A sensitive electrochemical immunosensor was proposed by functionalizing single-walled carbon nanohorns (SWNHs) with analyte for microcystin-LR (MC-LR) detection. The functionalization of SWNHs was performed by covalently binding MC-LR to the abundant carboxylic groups on the cone-shaped tips of SWNHs in the presence of linkage reagents and characterized with Raman spectroscopy, X-ray photoelectron spectroscopy, scanning electron microscopy, and a transmission electron micrograph. Compared with single-walled carbon nanotubes, SWNHs as immobilization matrices showed a better sensitizing effect. Using home-prepared horseradish peroxidase-labeled MC-LR antibody for the competitive immunoassay, under optimal conditions, the immunosensor exhibited a wide linear response to MC-LR ranging from 0.05 to 20 µg/L with a detection limit of 0.03 µg/L 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 strategy provided a biocompatible immobilization and sensitized recognition platform for analytes as small antigens and possessed promising application in food and environmental monitoring.

Cyanobacterial bloom has significant hazard to public health and the environment due to the release of cyanotoxins into water supplies. One of the most common cyanotoxins is cyclic hepatotoxin toxin of microcystins (MCs), which may cause mainly functional and structural disturbances of the liver due to a marked inhibition of protein phosphatases 1 and 2A. 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 toxic species. In 1998, the World Health Organization (WHO) set up a provisional guideline limit of 1 µg/L for MC-LR in drinking water. Thus, the recognition and quantification of MC-LR are of great importance in the analysis of environmental samples.

The analytical techniques for MC-LR detection usually involve thin-layer chromatography, high-performance liquid chromatography (HPLC), liquid chromatography/mass spectrometry, and protein phosphatase inhibition assays. Although these methods are well-proven and widely accepted, they require relatively expensive equipment, advanced technical expertise, and 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, ease of miniaturization, and low cost of both the sensors and the instrumentation. The first electrochemical immunosensor...
for MC-LR analysis was designed using a screen-printed graphite electrode as support for antibody adsorption in 2007.\textsuperscript{23} An immunosensor for the electrochemical enzyme immunoassay of MC-LR was also proposed by immobilizing MC-LR antibody on a double-sided microporous gold electrode.\textsuperscript{24} These immunosensors used enzyme labeled MC-LR as signal element. The preparation and purification of enzyme labeled small antigen molecules are very complicated, and the physical adsorption may cause unacceptable precision, reproducibility, and stability. Alternately, searching a biocompatible nanomaterial for the effective immobilization and presentation of small antigen molecules to construct a highly sensitive immunosensor is of considerable interest for the detection of MC-LR.

Single-walled carbon nanohorns (SWNHs), as dahlia flowerlike spherical aggregates (diameters of about 80–120 nm), are composed of thousands of graphitic tubule closed ends with cone-shaped horns\textsuperscript{25,26} and have large surface area, excellent conductivity, plentiful inner nanospaces, and highly defective horns.\textsuperscript{27,28} The oxidation treatment of SWNHs can produce extensive oxygen-functionalized sites exposed on the cone-shaped tips\textsuperscript{29} for immobilization of biomacromolecules\textsuperscript{30} and drug delivery in cancer phototherapy.\textsuperscript{31} This work used SWNHs as an immobilization scaffold of small antigen molecules to propose a novel immunosensor for MC-LR.

The immobilization of MC-LR on SWNHs could be conveniently performed on an electrode surface by covalently binding MC-LR to the carboxylic groups on the cone-shaped tips of SWNHs. Due to the three-dimensional recognition, SWNHs showed an efficient sensitizing action, leading to a high sensitivity and a wide linear range for rapid immunoassay of MC-LR. The designed immunosensor for MC-LR had acceptable precision and reproducibility. The good biocompatibility of SWNHs and simple preparation resulted in excellent stability of the immunosensor. It could be successfully applied in the detection of MC-LR in Tai Lake water without any pretreatment. These results demonstrated SWNHs are an excellent matrix for immobilization of small toxin residues and then sensitive recognition to labeled antibody for immunoassay of toxins. This strategy provided a useful tool for monitoring the hazard components in biological, food, and environmental fields.

**EXPERIMENTAL SECTION**

**Materials and Reagents.** SWNHs were kindly provided by Professor Sumio Iijima, who leads the carbon nanotube project in the Japan Science and Technology Agency. Single-walled carbon nanotubes (SWNTs) (<2 nm diameter) were purchased from Shenzhen Nanotech Port Ltd. Co. (China). N-Hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethyaminopropyl) carbodiimide (EDC), and horseradish peroxidase (HRP) were purchased from Sigma-Aldrich Inc. (U.S.A.). Bovine serum albumin (BSA) was obtained from Sigma (St. Louis, MO). All other reagents were of analytical grade. The blocking buffer was 0.01 M, pH 7.4 phosphate buffer saline (PBS) containing 2% BSA. To minimize unspecific adsorption, 0.05% Tween-20 was spiked into PBS as wash buffer (PBST). The detection buffer was 0.2 M, pH 7.4 PBS. Ultrapure water obtained from a Millipore water purification system (≥18 MΩ, Milli-Q, Millipore) was used in all runs. MC-LR was purchased from Express Technology Co., Ltd. (China). MC-LR stock solution (1 mg/mL) was prepared in 0.01 M, pH 7.4 PBS and stored at 4 °C. The polluted water samples were from Tai Lake in Wuxi, China.

SWNHs (40 mg) were dispersed in 60 mL of 30% HNO\textsubscript{3} and then refluxed for 24 h at 140 °C to obtain carboxylic group functionalized SWNHs. The resulting suspension was centrifuged, and the precipitate was washed thoroughly with water until the pH value was about 7.0. The black solid was collected and dried in a vacuum at 80 °C. Then, the oxidized SWNHs were dispersed in deionized water to a concentration of 0.5 mg/mL. As a control, SWNTs were treated with the same procedure.

**Apparatus.** Scanning electron microscopic (SEM) images were obtained using a Hitachi S-4800 scanning electron microscope (Japan). The transmission electron micrograph (TEM) was gained on a JEM-2100 TEM (JEOL, Japan). Infrared spectra were recorded on a Nicolet 400 Fourier transform infrared spectrometer (Madison, WI). Resonance Raman spectra were measured with the excitation wavelength of 514 nm on a Renishaw-inVia Raman microscope (Renishaw, United Kingdom). X-ray photoelectron spectroscopic (XPS) measurements were performed with an ESCALAB 250 spectrometer (Thermo VG Scientific, U.S.A.) with an ultrahigh vacuum generator. High-performance liquid chromatographic determination of MC-LR in polluted water was carried out on a Waters 1525 high-performance liquid chromatograph (U.S.A.). Electrochemical measurements were performed on a CHI 610C electrochemical analyzer (Co., CHI, U.S.A.) with a conventional three electrode system with a modified glassy carbon electrode (GCE) as working, a platinum wire as auxiliary, and a saturated calomel electrode as reference electrode, respectively. All measurements were carried out at 25 °C.

**Preparation of HRP-Labeled MC-LR Antibody (HRP-anti-MC-LR).** First, MC-LR antibody was prepared according to our previous method.\textsuperscript{32} Fresh NaI\textsubscript{2}O solution (0.5 mL of 0.06 mol/L) was mixed with 0.5 mL of 10 mg/mL HRP solution and kept for 30 min at 4 °C, followed by adding 0.5 mL of 0.16 mol/L glycol. Then, 1 mL of 5 mg/mL MC-LR antibody was dispersed into the mixture, and the pH of the mixture was adjusted to 9 using 0.05 M pH 10.0 carbonate buffer saline. After reacting for 16–24 h at 4 °C, 0.2 mL of 5 mg/mL NaBH\textsubscript{4} solution was added and incubated for 2 h at 4 °C to produce HRP-anti-MC-LR, followed by dropwise addition of 0.2 mL of saturated NaI\textsubscript{2}O solution.
ammonium sulfate to precipitate HRP-anti-MC-LR. After centrifugation at 4000 rpm for 15 min, the obtained bioconjugate was redispersed in 0.02 mol/L, pH 7.4 PBS and further purified by dialysis overnight to obtain a solution containing 765 µg/mL HRP-anti-MC-LR.

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, acetone, and deionized water, the electrode was rinsed with deionized water and allowed to dry at room temperature. A carboxylic group functionalized SWNH suspension (6 µL of 0.5 mg/mL) was dropped on the pretreated GCE and dried in a desiccator. The SWNHs-coated GCE was then immersed in a solution containing 2 mM EDC and 5 mM NHS for 1 h. After the activated SWNHs/GCE was thoroughly rinsed with deionized water, 5 µL of 200 µg/mL MC-LR was immediately dropped on its surface and then incubated for 2 h to form MC-LR-SWNHs/GCE. Following a rinse with 0.01 M pH 7.4 PBST, the formed immunosensor was blocked with 0.01 M pH 7.4 PBS containing 2% BSA. The preparation of the MC-LR immunosensor is shown in Scheme 1. After being thoroughly rinsed with 0.01 M pH 7.4 PBST, the obtained immunosensor was stored at 4 °C prior to use. As a control, MC-LR-SWNHs/GCE was prepared with the same procedure. A control electrode was also prepared by simply dropping 5 µL of 200 µg/L MC-LR on SWNH-coated GCE without activation step or the presence of any activated reagent (EDC or NHS) for blank experiments.

Analytical Procedure. MC-LR solutions (5 µL) with different concentrations or water samples were mixed with 5 µL of HRP-labeled 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 30 min at 25 °C and then washed carefully with 0.01 M, pH 7.4 PBST to obtain HRP-anti-MC-LR/MC-LR-SWNHs/GCE. During the incubation process, the immunosensor was placed in a container to avoid the evaporation of incubation solution. The electrochemical measurement was recorded in a 0.2 M PBS solution containing 8.0 mM H₂O₂ and 10 mM o-phenylenediamine (o-PD). The detection solution was bubbled thoroughly with high purity nitrogen for 10 min and maintained in a nitrogen atmosphere. The differential pulse voltammetric (DPV) measurements were from −300 to −850 mV with pulse amplitude of 50 mV and width of 50 ms. The data for condition optimization and the calibration curve were the average of three measurements.

RESULTS AND DISCUSSION

Cyclic Voltammetric Behavior of Modified Electrodes. In 0.2 M PBS, HRP-anti-MC-LR/MC-LR-SWNHs/GCE did not show any detectable signal in the working potential range (Figure 1a), though it showed a larger background current than HRP-anti-MC-LR/MC-LR-SWNHs/GCE exhibited a pair of stable and well-defined redox peaks at −0.546 and −0.615 V (Figure 1c), which corresponded to the redox of 2,2′-diaminoazobenzene, the enzymatic product.33,34 Although these peaks also occurred at HRP-anti-MC-LR/MC-LR-SWNHs/GCE (Figure 1d), the peak currents were 2 times lower than those at HRP-anti-MC-LR/MC-LR-SWNHs/GCE, indicating the significant sensitizing effect of SWNHs, which would lead to enhanced sensitivity for the proposed MC-LR immunosensor. The sensitizing effect benefited from the abundant oxygen-functionalized sites exposed on the cone-shaped tips of SWNHs for MC-LR binding and easy recognition of bound MC-LR to HRP-anti-MC-LR.

Characterization. The covalent modification of MC-LR on oxidized SWNHs was confirmed by Raman and FT-IR spectra (Figure 2). The pristine SWNHs showed the characteristic peaks with a disorder-induced D-band at 1355 cm⁻¹ and a tangential stretch G-band at 1584 cm⁻¹ (Figure 2A, curve a),35 while the oxidized SWNHs showed the two bands at 1352 and 1581 cm⁻¹ (Figure 2A, curve b), respectively. The ratios of D-band to G-band for pristine SWNHs and oxidized SWNHs were 0.73 and 0.94, respectively, which was due to the generation of defect sites for pristine SWNHs and oxidized SWNHs was confirmed by Raman and FT-IR spectra (Figure 2A, curve a),35 while the oxidized SWNHs showed the two bands at 1352 and 1581 cm⁻¹ (Figure 2A, curve b), respectively. The ratios of D-band to G-band for pristine SWNHs and oxidized SWNHs were 0.73 and 0.94, respectively, which was due to the generation of defect sites arising from the oxidation process. After conjugation of MC-LR on oxidized SWNHs, those spectra slightly shifted to 1356 and 1580 cm⁻¹, respectively.

1588 cm$^{-1}$, respectively (Figure 2A, curve c). Moreover, the ratio of D-band to G-band increased to 1.02 due to the further introduction of defects in the SWNHs after the grafting of MC-LR, which provided the evidence of the covalent modification of SWNHs.

The FT-IR spectrum of oxidized SWNHs displayed two peaks at 1734 and 1588 cm$^{-1}$ (Figure 2B, curve a), which were assigned to the carbonyl stretch mode of COOH and COO$^-$. After covalent conjugation of the MC-LR to the SWNHs, the vibration of amide I and amide II of the MC-LR could be observed at 1637 and 1542 cm$^{-1}$ (Figure 2B, curve b), while the peaks assigned to the carbonyl stretch mode decreased greatly, indicating the effective reaction of the carboxylic group of SWNHs with the amino group of the MC-LR.

The O1s XPS spectrum of SWNHs consisted of two peaks assigned to carbon–oxygen double bonding (C=O, 531.9 eV) and single bonding (C–O, 533.7 eV) due to the presence of a –COO group on SWNHs produced during the oxidation treatment (Figure 3A). Moreover, after binding with MC-LR, the intensity ratio of C=O to C–O O1s for MC-LR-SWNHs increased from 1.07 to 1.21 (Figure 3B). Those changes should be attributed to the high ratio of C=O to C–O in the MC-LR molecule, indicating that MC-LR was successfully immobilized onto SWNHs.

Optimization of Conditions for Electrochemical Detection. The analytical performance of the electrochemical enzymatic analysis was related to the concentration of o-PD and H$_2$O$_2$ in the measuring system. As seen in Figure 5, the DPV peak current of the MC-LR immunosensor after an immunoreaction with HRP-anti-MC-LR reached the similar maximum values at 8.0 mM H$_2$O$_2$ and 10 mM o-PD, respectively.
mM (Figure 5B), respectively. Therefore, the optimal concentrations of o-PD and H2O2 were selected at 10.0 and 8.0 mM, respectively.

**Optimal Conditions for Immunoreaction.** Under optimal detection conditions, the electrochemical response depends on the formation of immunocomplex on the electrode surface. The latter is decided by the concentration of HRP-labeled MC-LR antibody in the incubation solution and incubation time. In order to obtain the optimal concentration of the HRP-labeled MC-LR antibody, the MC-LR-SWNHs/GCEs were incubated in HRP-labeled MC-LR antibody solutions with different concentrations. As shown in Figure 6A, the peak currents increased with the increasing HRP-labeled MC-LR antibody concentration and tended to a plateau at 42.1 µg/mL, indicating that all the available recognition sites of immobilized MC-LR were matched with the enzyme conjugate. Thus, 42.1 µg/mL of HRP-labeled MC-LR antibody was used for the incubation step.

At the optimized HRP-labeled MC-LR antibody concentration, with the increasing incubation time from 5 to 60 min, the immunosensor showed the increasing response until an incubation time of 30 min (Figure 6B). Longer incubation time did not obviously improve the response. Therefore, the incubation time of 30 min was chosen as the optimal incubation condition for the immunoassay of MC-LR.

**Electrochemical Response of 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 at a known concentration was added into the incubation solution containing a certain concentration of HRP-labeled 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 HRP-labeled MC-LR antibody to form the immunocomplex. The captured HRP-labeled MC-LR antibody on the immunosensor surface then catalyzed the oxidation of o-PD by H2O2 to produce 2,2′-diaminoazobenzene. The DPV peak current of 2,2′-diaminoazobenzene decreased with the increasing MC-LR concentration in the incubation solution (Figure 7A). As shown in Figure 7B, the decrease of DPV peak current was proportional to MC-LR concentration in the range of 0.05–20 µg/L with a correlation coefficient of 0.993. This result was much wider than those of 0.03–3.16 µg/L for a competitive binding electrochemical enzyme immunoassay, 0.06–0.65 µg/L for a chemiluminescence enzyme immunoassay, and 0.1–10.1 µg/L for a portable optical immunosensor. The detection limit was calculated to be 0.03 µg/L at a signal-to-noise ratio of 3, which was much lower than the WHO provisional guideline limit of 1 µg/L for MC-LR in drinking water. According to the analytical process, the detectable linear ranges in the sample with a dilution factor of 2 by adding 5 µL of HRP-labeled MC-LR antibody were 0.1–40 µg/L with the detection limit of 0.06 µg/L. Thus, the proposed method was enough for practical application.

**Blank Experiments.** At a control electrode prepared in the absence of activated reagent, upon an incubation step in 42.1 µg/mL HRP-labeled MC-LR antibody, the DPV curve showed a peak current of 6.9 µA, which was about 6 times lower than that of the proposed immunosensor, which resulted from the nonspecific adsorption of HRP-labeled MC-LR antibody, as observed at the SWNHs-coated GCE (Figure 8), and the immunoreaction between adsorbed MC-LR and the labeled MC-LR antibody. Obviously, the amount of adsorbed MC-LR was much lower than that of the covalently bound MC-LR on the electrode surface. After 0.5 µg/L MC-LR was added into the incubation solution, the peak current of the control electrode did not show any change, while 15 µg/L MC-LR in the incubation solution led to a current decrease from 6.9 to 4.7 µA due to the complexation of HRP-labeled MC-LR antibody with MC-LR in the solution. However, the change was about 10 times smaller than that of the proposed immunosensor. These results demonstrated a good performance of the immunosensor prepared via covalent immobilization of MC-LR on activated SWNHs-coated GCE.

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**Figure 6.** Effects of HRP-labeled MC-LR antibody concentration (A) and incubation time (B) on amperometric response of HRP-anti-MC-LR/MC-LR-SWNHs/GCE under other optimal conditions.

**Figure 7.** DPV response of the immunosensor in 0.2 M, pH 7.0 PBS containing 10 mM o-PD and 8.0 mM H2O2 after incubation with 0.05, 0.25, 0.5, 2.5, 5, 10, 15, and 20 µg/L MC-LR (from highest to lowest peak currents) (A) and calibration curve for MC-LR immunoassay (B).

**Figure 8.** Comparison of DPV peak currents at the proposed immunosensor, a control electrode prepared by dropping MC-LR on SWNH-coated GCE without absence of activated reagent and SWNH-coated GCE after incubation with 0, 0.5, and 15 of MC-LR µg/L.
Precision, Reproducibility, Stability, and Reusability of MC-LR Immunosensor. The intra-assay precision of the immunosensor was evaluated by assaying MC-LR at two levels for five replicative measurements. The intra-assay variation coefficients with this method were 2.7% and 7.5% at MC-LR concentrations of 0.25 and 10 µg/L, respectively, showing a good repeatability. While the interassay variation coefficients at these concentrations on five immunosensors made independently were 5.9% and 7.7%, respectively, indicating acceptable fabrication reproducibility. When the immunosensor was not in use, it was stored at 4 °C. 95.6% of the initial response of the immunosensor for MC-LR remained after 1 week, and 85.3% of the initial response remained after 4 weeks. These results indicated the immunosensor had acceptable stability. The regeneration of the immunosensor could be realized by rinsing with pH 2.8 glycine–HCl solution to dissociate the antigen–antibody complex. The as-renewed immunosensor could restore 91.2% of the initial value after five assay runs, showing accepted reusability.

Analysis of Polluted Water Samples. To evaluate the analytical reliability and application potential of the proposed method, the assay results of polluted water samples using the MC-LR immunosensor were compared with the results obtained from HPLC. Water samples (5 µL) were mixed with 5 µL of HRP-labeled MC-LR antibody, and the mixtures were dropped on the MC-LR immunosensor. The MC-LR concentrations determined with this method were 1.4 ± 0.05 and 5.5 ± 0.2 µg/L, which is consistent with the values of 1.5 and 5.7 µg/L from HPLC. Thus, the present method could satisfy the need for the immunoassay of MC-LR in water samples.

CONCLUSION
A sensitive electrochemical immunoassay method was proposed for MC-LR detection in environmental samples by the sensitizing effect of SWNHs. The small analyte molecule could be easily covalently bound to SWNHs to functionalize the carbon nanoparticles for preparation of the immunosensor. The abundant carboxylic groups on the cone-shaped tips of SWNHs enhanced the immobilization capability of MC-LR and provided a three-dimensional recognition of HRP-anti-MC-LR to the binding sites of MC-LR. With a competitive immunoassay format, the designed immunosensor for MC-LR had good performance with high sensitivity, a wide linear range, acceptable precision and fabrication reproducibility, and excellent stability. It had been successfully applied in the detection of MC-LR in practical samples. The biocompatible recognition and immobilization strategy provides a useful platform for preparation of immunosensors for wide-range small toxin molecules and could be readily extended toward the on-site monitoring of the hazard components in food and environmental matrixes.

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