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Electrochemical detection of scDNA cleavage in the presence of macrocyclic hexaaza–copper(II) complex

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Abstract

The hexaaza macrocyclic copper(II) complex Cu(II)L(L═1,8-Dihydroxyethyl-1,3,6,8,10,13-hexaazacyclotetradecane), which has octahedral structure similar to some natural complexes, is synthesized and purified. In this study, oxidative breakage DNA by the reaction of Cu(II)L with H_2O_2 and ascorbate has been investigated by gel electrophoresis experiments. In electrochemical experiments, the on scDNA-modified glassy carbon electrode(GCE) is cleaved by the Cu(II)L and redox changing of the metal catalyst without adding any other reagents. Above all, the need for concentration of scDNA is much lower than that of gel electrophoresis experiments and the process of the performance is easy. Furthermore, Cyclic Voltammetry (CV) and A.C. Impedance, which are performed to monitor scDNA cleavage at the scDNA-modified glassy carbon electrode (GCE), are fast, simple and highly efficient. The mechanism of the damage can be suggested: Fenton.

Keywords: pBR322 supercoiled DNA; scDNA-modified GCE; Cu(II)L; Cleave; Cyclic Voltammetry; A.C. Impedance

1. Introduction

Deoxyribonucleic acid (DNA) is the primary target molecule for most of anticancer and antiviral therapies according to the cell biology. Over the last two decades, DNA cleavage induced by oxygen radicals has been implicated to be important in ageing, mutagenesis and carcinogenesis [1–4]. Some metal ions and complexes can undergo one-electron redox reactions (such as copper, iron or manganese) producing reactive oxygen species, the latter then damage DNA, frequently yielding strand breaks [5]. Copper is recognized as an essential metal element widely distributed in the biological system such as cells and body. It is a bioessential element with relevant oxidation states +1 and +2. Coordination compounds of copper have been extensively used in metal-mediated DNA cleavage [6–11].

There are several strategies to induce redox reaction of copper ion. Copper ion can be reduced in the presence of reducing agents such as thiols, ascorbate, NADH or phenol compounds, the reduced copper ion react with hydrogen peroxide producing hydroxyl radicals in a one-electron redox reaction. These hydroxyl radicals attack the DNA double strands to cause strands cleavage. However, extra reductant and H_2O_2 are required in the reaction system. It caused the conditions of DNA cleavage reaction which were obviously different from physiological conditions. Since copper ion can react with oxygen producing reactive oxygen in redox reactions, using electrochemical methods to cleave DNA has reached much attention [12]. Fojta and co-workers have already reported that supercoiled (sc) DNA immobilized at the surface of a hanging mercury drop electrode is cleaved by reactive oxygen species generated by an electrochemically modulated reaction of hydrogen peroxide or oxygen [13]. Labuda et al. [14] studied DNA cleavage using a DNA modified glassy carbon electrode in the presence of a copper-1,10-phenanthroline complex by examining oxidative current of base, the break of DNA strands was observed.

Thereby, in this paper, firstly we synthesize Cu(II)L (the structure is in Scheme 1) which has octahedral structure similar to some natural complexes, such as chlorophyll, hemoglobin, vitamin B_{12} and other bioenzymes. These substances can possess antiviral, antitumor activities, and even protective

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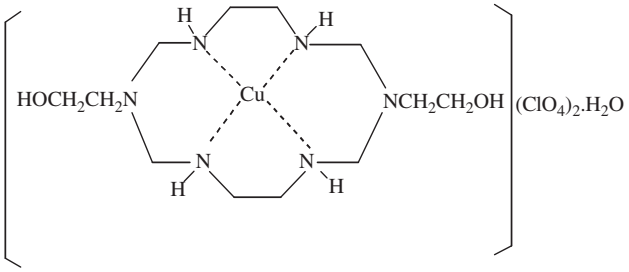

Scheme 1. The structure of 1,8-Dihydroxyethyl-1,3,6,8,10,13-hexaazacyclotetra-decane copper(II) perchlorate monohydrate(Cu(II)L).

effects against chronic diseases. The synthetic method has been reported in our laboratory previously [15], and then we study the Cu(II)L cleavage ability in absence or presence of H_2O_2 and ascorbic acid by electrophoresis. Then, a scDNA-modified electrode has been suggested a sensor for detection of damage to DNA strand. The electrochemical method, Cyclic Voltammetry, is found for highly sensitive detection of DNA strand breaks based on a supercoiled (sc) plasmid DNA-modified glassy carbon electrode without adding any reductant and oxidant. The method for monitoring DNA cleavage is fast, simple, highly efficient and the concentration of DNA required for the analysis is below 0.12 μg/mL. A.C. impedance is employed to validate accessorily the results of the Cyclic Voltammetry.

2. Experimental

2.1. Reagents and apparatus

Cu(II)L complex was synthesized and purified according to the method reported in our laboratory previously [15]. pBR322 (0.50 μg/μL) were obtained from Sino-American Biotechnology Co. (Beijing China). Native pBR322 was dissolved in Tris–HCl solution. Other chemical and reagents used were of analytical grade without further purified unless otherwise noted. All the experiments were carried out in Tris–HCl buffer (10 mM Tris, 50 mM NaCl, pH 7.6).

Cyclic Voltammetry and A.C. Impedance were carried out by CHI660 electrochemical workstation (CH Instruments Ltd. Co. USA). A standard three-electrode system comprising a glassy carbon or scDNA-modified glassy carbon working electrode, platinum-wire auxiliary electrode and Ag/AgCl reference electrode was used. Experiments were carried out at the laboratory temperature.

2.2. Preparation of scDNA-modified GCE

A glassy carbon electrode was first polished successively with 0.1 and 0.05 μm alpha alumina powder and then cleaned ultrasonically in water and acetone for 5 min, respectively. Supercoiled pBR322 DNA was adsorbed at the freshly-pretreated glassy carbon electrode by transferring a drop of 6 μL solution containing 0.12 μg/μL scDNA in 0.2 M NaCl and 10 mM Tris–HCl pH 7.6 for 15 min, followed by rinsing in double distilled water. A scDNA-GCE was obtained.

2.3. Procedure

2.3.1. The agarose gel electrophoresis experiments

For the gel electrophoresis experiments, supercoiled pBR 322 DNA 0.5 μg/mL in Tris–HCl buffer was treated with different concentrations of Cu(II)L; and in presence of peroxide hydrogen; followed the ascorbic acid was added in reaction solution. The samples were incubated for 2 h at 37 °C, a loading buffer containing 25% bromophenol blue (2 μL) was added and electrophoresis was performed at 80 V for 2 h in TAE buffer using 0.8% agarose gel containing 1.0 μg/mL ethidium bromide. Bands were visualized by UV light and photographed on a capturing system (Gelprinter plus TDI).

2.3.2. Electrochemical methods monitor scDNA modified on the electrode

Firstly, Cyclic Voltammetry was used to investigate if the electrode was modified by scDNA in 5 mM $K_3Fe(CN)_6$ and 0.5 M KCl solution, the scan rate was 50 mv/s. Then, A.C. impedance was employed to validate the results of CV under the same solution, the measurement was conducted in the frequency range from 100 kHz to 1 Hz.

2.3.3. Electrochemical methods monitor scDNA cleavage on the electrode

After Ec = −0.3 v (potential applied at GCE during the scDNA cleavage reaction) was adjusted in different reaction solutions. Unless otherwise noted, which oxygen was passed through the solutions to saturate them. After 60 s, the reaction was stopped (by opening the current circuit) and the electrode was washed in double distilled water then placed in the blanket back ground solution to record the voltammogram, followed by the electrode washed in double distilled water and was transferred into 5 mM $K_3Fe(CN)_6$ and 0.5 M KCl solution

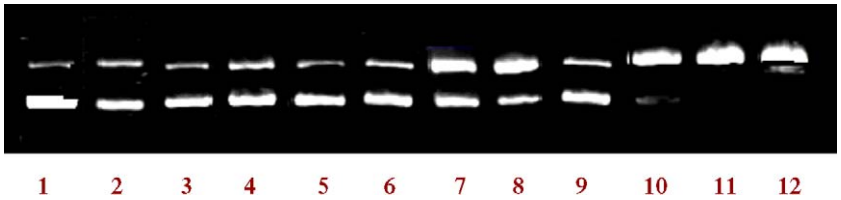

Fig. 1. Agarose gel electrophoresis of pBR322 DNA treated with copper(II) complex in the presence of H_2O_2 or H_2O_2 and ascorbate in a buffer containing 10 mM Tris and 50 mM NaCl at 37 °C. (1)DNA alone; (2–4) in the presence of different concentration of Cu(II)L,(2)20 μM, (3)40 μM, (4)60 μM; (5)DNA + 0.5 mM H_2O_2; (6–8) in presence of 0.5 mM H_2O_2 and different concentration of Cu(II)L, (6)20 μM, (7)40 μM, (8)60 μM; (9)DNA + 0.5 mM H_2O_2 + 1 mM ascorbic acid; (10–12) in the presence of 0.5 mM H_2O_2 and 1 mM ascorbic acid and different concentration of Cu(II)L, (10)20 μM, (11)40 μM, (12)60 μM.

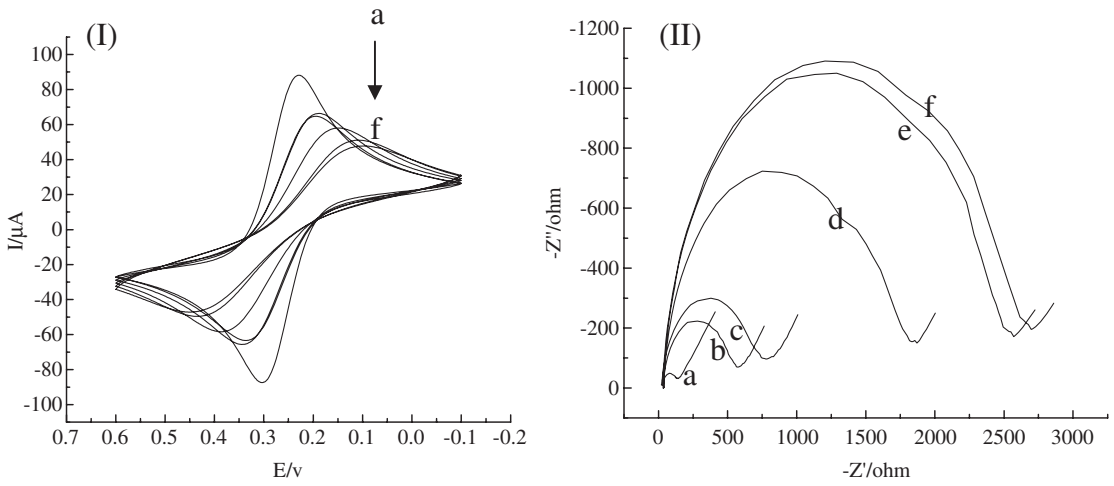

Fig. 2. (I) Cyclic Voltammetry of 5 mM $K_3Fe(CN)_6$ solution and (II) Nyquist plot of 5 mM $K_3Fe(CN)_6$ solution with 0.5 M KCl support electrolyte at (a) bare glassy carbon electrode; (b) scDNA modified-GCE; (c–f) the modified-GCE after cleavage by Cu(II)L: (c)10 μM, (d)30 μM, (e)60 μM, (f)80 μM.

again to measure the scDNA cleavage with Cyclic Voltammetry and A.C. Impedance at room temperature.

3. Results and discussion

3.1. Agarose gel electrophoresis

The ability of Cu(II)L complex in effecting DNA cleavage has been studied by gel electrophoresis using supercoiled pBR 322 DNA in Tris–HC*l* buffer. We incubate of the Cu(II)L complex alone with pBR322 DNA and subsequent electrophoresis, Fig. 1 (lanes 2–4) shows the gel electrophoretic separations of plasmid pBR322 DNA after incubation 2 h in the presence of varying concentrations of Cu(II)L complex alone. It can be seen that with increasing the concentration of Cu (II)L complex, Form II(nicked circular DNA)increase gradually, while Form I (supercoiled circular pBR 322 DNA) diminish gradually, but the Form III (linear DNA) do not occur. It is suggested that the Cu(II)L complex presents weak nuclease activity. As it is well known, many copper complexes have been shown to cleave DNA in presence of H_2O_2, which is the DNA cleaving species formed in Fenton reaction [8]. Under the same complex concentrations containing H_2O_2, the electrophoresis experiments are carried out. From Fig. 1 (lanes 6–8), it is clear that the scDNA is not also cleaved apparently. This result indicates that the complex to damage DNA in presence of H_2O_2 produced small amount hydroxyl radicals and they are insufficient to cleave DNA well because without reduction agent, it is difficult to produce the Cu(I) species to reduce H_2O_2. So under the same experimental conditions, adding an excess of reduction agent ascorbate (Fig. 1, lanes 10–12), ascorbate is added to reduce Cu(II) to more Cu(I) which then reacts to produce more hydroxyl radicals [13]. In Fig. 1 lane 12, it can be seen that the scDNA can even be converted to linDNA when the Cu(II)L concentration is increased. It is likely that the reduction is the important step leading to DNA cleavage. Control experiments were performed by pBR322 DNA (lanes 1, 5, 9): (lane 1) in absence of Cu(II)L, (lane 5) in presence of H_2O_2, (lane 9) in presence of H_2O_2 and ascorbic, no obvious change occurred.

From Fig. 1, we can observe that increasing of the complex concentration also cause increasing DNA cleavage and the cleavage efficiency of the complex in presence of H_2O_2 and ascorbate is found to be much better than that of only Cu(II)L or Cu(II)L and H_2O_2. Hence, it is clear that the Cu(II)L complex in the presence of H_2O_2 and ascorbate shows nuclease activity.

However, the disadvantage of this method is that the analysis takes at least several hours and that quantitation of the ethidium bromide stained is rather difficult. So then electrochemical methods have been developed, Cyclic Voltammetry and A.C.

Table 1
The Voltammetric behaviours of 5 mM $K_3Fe(CN)_6$ solution with 0.5 M KCl support electrolyte in presence or absence of the scDNA are cleaved by Cu(II)L at the scDNA-modified GCE

	$C_{(Cu(II)L)}$	E_{pa} (V)	E_{pc} (V)	ΔE_p (mV)	$E_{1/2}$ (V)
Bare GCE	0	0.303	0.229	74	0.266
scDNA-modified GCE	0	0.339	0.195	144	0.267
scDNA-modified GCE	10 μM	0.353	0.187	156	0.270
(after cleavage by Cu(II)L)	30 μM	0.396	0.149	237	0.273
	60 μM	0.439	0.108	331	0.274
	80 μM	0.450	0.097	365	0.274

Table 2
The EIS behaviours of 5 mM $K_3Fe(CN)_6$ solution with 0.5 M KCl support electrolyte in presence or absence of the scDNA are cleaved by Cu(II)L at the scDNA-modified GCE

	$C_{(Cu(II)L)}$	R_{et} (Ω)	I_0 (A)	K_{et} (cm s^{-1})
Bare GCE	0	128	2.00×10^{-4}	0.030
scDNA-modified GCE	0	562	4.57×10^{-5}	6.91×10^{-3}
scDNA-modified GCE	10 μM	746	3.44×10^{-5}	5.2×10^{-3}
(after cleavage by	30 μM	1823	1.41×10^{-5}	2.13×10^{-3}
Cu(II)L)	60 μM	2518	1.02×10^{-5}	1.54×10^{-3}
	80 μM	2703	9.49×10^{-6}	1.44×10^{-3}

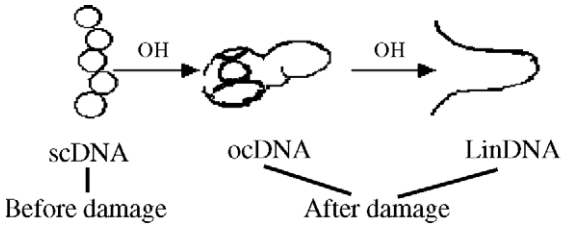

Fig. 3. Formation of DNA strand breaks by Cu(II)L in presence of H_2O_2 and ascorbic acid.

Impedance, for highly sensitive detection of DNA strand breaks based on a supercoiled(sc) plasmid DNA-modified GCE.

3.2. Electrochemistry methods detecting scDNA cleavage

Fig. 2 (I) shows Cyclic Voltammetry of the 5-mM $K_3Fe(CN)_6$ solution with 0.5 M KCl support electrolyte at different electrodes. It can be seen that a pair of redox peaks for $K_3Fe(CN)_6$ solution appear at 0.303 v (Epa) and 0.229 v (Epc), respectively, and the peak separation (ΔE) of 74 mV can be obtained at the bare glassy carbon electrode at scDNA-modified GCE the redox peak currents decrease, and $\Delta E \approx 144$ mV increase apparently, these electrochemical signals indicate glass carbon electrode surface has covered with DNA. The Voltammetric behaviours of the Cu(II)L cleavage scDNA at the scDNA-modified GCE have been studied (Table 1). It is clear to find out that the pairs of redox peaks current decrease, ΔE increase and reduction peak potential shifts to more negative values, oxidation peak potential shifts to more positive direction and the $E_{1/2}$ moves to more positive direction. These observations can be explained that the scDNA may be cleaved to open circular DNA and linear DNA because after damaging, the more phosphate backbone are exposed, the stronger the electrostatic repulsion between the DNA film and $K_3Fe(CN)_6$ due to the increasing of negative film charges block $Fe(CN)_6^{3-/4-}$ electron transfer.

As it is well known, electrochemical impedance spectroscopy (EIS) is an effective method for probing features surface-modified electrodes [16]. In order to demonstrate deeply the results of the CV, A.C. Impedance experiments are carried out. Fig. 2 (II) shows the EIS of the same procedures followed as described above; we can see from curve a, on the bare GCE, electron transfer resistance (R_{et}) is 0.128 kΩ that exists if a redox probe is present in the electrolyte solution, in the fact that $[Fe(CN)_6]^{3-/4-}$ is used as a redox probe [17] in this experiment; curve b, the scDNA is modified on the electrode, $R_{et} \approx 0.562$ kΩ; after the scDNA are damaged by various concentration Cu(II)L, the $R_{et} \approx 2.5$ kΩ. It is clear that the electron transfer resistance of $[Fe(CN)_6]^{3-/4-}$ redox couple before damage is different from that after the damage. Results from the change of the charge number holding on the modified GCE, we can see that after damaging, more and more negative charges of phosphate backbone accumulated on the electrode to block $k_3Fe(CN)_6$ electron transfer. The fact is consisted with the above experiment result. The electron transfer resistance R_{et} can be translated into the exchange current (I_0) under equilibrium according to Eq. (1), and then the heterogeneous electron transfer rate constant (K_{et}) can be evaluated from Eq. (2) [17].

$$R_{et} = RT(nFI_0)^{-1} \quad (1)$$

$$I_0 = nFAK_{et}[s] \quad (2)$$

where $R = 8.31$ J mol^{-1} K^{-1} is the gas constant, T is the temperature (K), $F = 9.65 \times 10^4$ C equiv^{-1} is the Faraday constant, A is the electrode area (cm^2), $[s]$ corresponds to the bulk concentration of the redox probe, and n is the number of electrons transferred per molecule of the redox probe.

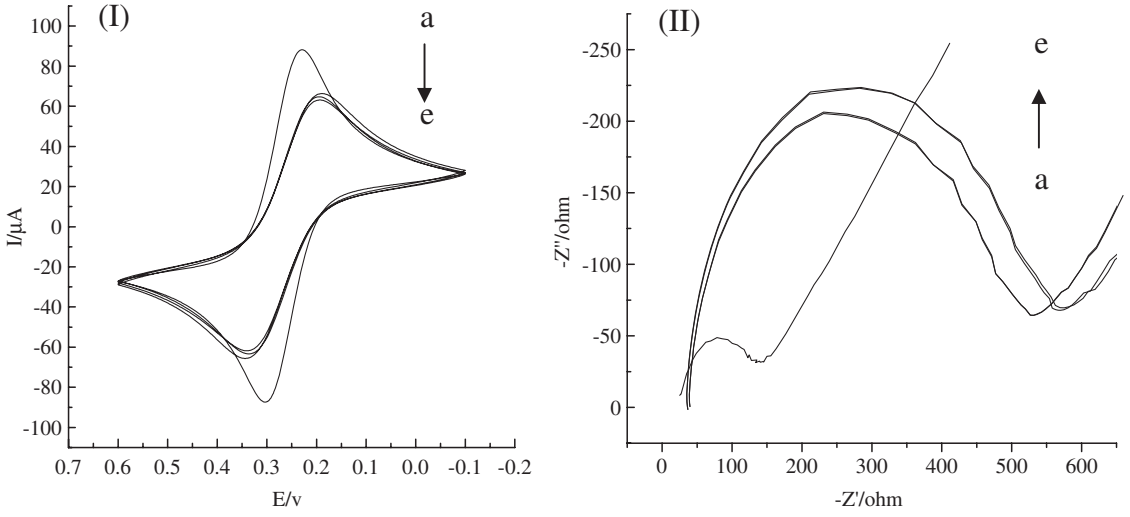

Fig. 4. Control experiment: (I) Cyclic Voltammetry of 5 mM $K_3Fe(CN)_6$ solution and (II) Nyquist plot of 5 mM $K_3Fe(CN)_6$ solution with 0.5 M KCl support electrolyte at (a) bare glassy carbon electrode; (b) scDNA modified-GCE; (c) the background electrolyte, in absence of Cu(II)L complex; (d) in presence of Cu(II)L complex, without air-saturated; (e) in presence of 60 μM Cu(II)L, without electrochemical reducing cleavage.

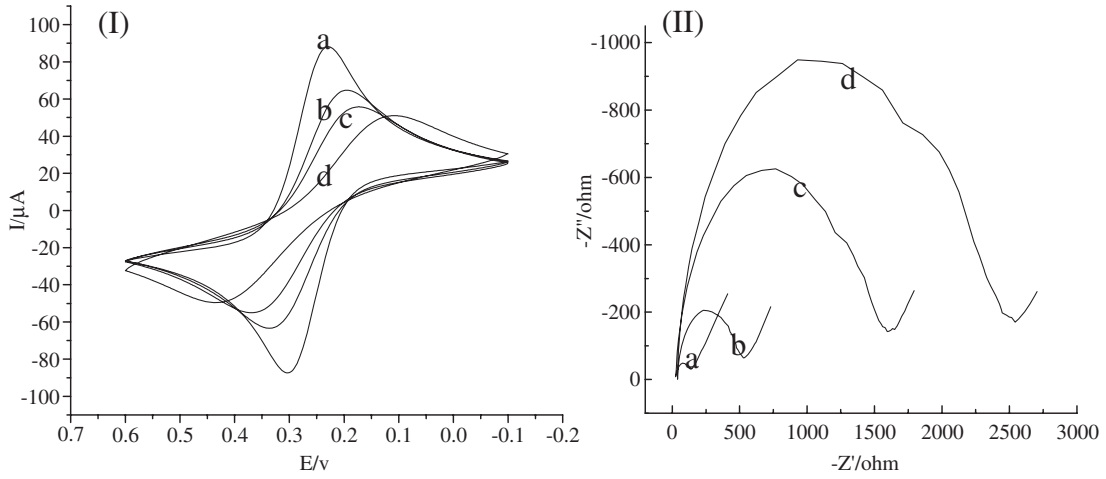

Fig. 5. (I) Cyclic Voltammetry of 5 mM $K_3Fe(CN)_6$ solution and (II) Nyquist plot of 5 mM $K_3Fe(CN)_6$ solution with 0.5 M KCl support electrolyte at (a) bare glassy carbon electrode; (b) scDNA modified-GCE; (c) the modified-GCE after cleavage by 60 μM $CuCl_2$ in presence of 0.5 mM H_2O_2; (d) the modified-GCE after cleavage by 60 μM Cu(II)L in presence of 0.5 mM H_2O_2.

In Table 2, the K_{et} are calculated according to the Eqs. (1) and (2), it is clear to see that R_{et}: 10 μM([Cu(II)L])<30 μM <60 μM<80 μM; K_{et}: 10 μM ([Cu(II)L])>30 μM>60 μM >80 μM. K_{et} become more and more slowly, it can be explained that with the increasing of the Cu(II)L concentration, the more scDNA has been cleaved and the more negative charges on the modified GCE, so the electron transfer rate constant K_{et} become slower. The results of the experiments are in good agreement with the results of CV experiment. The sketch map of the scDNA cleavage processes can be seen clearly from Fig. 3.

Furthermore, the control experiments are performed to study the reaction mechanism, Fig. 4 (I) shows Cyclic Voltammetry of the $K_3Fe(CN)_6$ solution with KCl support electrolyte at different working electrode. It can be seen clearly that the curves' change obscured, it means that the damage requires oxygen and electrode potential, otherwise, the cleavage reaction will not occur, so the mechanism of the cleavage can be deduced: Fenton catalyst [8]. Further A.C. Impedance is carried to do the control experiments and demonstrate the reaction mechanism. The electrochemical signals are also changed slightly (Fig. 4 (II)). The experiment results indicate that the Cu(II)L complex can cleave scDNA by electrochemical-modulated reaction, hydrogen peroxide is replaced by oxygen, while a sufficiently negative electrode potential is used to maintain the metal complex redox cycling [18]; therefore, there is no affection on the cleavage reaction in presence of H_2O_2 or H_2O_2 and ascorbate (the curves not shown), the mechanism of the oxidative cleavage can be demonstrated as well.

3.3. Application of the electrochemical methods to detect damage

Cyclic Voltammetry is employed to monitor the scDNA damage by measuring changes of electrochemical behaviors on glassy carbon electrode. As a means for further proving the feasibility, we incubate the scDNA-modified GCE in Tris–HCl buffer containing $CuCl_2$ and H_2O_2 because $CuCl_2$, in presence of H_2O_2, has already been proved to cleave scDNA [5], then potential Ec is adjusted and the cleavage reaction is started by an addition of H_2O_2, followed by the scDNA-modified GCE, it is placed into $K_3Fe(CN)_6$ solution, and then the CV experiment is carried out. From Fig. 5 (I), it shows that the change of electrochemical signals is similar and we also can see that Cu(II)L in presence of H_2O_2 damage DNA better than $CuCl_2$ and H_2O_2, the data of Cyclic Voltammetric has been listed in the Table 3. The experiments are carried out by A.C. Impedance under the same condition, after the scDNA is cleaved by $CuCl_2$ and H_2O_2, the $K_{et}=2.40\times10^{-3}$ cm s^{-1} is faster than the K_{et} 1.44×10^{-3} cm s^{-1} that by Cu(II)L and H_2O_2, the result agrees with the above conclusion.

The results not only indicate that the complex interacted with DNA stronger than Cu^{2+} in presence of H_2O_2, but also the methods of electrochemistry for monitoring DNA cleavage are fast, simple, highly efficient, feasible and the concentration of DNA required for the analysis is below 0.12 μg/mL. Moreover, the intact scDNA or the cleaved scDNA (ocDNA or linDNA) modified electrode can act as a sensor to detect the damaging agents.

Table 3
The Voltammetric behaviours of 5 mM $K_3Fe(CN)_6$ solution with 0.5 M KCl support electrolyte in presence or absence of the scDNA are cleaved by Cu(II)L or $CuCl_2$ at the scDNA-modified GCE

	C	E_{pa}(V)	E_{pc}(V)	ΔE_p (mV)	$E_{1/2}$(V)
Bare GCE	0	0.303	0.229	74	0.266
scDNA-modified GCE	0	0.339	0.195	144	0.267
scDNA-modified GCE (after cleavage by $CuCl_2$)	60 μM	0.376	0.161	215	0.269
scDNA-modified GCE (after cleavage by Cu(II)L)	60 μM	0.439	0.108	331	0.274

3.4. The mechanism of DNA cleavage

From the Agarose gel electrophoresis, the scDNA cannot be cleaved dramatically only by Cu(II)L complex; the scDNA can be cleaved apparently in the presence of H_2O_2 and ascorbate because there are more hydroxyl radicals. The process could be expressed as follows [8]:

$$Cu(II)L + e \rightarrow Cu(I)L(\text{reduction reaction})$$

$$2Cu(I)L + H_2O_2 \rightarrow OH^- + {}^{\cdot}OH + 2Cu(II)L$$

$$DNA + {}^{\cdot}OH^- \rightarrow ocDNA + LinDNA + \text{fragments}$$

According to testing DNA cleavage by the electrochemical experiments, DNA cannot be cleaved only by the controlling potential of the electrode (Ec = −0.3 v) in absence of Cu(II)L, and there are nearly no affection in presence or absence of H_2O_2 or H_2O_2 and ascorbate. Therefore, we demonstrate that DNA damage at the GCE surface in the presence of hydrogen peroxide (or dioxygen) and transition metal complex can be controlled by the electrode potential, thus providing an electrochemical nuclease-like activity. Based on the above experiment and theory results, the mechanism of the cleavage is possibly the following reactions [19]:

$$Cu(II)L + e \rightarrow Cu(I)L(\text{electrode reaction})$$

$$2Cu(I)L + 2H^+O_2 \rightarrow 2Cu(II)L + H_2O_2$$

$$2Cu(I)L + H_2O_2 \rightarrow OH^- + {}^{\cdot}OH + 2Cu(II)L$$

$$DNA + {}^{\cdot}OH^- \rightarrow ocDNA + LinDNA + \text{fragments}$$

In general, copper yields one reversible pair of peaks related to a two-electron reaction (Cu^{2+}/Cu^0) at bare GCE [20]. However, the Cu(II)L complex is reduced Cu(I)L, firstly. This phenomenon results in the splitting of the reduction/oxidation of coordinated copper at GCE in two one-electron steps. These processes suggest that a stabilization of Cu(I)L in the Cu(II)L, where it can undergo one-electron redox cycling, and then the copper(I) species, may react with oxygen in presence of H^+ to produce hydroxyl radicals, the hydroxyl radical cleaves DNA by abstracting a hydrogen atom from a sugar residue in the DNA back bone, the deoxyribose residue breaks down, leaving a gap with predominantly 3′- and 5′-phosphate ends, the scDNA was convert into a small amount of phosphorglycolate [13].

As mentioned above, regardless of the agarose gel electrophoresis or the redox changing of metal catalyst, the mechanism of the Cu(II)L complex cleavage reactions is both Fenton catalyst. We can conclude that hydrogen peroxide(and oxygen) can damage surface-attached DNA through a metal-independent mechanism involving H_2O_2 decomposition and oxygen electroreduction [18]. These processes of the cleavage reactions also indicate that the effectiveness of Cu(II)L as chemical nuclease depend on the reducing agent used for activation. The oxidation cleavage is produced by formation of reactive Cu(I)L complex with DNA, with subsequent generation of reactive oxygen species(${}^{\cdot}OH^-$).

4. Conclusion

The Cu(II)L complex has been found to promote cleave plasmid pBR 322 DNA in presence of H_2O_2 and ascorbate, which may be taken as the potential DNA cleavage reagent. The reports about the electrochemical methods detecting scDNA damage at modified electrode are few. In these experiments, a glassy carbon electrode modified with supercoiled DNA yields obvious voltammetric responses to agents generating DNA strand breaks. The methods are fast, simply and highly efficient. Using these approaches, the scDNA-modified electrode is capable not only to detect chemical or biochemical DNA cleaving agents, but also to modulate the DNA cleavage by generating the DNA damaging species electrochemically. This feature significantly improves our detection system. DNA cleavage mechanism has been demonstrated by the experiments that the Cu(II)L complex can oxidate cleaved scDNA.

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