electrolyte was 50 mmol/L phosphate buffer (pH 6.5). The solutions were deoxygenated with nitrogen gas for 15 min prior to measurements.

DNA cleavage. Potential-modulated DNA cleavage was carried out on a gold disk electrode (diameter 5 mm) in a thin layer electrochemical cell. The volume of the cell was 250 μL . The solution containing 2.5×10^{-4} mol/L Cu(salgly)^{2+} , 3.0×10^{-4} mol/L DNA and 10 mmol/L phosphate buffer (pH 6.5) was added to the thin layer cell and electrolyzed at -0.7 V (vs. Ag/AgCl) for 30 min. The solution was saturated by air except for special illustrations.

Detection of DNA fragments. After the electrolysis finished, 10 μL of electrolyzed solution was taken out by a micro-injector for chromatographic analysis. The eluent was 10 mmol/L phosphate buffer (pH 6.5) and the flow rate was 1.0 mL/min. The UV-Vis spectrophotometric detector was used; its detecting wavelength was at 260 nm, which was the maximum absorption wavelength of DNA.

Fig. 2 Chromatograms of the solution containing 2.5×10^{-4} mol/L Cu(salgly)^{2+} complex, 3.0×10^{-4} mol/L DNA and 10 mM phosphate buffer (pH 6.5) before electrolysis (A) after electrolysis (B), the solution was deoxygenated with N_2 before electrolysis (C), and the solution was aerated with O_2 during electrolysis (D). Conditions: the eluent, 10 mM phosphate buffer (pH 6.5); flow rate, 1.0 mL/min.

Results and Discussion

Interaction of Cu(salgly)^{2+} complex with DNA

Typical CV behaviors of 2.5×10^{-4} mol/L Cu(salgly)^{2+} complex in the absence and presence of 3.0×10^{-4} mol/L DNA are shown in Fig. 1. The CV of Cu(salgly)^{2+} complex in the absence of DNA (Fig. 1a) featured reduction of Cu(salgly)^{2+} to the Cu(salgly)^+ form at a cathodic peak potential, E_{pc} , of -0.54 V vs. SCE. Reoxidation of Cu(salgly)^+ occurred at -0.02 V. The separation of the anodic and cathodic peak potentials, $\Delta E_{\text{p}} = 0.52$ V, indicated a *quasi-reversible* redox process. The peak currents (i) were linear with scan rate (v) for $v < 100$ mV/s. The formal potential, E° (or voltammetric $E_{1/2}$), taken as the average of E_{pc} and E_{pa} , was -0.28 V in the absence of DNA. In the presence of DNA (Fig. 1b), E_{pc} occurred at -0.61 V and E_{pa} at -0.05 V. Thus, both the anodic and cathodic peak potentials shifted to more positive values vs. a solution without DNA ($E_{1/2}$). And $E_{1/2}$ shifted to more negative by 5 mV. Addition of CT-DNA to the Cu(salgly)^{2+} complex solution results in decreases of the anodic and cathodic peak currents. All of those experimental results showed that some interaction occurred between Cu(salgly)^{2+} complex and DNA.

Potential-modulated DNA cleavage

Figure 2 shows the chromatographic separation of the solution containing 2.5×10^{-4} mol/L Cu(salgly)^{2+} complex, 3.0×10^{-4} mol/L DNA and 10 mM phosphate buffer (pH 6.5) before (A) and after electrolysis (B), respectively. Before the solution electrolysis, the peak of DNA appeared in the chromatogram at a retention time of 4.0 min (see Fig. 2A). After the solution was electrolyzed, the large and broad peak of DNA became small and narrow, and at the same time, some new chromatographic peaks were observed (see Fig. 2B). The retention times of these new peaks were 5.1, 7.2, 10, 12.6 and 14.2 min, respectively.

Fig. 3 (A) The dependence of percentage of DNA cleavage on working potential. (B) Dependence of DNA cleavage percentage on electrolysis time. (C) Dependence of DNA cleavage percentage on the ratio of Cu(salgly)²⁺ complex to DNA concentration.

After electrolysis, a peak at 4.6 min was attributed to the uncleaved long strand DNA. The changes of the DNA chromatograms revealed that the DNA molecules were destroyed after the test solution was electrolyzed. As a comparison, the solution without Cu(salgly)²⁺ complex was electrolyzed under the same conditions. A slight change of the DNA chromatogram was observed before and after electrolysis. This indicated that the extent of DNA cleavage was very small in the absence of a copper complex. DNA shows no direct electrochemical response on a gold electrode at -0.7 V. It could not be oxidized or reduced by electrochemistry and its structure cannot be destroyed. Hence, the cleavage of DNA molecules is attributed to the electrolysis (*i.e.* potential-modulation) in the presence of Cu(salgly)²⁺ complex, which produces small DNA fragments. Because the Shim-pack Diol-300 column was a size-exclusion chromatograph column, its separation was based on the size of the molecules separated. In the exclusion

chromatography, the shorter the strand of DNA is, the longer the retention time of the components is.^{28,29} After electrolysis, some new peaks in the chromatogram appeared at long retention times, which revealed that the strand of partial DNA molecules was broken from the longer to shorter strands. Undoubtedly, the components of the retention time at 12.6 and 14.2 min were much smaller DNA molecular fragments.

The above experimental results showed that DNA can be cleaved efficiently by potential-modulation in the presence of Cu(salgly)²⁺ complex. Obviously, the system for the DNA cleavage reaction was simple and mild, for it needs no extra reductive agents or H₂O₂ to produce electron transfer.

Effect of experimental conditions on DNA cleavage

The extent of DNA cleavage was obviously affected by the working potential, electrolysis time and the concentration of Cu(salgly)²⁺ complex. In order to characterize the effect, we examined how the chromatographic peak height of DNA at retention time of 4.0 min changed with experimental conditions. If the peak heights of DNA were H and H_i before and after electrolysis, respectively, $(H-H_i)/H$ could be used to indicate the percentage of DNA cleavage. Figure 3A showed the percentage of DNA cleavage changing with various potentials in the presence of Cu(salgly)²⁺ complex. An S-shaped curve with a sharp inflection at -0.55 V was obtained and no obvious DNA cleavage was detected at -0.20 V. When the working potential was adjusted to -0.50 V, the peak height of the DNA started to decrease obviously. At potentials more negative than -0.55 V, the peak height of the DNA decreased rapidly and a new chromatographic peak of DNA fragments appeared. As the working potential reached -0.70 V, the change of the percentage of DNA cleavage became slow.

Figure 3B showed the effect of electrolysis time on the percentage of DNA cleavage. It can be observed that the extent of DNA cleavage was enhanced with increasing electrolysis time. However, this change became slow. The reason may be as follows: after the solution has been electrolyzed for a certain time, the partial long strand of DNA was cleaved into short strand DNA and the amounts of short strand DNA gradually increase with increasing electrolysis time. This resulted in the probability of long strand DNA attack by hydroxyl radical being lessened. The result that the retention time of the cleaved DNA fragments lengthened with increasing electrolysis time indicated that these DNA fragments were further cleaved to form much smaller components.

The dependence of percentage of DNA cleavage on the ratio of Cu(salgly)²⁺ complex to DNA concentration is shown in Fig. 3C. It can be seen that when the electrolysis time was controlled for 30 min at -0.7 V, the percentage of DNA cleavage was enhanced with increasing ratio of Cu(salgly)²⁺ complex to DNA concentration. When the ratio was increased to 0.7, the percentage of DNA cleavage was changed slowly. At low Cu(salgly)²⁺ complex concentration, the amount of hydroxyl radical generated in the reaction system was increased with increasing the ratio and the percentage of DNA cleavage was enhanced. When the ratio was increased to 0.7, amounts of the generated radicals depended upon the concentration of dissolved oxygen. Hence, the percentage of DNA cleavage was not further increased.

The mechanism of DNA cleavage

In order to characterize the mechanism of DNA strand scission, we studied the effects of O₂, DMSO and glycerol on the extent of DNA cleavage. Oxygen was required for copper (II)-mediated DNA cleavage because it serves as a precursor of

hydrogen peroxide.^{30,31} For exploring the role of oxygen in potential-modulated DNA cleavage by Cu(salgly)²⁺ complex, we designed the experiments under three cases: 1) The solution was deoxygenated by N₂ for 15 min; 2) The solution was saturated by air; 3) The solution was aerated with a small flow O₂ during electrolysis. The electrolysis time was controlled for 30 min in each case. Figure 2C showed the separation result of the solution deoxygenated with N₂ before electrolysis. A slight split of the DNA chromatographic peak was observed in the chromatograms of DNA after electrolysis at -0.7 V. This observation indicated that DNA could be cleaved slightly by potential-modulation in the absence of oxygen in solution. Dramatic changes were observed in the chromatogram of DNA when the solution was bubbled with a small flow of O₂ during electrolysis. Figure 2D showed the chromatographic separation of the electrolyzed solution. Compared to Fig. 2B (in case (2)), the height of long strand DNA lessened strongly and a new small peak of DNA component emerged at a retention time of 15.2 min, and the peak heights at retention times of 7.2 and 12.6 min increased obviously. All such experimental results revealed the oxygen was essential to potential-modulated DNA cleavage by Cu(salgly)²⁺ complex.

When hydroxyl radical scavengers, such as DMSO, glycerol,³² were added to the studied solution, the solution was electrolyzed at the same condition. The results of chromatographic analysis showed that the chromatogram changed slightly before and after electrolysis. It indicated that potential-modulated DNA cleavage by Cu(salgly)²⁺ complex was inhibited by radical scavengers.

According to the above experimental results, a mechanism of the cleavage was proposed as follows: the Cu(salgly)²⁺ complex was first electrochemically reduced to form Cu(salgly)⁺ complex on the gold electrode surface. Secondly, the Cu(salgly)⁺ complex was reoxidized by the dissolved oxygen to reform Cu(salgly)²⁺ complex, while hydrogen peroxide was generated in the process. It was this hydrogen peroxide that initiated a series of reaction to form ·OH radicals. Thirdly, the formed hydroxyl radical attacked the DNA strand and resulted in the breakage of DNA strands. The processes could be expressed as follows:

Conclusion

DNA can be cleaved by potential modulating in the presence of Cu(salgly)²⁺ complex. The extent of DNA cleavage was dependent on the working potential, on the electrolysis time and on the concentration of Cu(salgly)²⁺ complex. The cleavage proceeds *via* electrochemically inducing a redox reaction and producing ·OH radicals and resulting in the breakage of DNA strand. The process may give a new application in the design of more effective and useful methods for DNA cleavage.

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