An electrochemical non-enzymatic immunosensor for ultrasensitive detection of microcystin-LR using carbon nanofibers as the matrix

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Abstract:

This work designed an ultrasensitive non-enzymatic immunosensor for microcystin-LR (MC-LR) detection using carbon nanofibers (CNFs) as immobilization matrix and gold nanoparticles (AuNPs) as an electrochemical label. The carboxylic group functionalized CNFs could well disperse in water for at least three months, and CNFs modified electrode showed high conductivity and enhanced electron transfer. The immunosensor was constructed by immobilizing MC-LR antigen on CNFs modified electrode. With a competitive immunoassay format, the coated MC-LR antigen competed for MC-LR antibody with added target MC-LR to form antibody-antigen immunocomplex. Subsequently, the immunocomplex reacted with AuNPs labeled secondary antibodies, and the bound AuNPs were finally electro-oxidized in 0.1 M HCl to produce AuCl_4^- for differential pulse voltammetric detection of MC-LR. Under optimal conditions, the immunosensor exhibited a wide linear response to MC-LR ranging from 0.0025 to 5 $\mu\text{g L}^{-1}$ with a detection limit of 1.68 ng L^{-1} at a signal to noise of 3. This method showed good accuracy, acceptable precision and reproducibility. The assay results of MC-LR in polluted water were in a good agreement with the reference values. The proposed ultrasensitive immunoassay showed the promising application in environmental monitoring and public health.

Keywords: Microcystin-LR; Non-enzymatic immunosensor; Carbon nanofibers; Gold nanoparticles; Electrochemical detection

1. Introduction

Microcystins (MCs), as a family of cyclic heptapeptide toxins, are produced mainly by the common cyanobacteria and found in water source at various times all over the world [1,2]. MCs consist of five constant amino acids and two varying *L*-amino acids, and form a family of congeners which have different toxicities [3]. MCs have shown a tumor promotion effect and potent inhibition of protein phosphatases 1A (PP1A) and 2A (PP2A), two key enzymes in cellular processes [4,5]. To date, more than 90 congeners of MCs have been identified from lowly toxic to highly toxic with molecular weights from 900 to 1120 Da. Microcystin-LR (MC-LR), containing five nonproteinogens and two substitutions of leucine (L) and arginine (R) at positions 2 and 4, is the most common and hepatotoxic congener among all microcystin variants [6-8], and the LD₅₀ of MC-LR is 43 $\mu\text{g kg}^{-1}$ [9]. In 1998, the World Health Organization (WHO) had set a maximum permitted level of 1 $\mu\text{g L}^{-1}$ for MC-LR in drinking water, and the acceptable daily intake (ADI) value for MC-LR was between 0 and 0.04 $\mu\text{g kg}^{-1}$ [10]. Therefore, building more sensitive, practicable and convenient analytic method for the recognition and quantification of MC-LR is of great importance for the public health and environmental monitoring.

The analytical methods for MC-LR mainly involve high-performance liquid chromatography (HPLC) [11], liquid chromatography tandem mass spectrometry (LC-MS/MS) [12] and protein phosphatase inhibition assays PPIA [13]. Although these methods are well-proven and widely accepted, they require relatively expensive equipments, advanced technical expertise, high cost and are time-consuming. Thus, immunoassay techniques are of great interest in qualitative and quantitative detection of MC-LR due to their highly specific molecular recognition without the need for prior sample concentration or pretreatment. The best candidates for the on-site

immunoassay of MC-LR are the electrochemical immunosensors due to the high sensitivity, simplicity, easy to miniaturization, and low cost of both the sensors and the instrumentation. The first electrochemical immunosensor for MC-LR analysis was designed using screen-printed graphite electrode as support for antibody adsorption in 2007 [14]. In order to improve the sensitivity of MC-LR immunosensor, biocompatible carbon nanomaterials such as multiwalled carbon nanotubes [15], nitrogen-doped carbon nanotubes (CNTs) [16], carbon nanohorns [17], and grapheme [18] had been used to construct highly sensitive immunosensors for the detection of MC-LR.

Carbon nanofibers (CNFs), as a one-dimensional (1D) nanostructured carbon material, has been proven to be the most attractive nanomaterial due to the wide applications in the fields of nanoelectronics [19], nanolithography [20], photovoltaic devices and emission displays [21]. Recently, CNFs have been employed for the electrochemical analysis of biomolecules [22,23] and catalyst support [24,25] owing to their advantageous properties including high conductivity, wide potential window and low cost. Compared with CNTs, CNFs possesses much larger functionalized surface area and more edge sites on the outer wall [26], and CNFs is more suitable for immobilization and stability of biomolecules [27]. This work used CNFs as the immobilization matrix of small antigen molecules to propose a novel immunosensor for MC-LR.

In immunosensing and immunoassay, various materials such as enzymes [16,17], quantum dots [28], oligonucleotides [29] and dyes [30], have been used as signal tags for signal enhancement. Utilizing the direct electrochemical signal of metallic nanoparticles including gold nanoparticles (AuNPs) [31,32] and silver NPs [33,34] as labels, several substitute strategies have also been developed for ultrasensitive

detection for protein marker and DNA hybridization. AuNPs can be chemically oxidized in Br_2 or electrochemically oxidized in HCl to produce electroactive AuCl_4^- , which is then reduced to give the detectable signal. Moreover, AuNPs have shown extraordinary performance in biosensor preparation and signal amplification due to their rapid and simple chemical synthesis, narrow size distribution, and convenient labeling to biomolecules. This work employed AuNPs as the tracing tag to label second antibody by electrostatic interaction for ultrasensitive immunosensing of MC-LR.

By combining the high immobilization capability of CNFs with signal amplification of AuNPs tracing tag, this work proposed a new electrochemical non-enzymatic immunosensor for ultrasensitive detection of microcystin-LR. The immunosensor was constructed by immobilizing MC-LR antigen on CNFs modified electrode with the aid of polyethylene glycol (PEG) film, which not only assisted antigen immobilization, but also served as a potential stabilizer for binding sites of the antigen structure. After a competitive immunoassay format, the high-content AuNPs on AuNPs-labeled secondary antibodies reacted with immune complexes were electro-oxidized to produce AuCl_4^- in HCl for electrochemical measurement. The proposed method could detect MC-LR with a linear range of three orders and the detection limit down to 1.68 ng L^{-1} , and successfully detect MC-LR in polluted water samples. This strategy provided a sensitive detection platform for monitoring the toxins in biological, food and environmental fields.

2. Experimental

2.1. Materials and reagents

CNFs was a gift from WPI (Sarasota, USA). PEG ($M_w=20000 \text{ g}\cdot\text{mol}^{-1}$),

Chloroauric acid ($\text{HAuCl}_4 \cdot 4\text{H}_2\text{O}$) and Tween-20 were obtained from Sinopharm Chemical Reagent Co. Ltd. (China). Trisodium citrate was purchased from Shanghai Reagent Company (Shanghai, China). MC-LR and MC-LR antibody (anti-MC-LR) solution with the concentration of 1 mg mL^{-1} were purchased from Express Technology Co., Ltd. (Beijing, China). Bovine serum albumin (BSA) and goat anti-mouse IgG (GaMIgG) were obtained from Wuhan Boster Biological Technology Co., Ltd. (Wuhan, China). All other reagents were of analytical grade. The blocking buffer was 0.01 M pH 7.4 phosphate buffer saline (PBS) containing 5% BSA. To minimize unspecific adsorption, 0.05% Tween-20 was spiked into PBS as wash buffer (PBST). The polluted water samples were from Tai Lake in Wuxi, China.

2.2. Apparatus

Scanning electron microscopy (SEM) images were obtained on a SUPRA55 scanning electron microscope (Germany), and TEM images were gained on a JEM-2100 transmission electron microscope (JEOL, Japan). The UV-vis absorption spectra were obtained on a Shimadzu UV mini-1240 spectrometer (Japan). Fourier transform infrared spectroscopy (FTIR) was recorded on a Nicolet FTIR iN10 spectrometer (Thermo Fisher Scientific, USA). Electrochemical impedance spectroscopy (EIS) was carried out with a VersaSTAT3 electrochemical workstation (Princeton Applied Research, USA) using the three-electrode setup in 0.1 M KCl containing 5 mM $\text{K}_3[\text{Fe}(\text{CN})_6]/\text{K}_4[\text{Fe}(\text{CN})_6]$, and the impedance spectra were recorded within the frequency range of 10^{-1} - 10^5 Hz. HPLC determination of MC-LR in polluted water was carried out on a Waters 1525 high-performance liquid chromatograph (U.S.A.). Cyclic voltammetric and differential pulse voltammetry (DPV) experiments were performed with CHI660D electrochemical workstation (CH

Instruments Inc., USA) with a conventional three-electrode system with a modified GCE as working, a platinum wire as auxiliary and a saturated calomel electrode as reference electrode, respectively. All the electrochemical measurements were carried out at 298 K.

2.3. Preparation of well-dispersed CNFs solution

40 mg CNFs was dispersed in 60 mL 30% HNO₃ solution, and the resultant mixture was refluxed for 24 h at 140 °C [27]. The resulting suspension was centrifuged and the precipitate was washed thoroughly with water to get carboxylic group functionalized CNFs. The black solid was collected and dried in a vacuum at 80 °C. The resulting CNFs could well disperse in water for preparation of a 1 mg mL⁻¹ CNFs solution, and the black homogeneous solution was stable for a long time (at least 3 months).

2.4. Preparation of AuNPs and AuNPs labeled GaMIgG

The AuNPs of 13 nm diameter were obtained by reducing chloroauric acid with trisodium citrate according to a previous report [32]. 1 mL of 1% HAuCl₄ · 4H₂O solution was dissolved in 50 mL of Millipore purified water (1mM) and brought to vigorous boiling with stirring, and a 1.75 mL-portion of 1% sodium citrate was added to the solution, followed by stirring for 10 min. The solution changed from colorless to deep red. After 10 min, the resulting solution was rapidly cooled in an ice water bath with stirring. This procedure produced a stable gold nanoparticle colloid solution with an average diameter of 13 nm.

25 µL 1.0 mg mL⁻¹ secondary antibodies GaMIgG (Ab₂) solution was added to 1

mL AuNPs solution adjusted to pH 9.0 with 0.1 M K_2CO_3 , followed by incubation at room temperature with lightly shaking for 1 h. The conjugate was then centrifuged at 4500 g for 5 min. The supernatant was discarded and the soft sediment of immunocomplex was rinsed with PBS for three times, after centrifugation at 4500 g for another 5 min, the conjugate was resuspended in 300 μL PBS containing 1% BSA for immunoassay. The resulting AuNPs labeled Ab_2 (AuNPs-Ab_2) could be stored at 4 °C for more than 2 weeks without loss of activity.

2.5. Preparation of MC-LR Immunosensor

The glassy carbon electrode (GCE, 3 mm diameter) was polished successively with 0.3 and 0.05 μm alumina slurry (Beuhler), followed by rinsing thoroughly with doubly distilled water. After successive sonication in 1:1 nitric acid, ethanol, and deionized water, the electrode was rinsed with deionized water and allowed to dry at room temperature. 5 μL of 1.0 $\text{mg}\cdot\text{mL}^{-1}$ carboxylic group functionalized CNFs suspension was dropped on the pretreated GCE and dried in a desiccator. After drying, dropping 5 μL of 2.0 $\text{mg}\cdot\text{mL}^{-1}$ PEG solution onto the CNFs modified GCE. The modified GCE was incubated in water-vapor-saturated environment at 4 °C overnight to allow the passive adsorption of PEG onto CNFs modified GCE surface. After washing with water, 10 μL of 500 $\mu\text{g}\cdot\text{L}^{-1}$ MC-LR was immediately dropped on the modified GCE surface and then incubated for 1h to form MC-LR/PEG/CNFs/GCE. Then, the electrode surface was washed with 0.01 M pH 7.4 PBST, the formed immunosensor was blocked with 0.01 M pH 7.4 PBS containing 5% BSA for 1h to block possible remaining active sites against nonspecific adsorption. The preparation of the MC-LR immunosensor is shown in Scheme 1. After thoroughly washing with 0.01M pH 7.4 PBST, the obtained immunosensor was stored at 4 °C prior to use.

2.6. Analytical Procedure

5 μL MC-LR solutions with different concentrations or water samples were mixed with 5 μL of 1:120 diluted MC-LR antibody to obtain the incubation solution. As shown in [Scheme 1](#), the incubation solution was dropped on the MC-LR immunosensor and incubated for 1 h at 25 $^{\circ}\text{C}$ and then washed carefully with 0.01 M pH 7.4 PBST to obtain anti-MC-LR/MC-LR/PEG/CNFs/GCE. Secondly, 10 μL AuNPs-Ab₂ solution was casted on the immunosensor incubated for 1 h at 25 $^{\circ}\text{C}$ to form AuNPs-Ab₂/anti-MC-LR/MC-LR/PEG/CNFs/GCE. During the incubation process, the immunosensor was placed in a container to avoid the evaporation of incubation solution. After washing with PBST, the electrochemical oxidation of AuNPs was performed in 2 mL 0.1 M HCl at a constant potential of 1.40 V for 30 s, and immediately the DPV detection was obtained from 0.6 to 0.1 V with a pulse amplitude of 50 mV and a width of 0.1s. The data for condition optimization and the calibration curve were the average of three measurements.

3. Results and discussion

3.1. Characterization

The morphologies of CNFs were characterized by SEM and TEM. As shown in [Fig. 1](#), the morphologies of the CNFs film displayed a chemically clean, three-dimensional homogeneous incompact structure. The diameter of the CNF was in the range from 30 to 100 nm. The CNFs could be used as excellent matrix for loading of biomolecules because of good monodispersity, the significant increase of effective electrode surface and versatility for surface functionalization. Also, the remarkable conductivity of CNFs could provide a feasible pathway for electron transfer with

interconnected nanofibers to promote the diffusion of signal molecules. These properties of CNFs provided a desirable platform for biosensing.

The UV-vis spectra of AuNPs and AuNPs-Ab₂ were shown in Fig. 2A. The UV-vis absorption spectrum of the Au colloidal suspension featured a sharp peak at 518 nm, consistent with the literature-reported peak for 13.0 nm diameter AuNPs [35]. Aggregation of the AuNPs after Ab₂ caused a red shift (524 nm), which could be attributed to the bonding between the AuNPs and the amino groups of the antibody molecules caused by the simple delocalization of the lone pair of electrons of an amino nitrogen atom to coordinatively unsaturated surface Au atoms [36]. The conjugation of Ab₂ on AuNPs was further confirmed by the IR spectrum (Fig. 2B). After conjugation of Ab₂ to AuNPs the vibration of amide I and amide II of Ab₂ could be observed at 1660 and 1540 cm⁻¹, indicating that Ab₂ was successfully conjugated onto AuNPs.

The electrochemical behaviors of bare GCE and CNFs/GCE were investigated using CV with Fe(CN)₆^{3-/4-} as a redox probe (Fig. 3A). A redox peak shapes was showed at the bare GCE and the peak current was 131 μA (curve a). The currents of ferricyanide anodic and cathodic peaks at CNFs/GCE largely increased and the peak current increased to 237 μA (curve b), indicating improved electron transfer and mass transfer, which could be contributed to the high surface area providing by the three dimensional structure of CNFs.

Fig. 3B shows the Nyquist plots of bare GCE and CNFs/GCE in the frequency range from 10⁻¹ to 10⁵ Hz using Fe(CN)₆^{3-/4-} redox couple as the electrochemical probe. To analyze the electrode's impedance characteristics a modified Randles equivalent circuit (Fig. 3B inset) was chosen to fit the measured results. The circuit contains a constant phase element (CPE), charge-transfer resistance (R_{ct}), the

Warburg impedance (Z_w), and electrolyte resistance (R_s). At bare GCE the redox process of the probe showed an electron transfer resistance (R_{ct}) of about 80 Ω (curve a). After CNFs was coated on the electrode, the resistance decreased to about 25 Ω (curve b), further implying that CNFs was an excellent electric conducting material. The result is consistent with the CV test.

3.2. Electrochemical performance of immunosensor

In order to obtain the minimum nonspecific adsorption and good biocompatibility, PEG was selected as a bridge or antigen immobilization because of its known biocompatibility and capacity to undergo a variety of interactions with proteins including bovine serum albumin (BSA), which acted to suppress the possible remaining active sites against nonspecific adsorption [37,38]. After MC-LR immunosensor incubated MC-LR antibody and AuNPs-Ab₂ solution successively, MC-LR immunosensor binds MC-LR antibody to form the immunocomplex. Subsequently, the immunocomplex reacted with AuNPs labeled secondary antibodies, and the bound AuNPs were finally electro-oxidized in 0.1 M HCl to produce AuCl₄⁻. As displayed in the curve a of Fig. 4, the immunosensor showed a large DPV peak current at 0.40 V in 2 mL 0.1 M HCl, which derived from both effects of modified electrode to adsorb more capture antigen and AuNPs to produce electro-oxidized AuCl₄⁻ in 0.1 M HCl. As a control, in the absence of MC-LR antibody, it did not show any obvious response in 0.1 M HCl (Fig. 4, curve b), indicating the elimination of nonspecific adsorption on the electrode surface by BSA. Thus, the proposing immunosensor showed an excellent performance with high sensitivity of DPV signal, good biocompatibility to retain their specific immunorecognition, and a feasible ability against nonspecific adsorption.

After immunoreactions, the electrochemical activity of AuNPs-Ab₂/anti-MC-LR/MC-LR/PEG/CNFs/GCE was investigated by electro-oxidization and then by DPV measurement. After incubation with 5 μ L of 1:120 diluted MC-LR antibody and 10 μ L AuNPs-Ab₂ solution successively,

3.3. Optimization of Conditions for Electrochemical Detection and Immunoreaction

The conditions for electro-oxidization of the captured AuNPs are important parameters to the analytical performance of the immunosensor. Preoxidation potential was crucial because too low potential would not be able to oxidize gold in HCl to AuCl₄⁻, whereas too high potential would cause damage of the modified electrode surfaces and led to poor stabilization. The oxidation potential was investigated with DPV detection after the preoxidation of the captured AuNPs in 0.1 M HCl at a constant potential for 30 s. As shown in Fig. 5A, the peak current increased continuously as the oxidation potential increasing from +1.2 V to +1.4 V, and reached the maximum value at +1.40 V, following with a large decrease in the potential range of +1.4 V -+1.45 V. Therefore, +1.40 V was chosen as the optimal preoxidation potential.

The influence of preoxidation time on electro-oxidization of the captured AuNPs was investigated. As shown in Fig. 5B, the peak current increased as the preoxidation time increasing from 20 to 30 s, afterwards the response decreased as the preoxidation time further increasing in range of 30-45 s. An insufficient time would lead to the incomplete oxidation of gold and a redundant time would not increase the electrochemical signal, while AuCl₄⁻ might diffuse away slowly from electrode

surface. Thus, 30 s was selected as the optimal preoxidation time.

Under optimal detection conditions, the performance of the prepared immunosensor was closely related to the concentration of immobilized MC-LR. As displayed in Fig. 6A, the DPV signals increased with increasing concentration of immobilized MC-LR solution and trended to the plateau values at the concentration of $500 \mu\text{g}\cdot\text{L}^{-1}$ for MC-LR. Thus, $500 \mu\text{g}\cdot\text{L}^{-1}$ MC-LR was chosen as the working solution.

The electro-oxidize response of the captured AuCl_4^- depends on the formation of immunocomplex on the electrode surface, which is decided by the concentration of MC-LR antibody in the incubation solution. In order to obtain the optimal concentration of the MC-LR antibody, the MC-LR/PEG/CNFs/GCE was incubated in MC-LR antibody solutions with different concentrations. As shown in Fig. 6B, with the decreasing dilution or increasing concentration of MC-LR antibody the DPV signals increased and trended to the plateau values at the dilutions of 1:120 for MC-LR antibody, which indicated all of the specific sites of the immobilized antigen were occupied. Thus, 1:120 dilution of anti-MC-LR was chosen as the working antibody solution.

The concentration of AuNPs-Ab₂ solution had been investigated in this work. As shown in Fig. 6C, with the increasing dilution or decreasing concentration of AuNPs-Ab₂ solution the DPV signals decreased. Thus, the prepared AuNPs-Ab₂ solution without any dilution was chosen as the working solution.

3.4. Effect of scan rate on the reduction response of AuCl_4^- at the electrode

The effect of scan rate on the reduction response of AuCl_4^- at AuNPs-Ab₂/anti-MC-LR/MC-LR/PEG/CNFs/GCE was investigated in 0.1 M HCl by

cyclic voltammetry (Fig. 7A). As shown in Fig. 7B, the reduction peak current of the proposed electrode increased linearly with the scan rate in the range of 40-160 mV s⁻¹ and can be expressed as the equation of $I_{pa} (\mu A) = 0.059 v (mV s^{-1}) - 1.96$ ($R^2 = 0.987$), indicating that the reduction of AuCl₄⁻ is an adsorption controlled process.

3.5. Immunoassay performance of the immunosensor to MC-LR

For the measurement of MC-LR, a competitive assay configuration was applied under optimized conditions. A standard solution of MC-LR with a known concentration was added into the incubation solution containing a certain concentration of MC-LR antibody. The MC-LR in the incubation solution competed with the immobilized MC-LR on the immunosensor surface to bind the limited binding sites of the MC-LR antibody to form the immunocomplex. Subsequently, the immunocomplex reacted with AuNPs labeled secondary antibodies, and the bound AuNPs were finally electro-oxidized in 0.1 M HCl to produce AuCl₄⁻. DPV peak current of AuNPs decreases with the decreasing immunocomplex amount. Increased MC-LR in the incubation solution causes decreased immunocomplex of MC-LR antibody and immobilized MC-LR at the electrode. Therefore, DPV peak current of AuNPs decreased with the increasing MC-LR concentration in the incubation solution (Fig. 8A). As shown in Fig. 8B, the decrease of DPV peak current was proportional to the logarithm value of MC-LR concentration in the range of 0.0025-5 μg L⁻¹ with a linear regression equation of $I_{pa} (\mu A) = -0.348 \lg C (\mu g L^{-1}) + 2.735$ ($R^2=0.996$). The detection limit was calculated to be 1.68 ng L⁻¹ at a signal-to-noise ratio of 3, at which the amperometric response and the noise were 17 and 5.5 nA (S/N~3), respectively. Comparison of different immunosensor techniques used for MC-LR detection was shown in Table 1, and the proposed MC-LR immunosensor had the lowest detection

limit. The detection limit of this immunosensor was also much lower than the WHO provisional guideline limit of $1 \mu\text{g L}^{-1}$ for MC-LR in drinking water. According to the analytical process, the detectable linear ranges in the sample with a dilute factor of 2 by adding $5 \mu\text{L}$ MC-LR antibody were $0.005\text{--}10 \mu\text{g L}^{-1}$ with the detection limit of 3.36 ng L^{-1} . Thus the proposed method was enough for practical application. The good performance of the proposed MC-LR immunosensor could be attributed to the combination of enhanced electrocatalytic activity of CNFs, good biocompatibility of PEG, and signal electrochemical amplification of AuNPs.

3.6. Precision, reproducibility, stability, and reusability of MC-LR Immunosensor.

The intra-assay variation coefficients for five replicative measurements with this method at MC-LR concentrations of 0.5 and $5 \mu\text{g L}^{-1}$ were 3.4% and 3.2% (Fig. 9A), respectively, showing a good repeatability. While the inter-assay variation coefficients at these concentrations on five immunosensors made independently were 1.3% and 1.5% (Fig. 9A), respectively, indicating excellent fabrication reproducibility. When the immunosensor was not in use, it was stored at 4°C . 93.4% of the initial response of the immunosensor for MC-LR was remained after one week and 86.7% of the initial response was remained after four weeks (Fig. 9B). These results indicated the immunosensor had acceptable stability. The regeneration of the immunosensor was developed by rinsing with stripping buffer of pH 2.8 Glycine–HCl solution to dissociate the antigen–antibody complex. The as-renewed immunosensor could restore 96.5% of the initial value after five assay runs (Fig. 9C), showing high reusability.

3.7. Analysis of polluted water samples.

To evaluate the analytical reliability and application potential of the proposed

method, 5 μL of water samples without any need of sample pretreatment appropriate dilution except for an appropriate dilution was mixed with 5 μL MC-LR antibody, and the mixture was dropped on the MC-LR immunosensor, subsequently, 10 μL AuNPs-Ab₂ solution was casted on the immunosensor. The MC-LR concentrations determined with this method were 1.14 ± 0.05 and $5.22 \pm 0.02 \mu\text{g L}^{-1}$, which is consistent with the values of 1.17 ± 0.03 and $5.33 \pm 0.05 \mu\text{g L}^{-1}$ from HPLC. Recovery testing was carried out to demonstrate the validity of the proposed method, after 1 $\mu\text{g L}^{-1}$ of MC-LR were added to two samples, and the obtained recoveries of MC-LR were $98.0 \pm 1.4\%$ and $99.2 \pm 2.3\%$, respectively (five measurements), which indicated acceptable accuracy. Thus, the present method could satisfy the need for immunoassay of MC-LR in water samples.

4. Conclusions

An electrochemical immunosensor based on CNFs as a sensor platform and AuNPs as the label was developed for MC-LR detection. The electro-reduction of AuCl_4^- produced by the oxidation of AuNPs was monitored for the detection of MC-LR. The modification of CNFs on the electrode increased the conductivity of the sensor probe and accelerated the electron transfer reaction, thus enhanced the immunosensor's response. With a competitive immunoassay format, the designed immunosensor for MC-LR had good performance with high sensitivity, wide linear range, acceptable precision and fabrication reproducibility, and excellent stability. It had been successfully applied in the detection of MC-LR in polluted water samples. The proposed immunosensor can be applied in toxin detection, and thus could be a

valuable tool for the detection of hazard components in environmental and food samples.

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Figure captions

Scheme 1. Schematic representation of the preparation and detection procedure of MC-LR immunosensor.

Fig. 1. SEM (A,B) and TEM (C) images of CNFs.

Fig. 2. UV-vis absorption spectra (A) of AuNPs and Ab₂-AuNPs, and IR spectrum (B) of Ab₂-AuNPs.

Fig. 3. Cyclic voltammograms (A) and EIS (B) in 0.1 M KCl containing 5 mM K₃[Fe(CN)₆]/K₄[Fe(CN)₆] at bare GCE (a) and CNFs modified GCE (b). Scan rate: 50 mV s⁻¹.

Fig. 4. DPV responses (A) and peak currents (B) of the immunosensor incubated with AuNPs-Ab₂ in presence of MC-LR antibody (a) and AuNPs-labeled GaMIgG in the absence of MC-LR antibody (b).

Fig. 5. Effects of preoxidation potential (A) and preoxidation time (B) on DPV peak currents of AuNPs-Ab₂/anti-MC-LR/MC-LR/PEG/CNFs/GCE obtained under optimal conditions in 0.1 M HCl.

Fig. 6. Effects of the concentration of immobilized MC-LR (A), MC-LR antibody (B) and AuNPs-Ab₂ (C) on DPV peak currents of AuNPs-Ab₂/anti-MC-LR/MC-LR/PEG/CNFs/GCE obtained under optimal conditions in 0.1 M HCl.

Fig. 7. Cyclic voltammograms of AuNPs-Ab₂/anti-MC-LR/MC-LR/PEG/CNFs/GCE with different scan rates with 40, 60, 80, 100, 120, 140 and 160 mV s⁻¹ in 0.1 M HCl (A), and the calibration curve of current and scan rate (B).

Fig. 8. DPV responses of the immunosensor in 0.1 M HCl after incubated with 0.0025, 0.025, 0.05, 0.25, 0.5, 2.5 and 5 µg L⁻¹ MC-LR (from highest to lowest peak currents) (A) and calibration curve for MC-LR immunoassay (B).

Fig. 9. DPV peak currents of the proposed immunosensor in intra-assay and inter-assay (A), stability (B) and reusability (C) experiments

Methods	Analytical ranges (LODs)	Comments	References
Electrode modification			
Electrochemical A multiwalled carbon nanotube/MC-LR/anti-MC-LR	0.05–20 $\mu\text{g L}^{-1}$ (0.04 $\mu\text{g L}^{-1}$)	High sensitivity, and good reproducibility and repeatability	15
Electrochemical HRP-MC-LR/anti-MC-LR/(Au/CNx-MWNTs)/GCE	0.005–1 $\mu\text{g L}^{-1}$ (0.002 $\mu\text{g L}^{-1}$)	Low detection limit and long-term stability	16
Electrochemical HRP-anti-MC-LR/MC-LR-SWNHs/GCE	0.05–20 $\mu\text{g L}^{-1}$ (0.03 $\mu\text{g L}^{-1}$)	High sensitivity, and good accuracy and precision	17
Electrochemical Pt ₇₀ Ru ₃₀ -Ab ₂ /MC-LR/GS-Ab ₁ /GCE	0.01–28 $\mu\text{g L}^{-1}$ (9.63 ng L ⁻¹)	Nonenzymatic detection and good reproducibility	18
electrochemical impedance MC-LR aptamers/Au	0.05–100 $\mu\text{g L}^{-1}$ (18 ng L ⁻¹)	Lable-free assay, wide linear range, and good selectivity and stability	39
Chemiluminescence MB/PEI/Ab ₁ -MC-LR/Si-Ab ₂	0.02–200 $\mu\text{g L}^{-1}$ (0.006 $\mu\text{g L}^{-1}$)	Low detection limit, and acceptable precision and accuracy	40
Electrochemical HRP-CNSs-Ab/MC-LR/GS-s-CS/GCE	0.05–15 $\mu\text{g L}^{-1}$ (0.016 $\mu\text{g L}^{-1}$)	High sensitivity and acceptable repeatability	41
QCM biosensor Gold-anti-MC-LR/ C18N3	(1 ng L ⁻¹)	Portability, simplicity, and cost-effectiveness	42
Photoelectrochemical CdS-graphene composite	0.01–25 $\mu\text{g L}^{-1}$ (0.021 $\mu\text{g L}^{-1}$)	Lable-free and rapid detection	43
Electrochemical MC-LR/BSA/anti-MC-LR/GNP/L-cysteine	0.05–15 $\mu\text{g L}^{-1}$ (20 ng L ⁻¹)	Lable-free assay and good stability	44
Electrochemical Au/anti-MC-LR/MC-LR/PE G/CNFs/GCE	0.0025–5 $\mu\text{g L}^{-1}$ (0.0017 $\mu\text{g L}^{-1}$)	Wide linear range and low detection limit	This work

Table 1 Comparison of different immunosensor techniques used for MC-LR detection

CNx-MWNTs: nitrogen-doped carbon nanotubes, SWNHs: single-walled carbon nanohorns, GS: grapheme Sheet, MB: magnetic beads, PEI: polyethyleneimine, CS: chitosan, QCM: quartz crystal microbalance, C18N3: bis (amidoethyl-carbamoyl ethyl) octadecylamine, BSA: Bovine serum albumin, GNP: gold nanoparticle

Graphical Abstract:

An electrochemical non-enzymatic immunosensor for ultrasensitive detection of microcystin-LR using carbon nanofibers as the matrix

By Jing Zhang, Yufeng Sun, Haifeng Dong, Xueji Zhang, Wenchang Wang, Zhidong Chen

We designed an ultrasensitive non-enzymatic immunosensor for microcystin-LR detection using carbon nanofibers as immobilization matrix and gold nanoparticles as an electrochemical label, and the proposed immunosensor successfully detected microcystin-LR in polluted water samples.