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Electrochemical detection of microcystin-LR based on its deleterious effect on DNA

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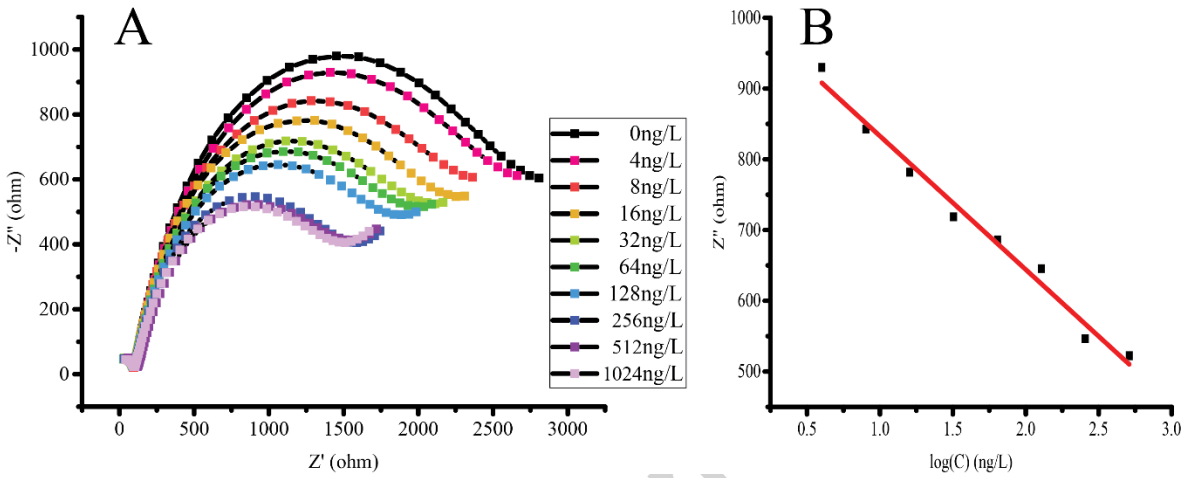
Abstract

This work reveals the deleterious effect of microcystin-LR (MC-LR) on the conformation of DNA and develops an electrochemical biosensor for detection of MC-LR. The biosensor is prepared by physically immobilizing calf thymus DNA (ctDNA) on gold electrode. In the presence of MC-LR, the conformation change of immobilized ctDNA decreases the electron transfer impedance, thus enhances the amperometric response. The proposed method shows a linear range of 4 to 512 ng/L and a detection limit of 1.4 ng/L, which is 700-fold lower than the guideline level suggested by the World Health Organization. The detection results are in good agreement with those from the conventional HPLC method. This biosensor possesses good stability against other components in wild water sample, and has been used for detection of MC-LR in local water bodies, indicating its application promise.

Graphic Abstract

Binding of microcystins by immobilized calf thymus DNA results in changes in impedance of the electrode surface. The DNA coated electrode can be used as a sensor to monitor water quality.



Keywords: Electrochemical sensor; Cyanobacteria blooms; Microcystin; DNA; Deleterious effect

1. Introduction

The harmful algal blooms (HABs) have been studied for more than a century (Araoz et al. 2005, Bickel et al. 2001, Fogg et al. 1973, Francis 1878). In the past decades, due to the climate change and increasing industrial and agricultural activities, the spatial and temporal incidences of HABs have increased tremendously (Annadotter et al. 2001, Ballot et al. 2005, Carmichael et al. 1997, Domingos et al. 1999, Foster 2013, Funari et al. 2000, Michalak et al. 2013, Wines 2013, Yan et al. 2017), posing risks to farming, fishing, industrial productions and human health (Carey et al. 2012). In China, due to the rapid economic development, the environment protection is more urgent and critical(Chen et al.

2017). Cyanobacteria can produce several classes of toxins, including microcystins, nodularin (van Apeldoorn et al. 2007), cylindrospermopsin (Chiswell et al. 1997, Evans and Murphy 2011). These toxins can cause various disorders and diseases including ALS (Amyotrophic Lateral Sclerosis), Parkinson's disease and Alzheimer's diseases (Holtcamp 2012). Among toxins, microcystin is the most common and toxic kind because of its high stability in addition to toxicity (Hudnell 2008).

Microcystins are cyclic hepta-peptides (Metcalf et al. 2000). More than 80 analogs have been identified so far (Zheng et al. 2016). Their presence can cause liver damage, vomiting, and nausea (Azevedo et al. 2002, Jochimsen et al. 1998). Due to their toxicity, thermal stability and resistance to chemical breakdown, microcystins have attracted much more attention compared with other cyanobacteria toxins (Butler et al. 2009). Microcystin-LR (MC-LR), which is produced by cyanobacteria, is the most poisonous and the most common member in microcystin family, and has been identified as one of the major toxins in Taihu Lake, China (Qin et al. 2007, Shen et al. 2003). Although conventional techniques such as thin-layer chromatography and high-performance liquid chromatography/mass spectrometry (Vasas et al. 2006, Xu et al. 2008, Zhang et al. 2011, Zhang et al. 2010, Zhang et al. 2004) and bioassays thus as including protein phosphatase inhibition assay (PPIA) (Bouaïcha et al. 2002), enzyme-linked immunosorbent assay (ELISA) (Foss and Aubel 2015, Pilotto et al. 1997), and mouse bioassay (MBA) (Campbell et al. 1994) have been used to detect microcystins (MC-LR), rapid in situ detection for these environmental toxins is still a challenge.

The widely used electrochemical techniques have recently showed their capabilities for rapid and portable detection (Das et al. 2015, Ojeda et al. 2012, Xu et al. 2017). Some

electrochemical methods for MC-LR detection have been developed (Gan et al. 2016, Zhang et al. 2011, Zhang et al. 2010). In spite of good sensitivities, these methods suffer from the drawback that the antibodies or nano-materials used are not easy to prepare which limits their application.

In this study, we develop an electrochemical biosensor based on the deleterious effect of MC-LR on calf thymus DNA (ctDNA). We reveal such effect using spectrometric methods including ultraviolet (UV) and circular dichroism (CD) in combination with electrochemical method. The resulting change in spectral data reflects the interaction mechanism between MC-LR and DNA. In the presence of MC-LR, the randomly folded ctDNA in solution undergoes conformational change, which leads to the decrease of electron transfer impedance, and thus an electrochemical method for sensitive detection of MC-LR. The proposed method and biosensor is successfully used for detection of MC-LR in water samples.

2. Materials and methods

2.1. Materials and reagents

Potassium ferricyanide ($K_3Fe(CN)_6$) (≥99.5%), potassium ferrocyanide ($K_4Fe(CN)_6$) (≥99.5%), and potassium chloride (KCl) (≥99.5%) were purchased from SinoPharm Chemical Reagent Co., Ltd. (Shanghai, China). Methanol (chromatography grade) was purchased from Merck (Darmstadt, Germany). Ethanol (99.7%, analytical grade) and sulfuric acid (95-98%, analytic grade) were purchased from SinoPharm Chemical Reagent Co., Ltd. All aqueous solutions were prepared with ultra-pure water (18 MΩ/cm).

Calf thymus DNA (ctDNA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). 1.0 g/L stock solution of ctDNA was prepared and stored at -20°C. Its concentration was determined by Nanodrop UV spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). Microcystin-LR (MC-LR) was purchased from Enzo (Farmingdale, NY, America). 100 mg/L (100 μM) solution of MC-LR was prepared in chromatographic grade methanol (≥99.9%). After sequential 10-fold dilutions with ultra-pure water, a series of stock solutions with different concentrations (100, 10, 1, and 0.1 mg/L) were obtained and stored at -20°C.

Real water samples were collected from tap water, Xuanwu Lake (Nanjing, China), Xianlin Lake (Nanjing, China) and Taihu Lake (Yixing, Jiangsu, China). The samples were filtered with qualitative grade (GB/T 1914-2007, China) filter paper (Shuangquan, Hangzhou Xinhua, Hangzhou, China) to remove large particles, and with 0.22 μm filter paper (Xinya, Shanghai, China) to remove fine particles sequentially.

2.2. Spectroscopic characterization of the interaction between MC-LR and ctDNA

UV absorption of ctDNA, MC-LR and ctDNA-MC-LR complex were performed on Nanodrop 2000 (Thermo Fisher Scientific, San Jose, CA, USA) for samples at 2 μL scale, and UV-Vis spectrophotometer Evolution 201 (Thermo Fisher Scientific) for samples at 200 μL scale at room temperature. Circular dichroism (CD) spectra were obtained with Chirascan (Applied Photophysics, Leatherhead, Surrey, UK). A standard quartz cell of 1 mm path length was used. The spectra were expressed in terms of molar ellipticity. CD spectra were recorded from 220 nm to 320 nm in Tris–HCl (30 mM, pH=7.4) buffer containing 0.1 g/L ctDNA and 10 to 300 μg/L MC-LR at room temperature under nitrogen

air flow.

2.3. Fabrication of biosensors

Electrochemical experiments were carried out on an electrochemical workstation (RST5200F, Suzhou, China) at room temperature (25°C), equipped with a three-electrode system comprising a gold working electrode or biosensor (3mm diameter), a platinum wire as the counter electrode, and a calomel electrode as reference (GaossUnion, Shanghai, China).

To fabricate the biosensor, a bare gold electrode was first polished with 0.05 mm α-Al_2O_3 powders on a polishing micro cloth, and then sonicated in ethanol and ultra-pure water for 5 min, respectively, followed by washing with ultra-pure water (3 times with total volume about 12 mL). It was activated through cyclic voltammetric scanning between -0.2 and +1.4 V in 0.5 M H_2SO_4 solution until steady and ideal cyclic voltammogram was observed. After the electrode was washed with ultra-pure water, it was re-tested in 0.1 M KCl containing 5.0 mM $K_3Fe(CN)_6$/$K_4Fe(CN)_6$ (1:1 molar ratio) to get the control result.

5 μL of ctDNA solution (1 g/L) was carefully dropped onto the pretreated gold electrode and incubated at 37 °C for 3 h to obtain ctDNA-immobilized electrode. The excess ctDNA was washed off by ultra-pure water (3 times with total volume about 12 mL). Under the optimal conditions, the amount of ctDNA immobilized on the electrode was evaluated by collecting and concentrating all washing solutions, followed by quantitation of ctDNA using Nanodrop.

2.4. Electrochemical detection of MC-LR

Cyclic voltammetric (CV), electrochemical impedance spectroscopic (EIS) and differential pulse voltammetric (DPV) measurements were performed on RST

electrochemical workstation (RST5200F, Suzhou, China). The electrolyte solution was 0.1 M KCl containing 5.0 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$. After MC-LR standards were added in the detection solution, the solution was stirred for 3 min and settled for 4 min. The CV data were recorded at the scan rate of 0.05 V/s. The EIS data were measured within the frequency range from 1 Hz to 0.1 MHz. The DPV measurements were performed between -0.4 and 0.7 V with a step of 4 mV, pulse height of 0.05V, width of 40 ms, period of 0.1 s and sampling width of 0.01 s.

3. Results and discussion

3.1. Interaction between MC-LR and ctDNA

The interaction between MC-LR and ctDNA can be characterized with UV absorption spectroscopy by monitoring changes in the absorption properties of ctDNA (Sirajuddin et al. 2013). As shown in Figure 1, with the increasing concentration of MC-LR from 10 μg/L to 300 μg/L, the absorbance at 260 nm increases significantly, suggesting a strong interaction between MC-LR and ctDNA. The presence of methanol, a less polar solvent, compromises such interaction. The absorbance increase induced by increased MC-LR concentration was less significant with 10% methanol (Fig. 1A) than with 5% methanol (Fig. 1B). The result indicates that the interaction between MC-LR and ctDNA is favored in hydrophilic environment.

Plotting UV absorbance against MC-LR concentration results in an exponential-like curve. The tilted nature of the otherwise plateau region is caused by the UV absorption by unbound MC-LR. The data points can be fitted using Equation 1 (Fig. 1C inset).

$$Abs.=0.223+7.70\times10^{-5}\times C-0.0436\times e^{-0.0597C} \qquad \text{Eq. 1}$$

Upon subtraction of the UV absorbance caused by MC-LR ($7.70\times10^{-5}\times C$), the corrected UV absorbance caused by the MC-DNA complex can be fitted using Equation 2 (Fig. 1C).

$$Abs.=0.223-0.0436\times e^{-0.0597C} \quad \text{Eq. 2}$$

From Equation 2, 11.6 μg/L MC-LR results in the median absorbance. Thus the apparent binding constant is determined as 86.2 L/mg (Cohlberg 1979).

CD measurement is generally used to monitor the chiral conformational change of DNA induced by the interaction between DNA and small molecules. In the far UV region (220–320 nm), the conformation of DNA skeleton shows a characteristic CD pattern of the right-handed B form. The change in CD patterns in this range can reflect the structural changes of DNA (Long et al. 2008). As shown in Figure S1, the CD spectrum of unbound ctDNA (10K DNA curve, no MC-LR present) showed a positive convex at 277 nm and a negative concave at 246 nm due to base stacking and right handed helicity of ctDNA, respectively. With the increasing concentration of MC-LR from 10 μg/L to 300 μg/L, the absolute intensities of both bands increase significantly. The growth of the 277-nm band is independent on the amount of MC-LR, suggesting that a trace amount of MC-LR suffices to switch on the compromise of base pair stacking of ctDNA. The band at 246 nm, on the other hand, showed strong dependence on the amount of MC-LR, suggesting that the interference of MC-LR to the right-handed helicity of ctDNA is not a two-state process.

The significant change in UV and CD absorbance unprecedentedly showed the deleterious effect of MC-LR to ctDNA (the strong interaction between MC-LR and ctDNA). Such an effect could be utilized to develop an electrochemical biosensor for detection of MC-LR in water samples by electrochemical approaches with ctDNA coated

gold electrode. Although ctDNA was immobilized on the gold electrode in the electrochemical measurements, MC-LR in solution could still interact with randomly folded ctDNA using the same mechanism. The change in its folding pattern caused the changes in the electrochemical properties in CV, EIS and DPV measurements. Thus the deleterious effect of MC-LR provided a promising method for construction of new biosensor for MC-LR.

3.2. Electrochemical response of ctDNA immobilized gold electrode to MC-LR

At the optimal conditions, around 50% ctDNA dropped on the electrode was immobilized. Compared with the blank solution, both the responses of oxidation and reduction peaks increased and the difference of peak potentials decreased when the concentration of MC-LR increased (Figure 2). As a control, the bare gold electrode did not show these changes (Figure S2). These phenomena indicated that MC-LR interacted with the ctDNA immobilized on the electrode. This led to changes in ctDNA structure and reduced the hindrance caused by ctDNA for electron transfer between $[Fe(CN)_6]^{3-/4-}$ and the electrode, thus enhanced the current response. When the concentration of MC-LR was above 512 ng/L CV, the CV curves did not change, suggesting that the binding between ctDNA and MC-LR reached saturation at around 512 ng/L.

3.3. Analytical performance of electrochemical biosensor

EIS measurements were further carried out to characterize the binding between immobilized ctDNA and MC-LR (Li et al. 2009). In the Nyquist plot, it was obvious that increasing MC-LR concentration caused the decrease of electron transfer impedance on the ctDNA-immobilized electrode (Figure 3A). In contrast, MC-LR did not show pronounced

effect on bare gold electrode (Figure S3). These results suggested that the interaction between MC-LR and ctDNA promoted the electron transfer between $[Fe(CN)_6]^{3-/4-}$ and the electrode. Once the binding between MC-LR and ctDNA reached saturation, the electron transfer could not be further enhanced. At the MC-LR concentration of 512 ng/L the impedance reached the minimum level. Such saturation concentration agreed with that derived from CV data.

The standard deviation (SD) for thrice impedance measurements was from 0.31% to 1.58%. The plot of -*Z*" versus logarithmic value of MC-LR concentration showed a good linearity in the concentration range of 4.0 to 512 ng/L with the equation of -*Z"* (ohm) = 1021.6 - 188.75×log *C* (ng/L) and R^2 of 0.9845 (Figure 3B). At the signal of 3SD measured in blank solution, the detection limit was calculated to be 1.4 ng/L. Thus, the ctDNA-immobilized gold electrode possessed the potential application in MC-LR impedance biosensing.

In order to develop a sensitive electrochemical method for MC-LR detection, DPV measurements was used to examine the effect of MC-LR on the response of $[Fe(CN)_6]^{3-/4-}$ at ctDNA-immobilized gold electrode. Under the optimal conditions, $[Fe(CN)_6]^{3-/4-}$ showed the well-defined anodic DPV peak, whose current increased and peak potential shifted negatively with the increasing MC-LR concentration (Figure 4A). The plot of the increased peak current versus the concentration of MC-LR in the range of 4 - 256 ng/L showed a linearity with a R^2 value of 0.9904 (Figure 4B). The regression equation was ΔIp(μA) = 0.389×*C*(ng/L) (R= 0.9904), from which the detection limit was estimated to be 1.7 ng/L at 3SD.

3.4. Stability and selectivity of the electrochemical biosensor

We evaluated the stability of the proposed biosensor by reproducibility and storage stability. Under the optimized conditions, the same modified electrode was tested for eight times with relative standard deviation (RSD) of the peak current (DPV) as 5.3% showing a good reproducibility. Furthermore, after being stored for 3 days at 4 °C in fridge, the biosensor could retain 94.4% of its original response, which suggested that ctDNA/Au electrode had excellent storage stability.

We assessed the selectivity of the proposed biosensor by evaluating the cross-reactivity of the biosensor with another coexisting variant, MC-RR. Microcystin-RR (MC-RR, the microcystin variant with both X and Y are arginines) were used to study the specificity of the proposed biosensor. As shown in Fig. 5, with the increasing of MC-RR concentration, the interferences will become big. The plot of -*Z*" versus logarithmic value of MC-RR concentration showed a good linearity in the concentration range of 4.0 to 256 ng/L with the equation of -*Z"* (ohm) = 746.3 – 111.53×log *C* (ng/L) and R^2 of 0.9579 (Figure 5B). The impedance changing rate of MC-RR at -111.53 was about 60% of that of MC-LR at 188.75 and the same concentration of MC-RR caused about 60% impedance changing rate. The results showed that high concentration of MC-RR may cause some interference. Since the concentration of MC-RR in real samples normally was much lower than MC-LR, little MC-RR interference would be found in real sample detection for proposed sensor. Thus, the proposed biosensor can be used to detect MCs in general.

The cross-reactivity with some ions that potentially coexisted with MCLR in water samples, such as K^+, Cl^-, etc., were evaluated as well. As shown in Fig. 6, some cross-reactivity with other interfering substances was observed, but the degree was limited.

Reasonable selectivity of the proposed electrochemical biosensor could be confirmed. Furthermore, for applications which are very sensitive to such substances or water samples potentially containing large amount of inorganic ions, solid-phase extraction (SPE) to remove all inorganic compounds was necessary.

3.5. Determination of MC-LR in real water samples

With a standard addition method, the ctDNA-immobilized gold electrode was used as a biosensor to detect the concentrations of MC-LR in real water samples, and the results were compared with those from a conventional HPLC-MS method with and without SPE in parallel (Table 1). No MC-LR was detectable in samples from tap water in Nanjing, Xuanwu Lake and Xianlin Lake with either methods, demonstrating that the ctNDA-based biosensor did not yield false positive response. However, the samples from Taihu Lake showed detectable MC-LR with both the proposed biosensor and SPE-LC-MS (Figure S4). It should be pointed out that the LC-MS methods require a pre-concentration operation to increase the MC-LR concentration to the μg/L level, and the method developed in this study could detect MC-LR at ng/L level. After MC-LR was added to all water samples, the recoveries provided by ctDNA-base biosensor were 95.1~107.6%. The high recovery excluded the masking effect of other components in real water samples. The results showed acceptable RSD and relative errors, indicating good accuracy of the proposed method for MC-LR detection.

Table 1. Signal responses in measurements of MC-LR in real water samples

	Tap Water	Xuanwu Lake	Xianlin Lake	Taihu Lake
HPLC-MS	N.D.*	N.D.	N.D.	N.D.
SPE-HPLC-MS	N.D.	N.D.	N.D.	26.5 ± 3.0 ng/L

(200 fold conc.)				
EIS	N.D.	N.D.	N.D.	9.6 ± 0.6 ng/L
DPV	N.D.	N.D.	N.D.	25.3 ± 5.2 ng/L

* N.D.: Not detected.

4. Conclusion

The interaction between MC-LR and ctDNA induces conformational changes of DNA, and opens channels for ion transportation. We characterized the deleterious effect of MC-LR to ctDNA, and constructed an electrochemical biosensor by immobilizing ctDNA on a gold electrode based on such effect. The ctDNA-based biosensor is suitable for rapid detection of MC-LR in a label-free manner. The method features low detection limit, wide linear range and low cost in terms of simple fabrication and elimination of pre-concentration. We also demonstrated that this method can be directly applied to the real water samples from wild water bodies, serving as a promising means to facilitate the evaluation and monitoring of local water pollution.

With the low detection limit (ng/L), this method was developed for fast and cheap detection of microcystins, especially for the high-diluted samples and for in-situ tests. It is not designed for heavy polluted water samples. For samples with lots of inorganic pollutants, SPE pre-treatment is required. For such purpose, the ability of reusing the electrode would be nice but not required.

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The authors declare no conflict of interest.

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Fig. 1. UV spectra of ctDNA at different concentrations of MC-LR containing (A) 5% and (B) 10% methanol. (C) UV absorbance of complex after subtracting MC-LR absorbance versus MC-LR concentration. Inset: without subtraction.

Fig. 2. Cyclic voltammograms of ctDNA immobilized gold electrode at different concentrations of MC-LR.

Fig. 3. (A) EIS graphs of ctDNA-immobilized gold electrode at different MC-LR concentrations. (B) Impedance -Z" versus logarithmic value of MC-LR concentration. Errors are invisible on the presented scale.

Fig. 4. (A) DPV responses of ctDNA-immobilized gold electrode at different MC-LR concentrations. (B) Plot of increased peak current versus the concentration of MC-LR.

Fig. 5. (A) EIS graphs of ctDNA-immobilized gold electrode at different MC-RR concentrations. (B) Impedance -Z" versus logarithmic value of MC-RR concentration. Errors are invisible on the presented scale.

Fig. 6. The response of biosensor (current change in DPV) with respective addition of potentially interfering substances. MC-LR at 8ng/L and others at 160ng/L.

Highlights

1. Calf thymus DNA modified electrode for detection of microcystins in lake water
2. Binding of microcystins with the DNA reduced electron transfer impedance
3. Modified electrode is easy and inexpensive to make
4. Highly sensitive and highly selective

Fig. 1

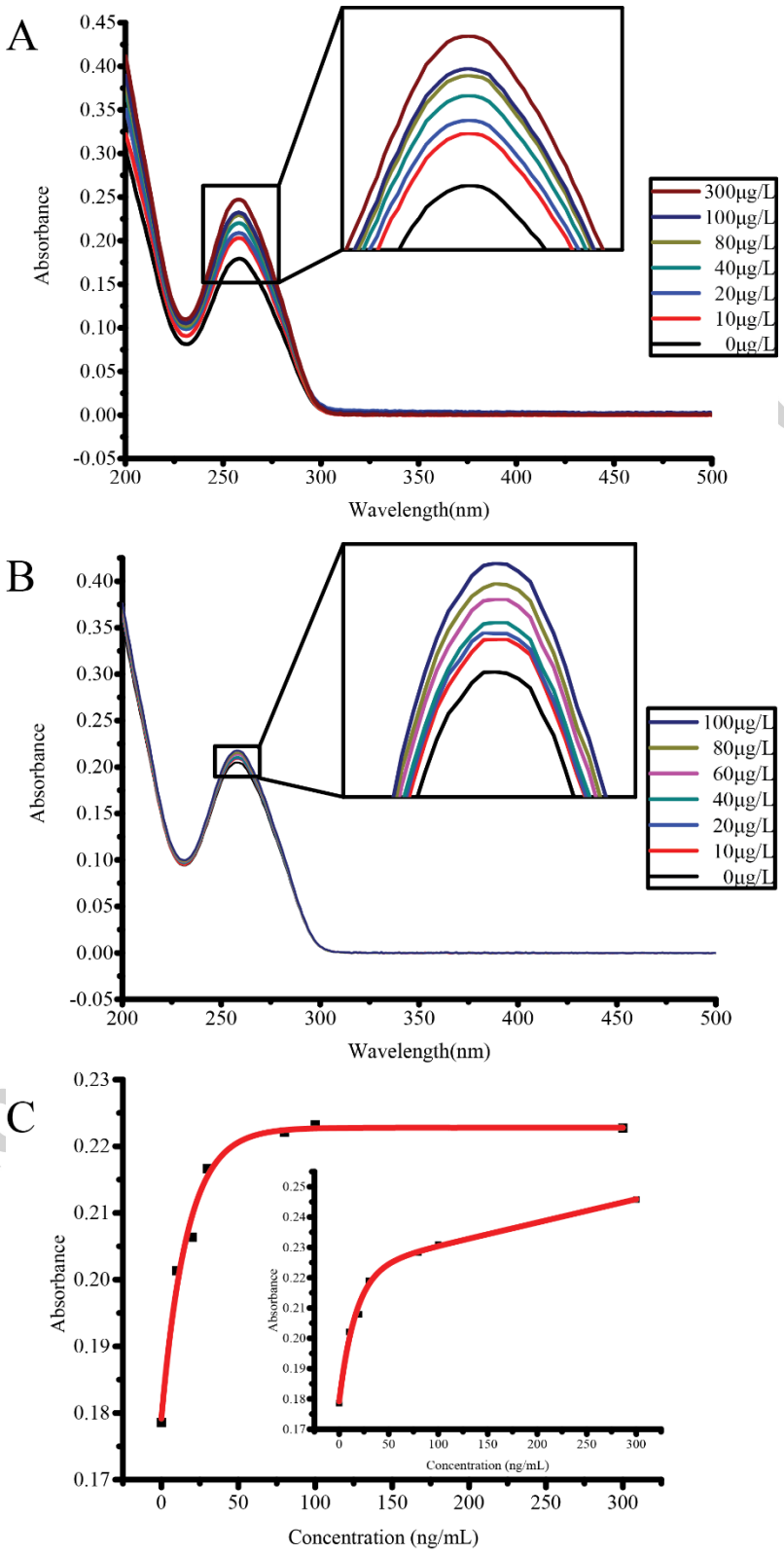

Fig. 2

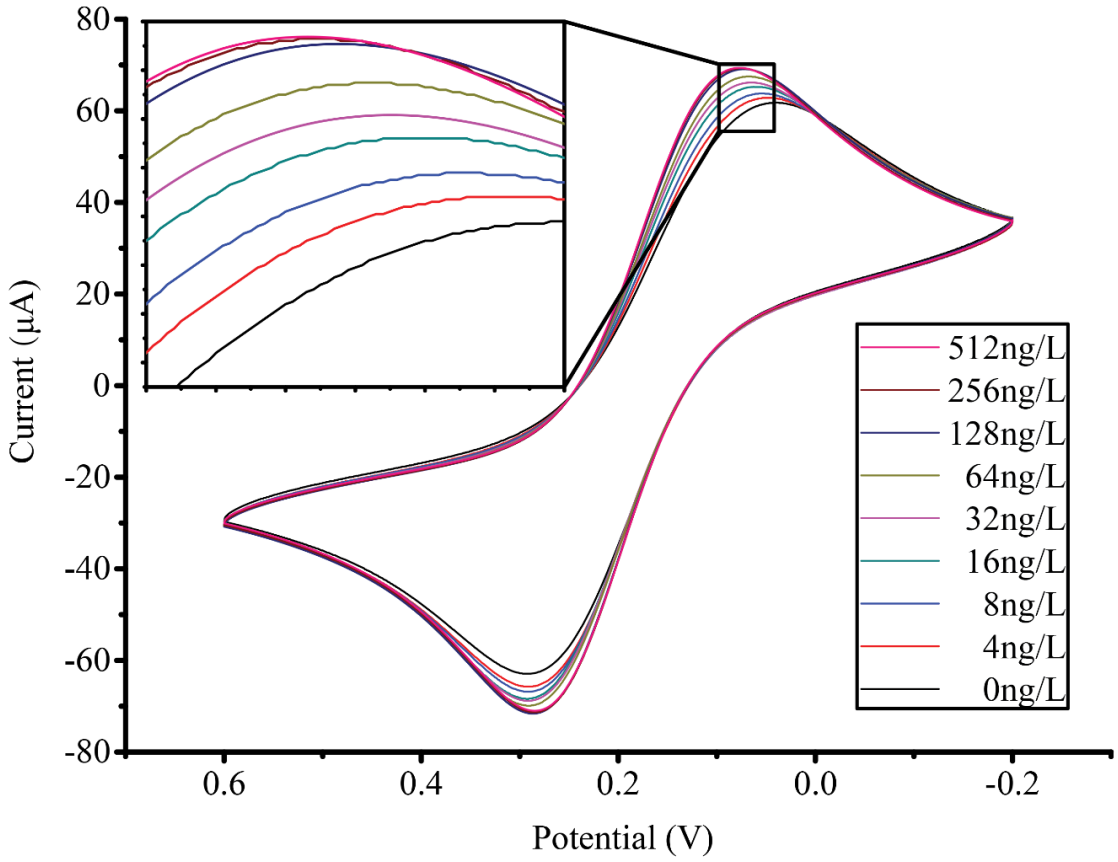
Current (μA)
Potential (V)
512ng/L
256ng/L
128ng/L
64ng/L
32ng/L
16ng/L
8ng/L
4ng/L
0ng/L

Fig. 3

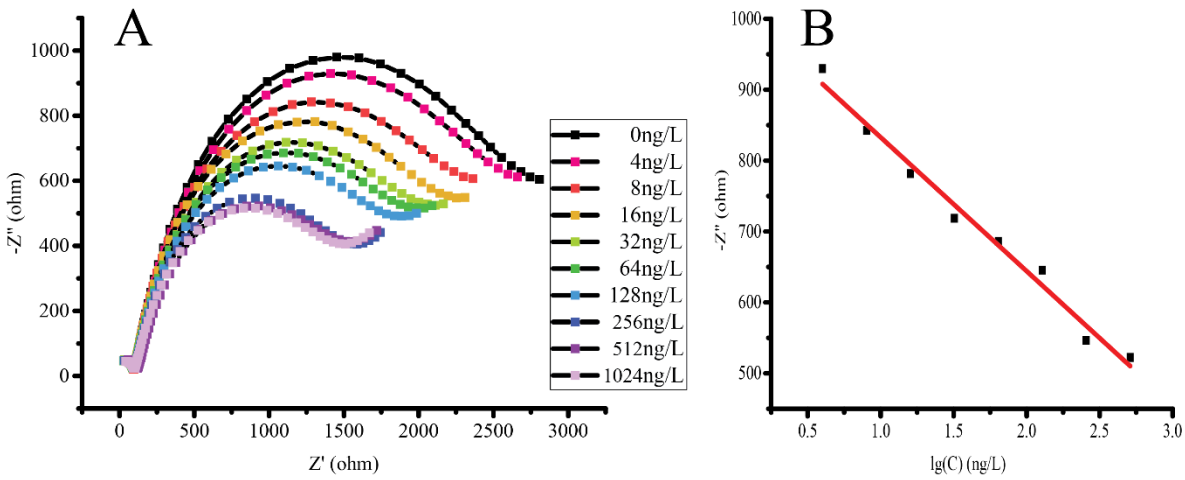
A
-Z'' (ohm)
Z' (ohm)
0ng/L
4ng/L
8ng/L
16ng/L
32ng/L
64ng/L
128ng/L
256ng/L
512ng/L
1024ng/L
B
-Z'' (ohm)
lg(C) (ng/L)

Fig. 4

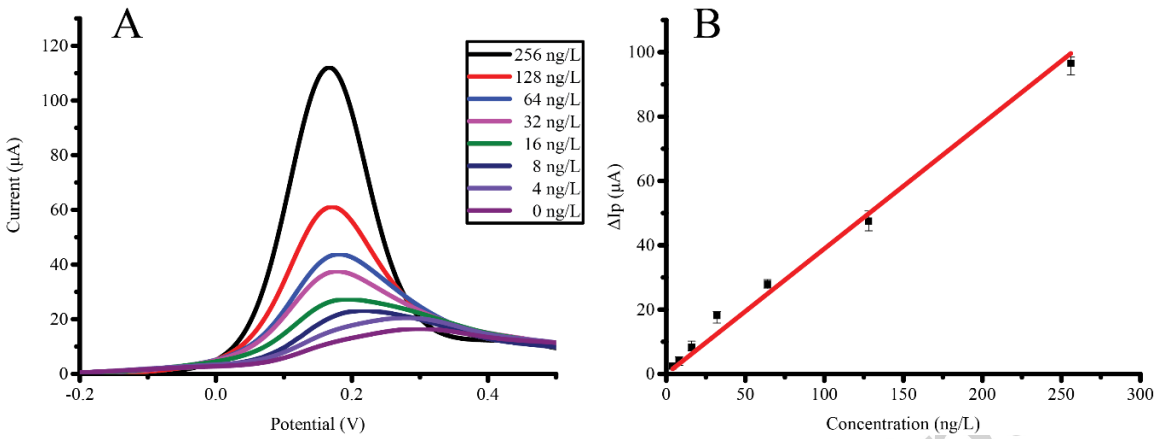

Fig. 5

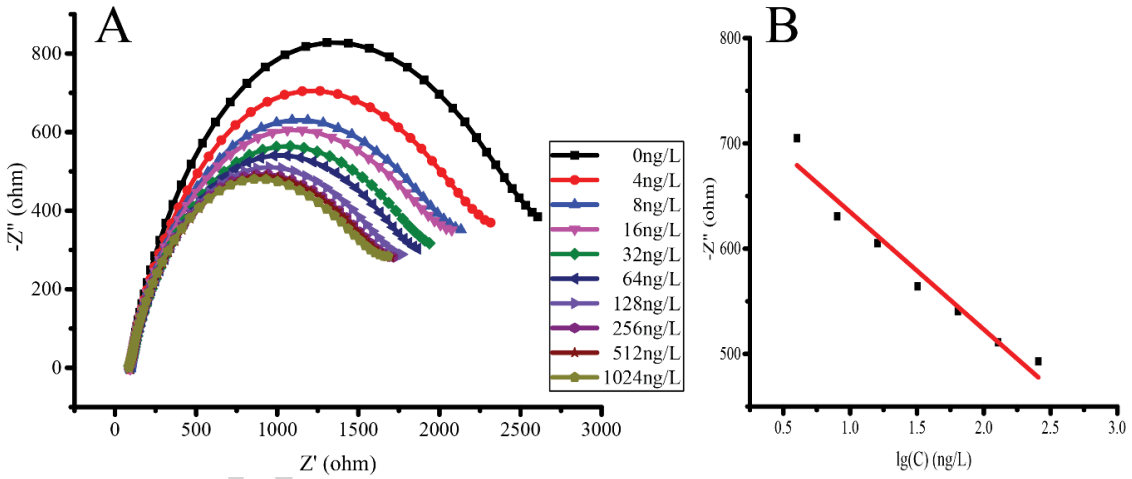

Fig. 6