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Simultaneous Multiplexed Stripping Voltammetric Monitoring of Marine Toxins in Seafood Based on Distinguishable Metal Nanocluster-Labeled Molecular Tags

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Supporting Information

ABSTRACT: Marine toxins from microscopic algae can accumulate through the food chain and cause various neurological and gastrointestinal illnesses for human health. Herein, we designed a new ultrasensitive multiplexed immunoassay protocol for simultaneous electrochemical determination of brevetoxin B (BTX-2) and dinophysistoxin-1 (DTX-1) in seafood using distinguishable metal nanocluster-labeled molecular tags as traces on bifunctionalized magnetic capture probes. To construct such a bifunctionalized probe, monoclonal mouse anti-BTX-2 (mAb_1) and anti-DTX-1 (mAb_2) antibodies were co-immobilized on a magnetic bead (MB−$mAb_{1,2}$). The distinguishable metal nanoclusters including cadmium nanoclusters (CdNC) and copper nanoclusters (CuNC) were synthesized using the artificial peptides with amino acid sequence CCCYYY, which were used as distinguishable signal tags for the label of the corresponding bovine serum albumin−BTX-2 and bovine serum albumin−DTX-1 conjugates. A competitive-type immunoassay format was adopted for the online simultaneous monitoring of BTX-2 and DTX-1 on a homemade flow-through magnetic detection cell. The assay was based on the stripping voltammetric behaviors of the labeled CdNC and CuNC at the various peak potentials in pH 2.5 HCl containing 0.01 M KCl using square wave anodic stripping voltammetry (SWASV). Under optimal conditions, the multiplexed immunoassays enabled simultaneous detection of BTX-2 and DTX-1 in a single run with wide working ranges of 0.005−5 ng mL^{-1} for two marine toxins. The limit of detection (LOD) and limit of quantification (LOQ) were 1.8 and 6.0 pg mL^{-1} for BTX-2, while those for DTX-1 were 2.2 and 7.3 pg mL^{-1}, respectively. No non-specific adsorption and electrochemical cross-talk between neighboring sites were observed during a series of procedures to detect target analytes. The covalent conjugation of biomolecules onto the nanoclusters and magnetic beads resulted in good repeatability and intermediate precision down to 9.5%. The method featured unbiased identification of negative (blank) and positive samples. No significant differences at the 0.05 significance level were encountered in the analysis of 12 spiked samples, including *Sinonovacula constricta*, *Musculista senhousia*, and *Tegillarca granosa*, between the multiplexed immunoassay and commercially available enzyme-linked immunosorbent assay (ELISA) for analysis of BTX-2 and DTX-1.

KEYWORDS: *Nanoparticle-based immunoassay, seafood, electrochemical immunoassay, brevetoxin B, dinophysistoxin-1*

INTRODUCTION

Marine toxins are naturally occurring chemicals that can contaminate certain seafood. Although seafood is rarely implicated in food poisoning compared to other food sources, it provides some specific human health hazards unique to this particular resource.[1] Various methods and strategies have been employed for detection of marine toxins, e.g., electrochemiluminescence-based immunoassay, radioimmunoassay (RIA), liquid chromatography−mass spectrometry (LC−MS), thin-layer chromatography, high-performance liquid chromatography (HPLC), and enzyme-linked immunosorbent assay (ELISA).[2−6] During the analysis and detection of food safety, however, the determination of a single toxin usually limits its screening value because marine toxins can be simultaneously caused by eating shellfish, including paralytic shellfish poisoning (PSP), red tide/neurotoxic shellfish poisoning (NSP), and diarrheic shellfish poisoning (DSP). Hence, exploring a sensitive and facile method for simultaneous detecting and quantifying of multiple marine toxins in a single run would be advantageous to simplify testing, throughout, and reduce overall cost per test for food safety monitoring.

Immunochemical methods mentioned are increasingly becoming routine methods.[7] Generally, they are performed as ELISA tests on microtiter plates. However, the conventional ELISAs have some limitations, such as a complicated washing procedure and a relatively long assay time.[8] Alternatively, an electrochemical method is suitable for sensor miniaturization and automated detection, owing to its high sensitivity, low cost, low power requirements, and high compatibility with advanced micromachining technologies.[9] Various strategies have been devised to realize simultaneous multianalyte analysis, using

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either multiple labels or spatial resolution to discriminate between the different analytes.[10−13] Although modification of the individual electrode with different biological recognition elements would enable construction of miniaturized biosensor arrays, the directed immobilization of functional proteins on individual microscopic regions is still a challenge.

The nanoparticle-based immunoassay involves the immobilization of the biomolecules on the nano-/microbeads and takes place in the solution, thus allowing for the integration of multiple liquid handling processes.[14] Especially in combination with a microfluidic device, the nanoparticle-based immunoassay can be used for the determination of complex samples without the large sample consumption and sample pretreatment, resulting in a relatively inexpensive and easy performance.[15] Magnetic beads (MBs) are attractive because they have good biocompatibility and can be separated very readily from reaction mixtures with an external magnet.[16] Magnetic-controlled molecular electronics can examine the effect of the external magnetic field on the electrochemical signals of functional MBs associated with the electrodes.[17] Magnetic sorting protein assay systems with varying throughputs have been built and employed for multiple immunoassays.[17] Recently, our group fabricated a MB-based immunoassay of carbohydrate antigen 125 using functional MBs as immunosensing probes and antibody-coated nanometer-sized enzyme-doped silica beads as signal tags.[18] During the process, the primary antibodies were covalently conjugated onto the MBs with high capture capacity for the determination of biomarkers. Later, the methodology was further extended for simultaneous detection of two biomarkers using magnetic graphene nanosheets as immunosensing probes.[19] Two corresponding primary antibodies were co-immobilized on the MB-coated graphene nanosheets. Unexpectedly, it was difficult for the large-sized nanostructures to homogenously disperse in the microfluidic device, although magnetic graphene nanosheets could increase the immobilized amount of biomolecules. In this work, our motivation is to directly immobilize two primary antibodies on single MB.

Another important issue for development of multiplexed immunoassays is signal amplification. A common resort is to exploit signal transduction labels, including ligand-conjugated enzymes and nanolabels.[20] In the past, our group reported an electrochemical immunosensor for the detection of biomarkers using horseradish-peroxidase-labeled secondary antibodies as traces.[9] One of the problems commonly associated with enzyme labels was to decrease their bioactivity when the biomolecules were exposed to reactive groups and harsh reaction conditions. To tackle this issue, various nanomaterials, including quantum dots (QDs),[21] metal nanoparticles,[22] and metal ions,[23] have been used as electroactive labels for the development of electrochemical immunoassays. Inspiringly, some metal ions comprising cadmium, copper, and zinc usually display various voltammetric characteristics at the different applied potentials.[24−27] Favorably, these metal ions can be stripped from the corresponding metal nanoparticles under the harsh conditions. In this work, cadmium and copper nanostructures would be employed as distinguishable signal tags for the construction of the multiplexed electrochemical immunoassay.

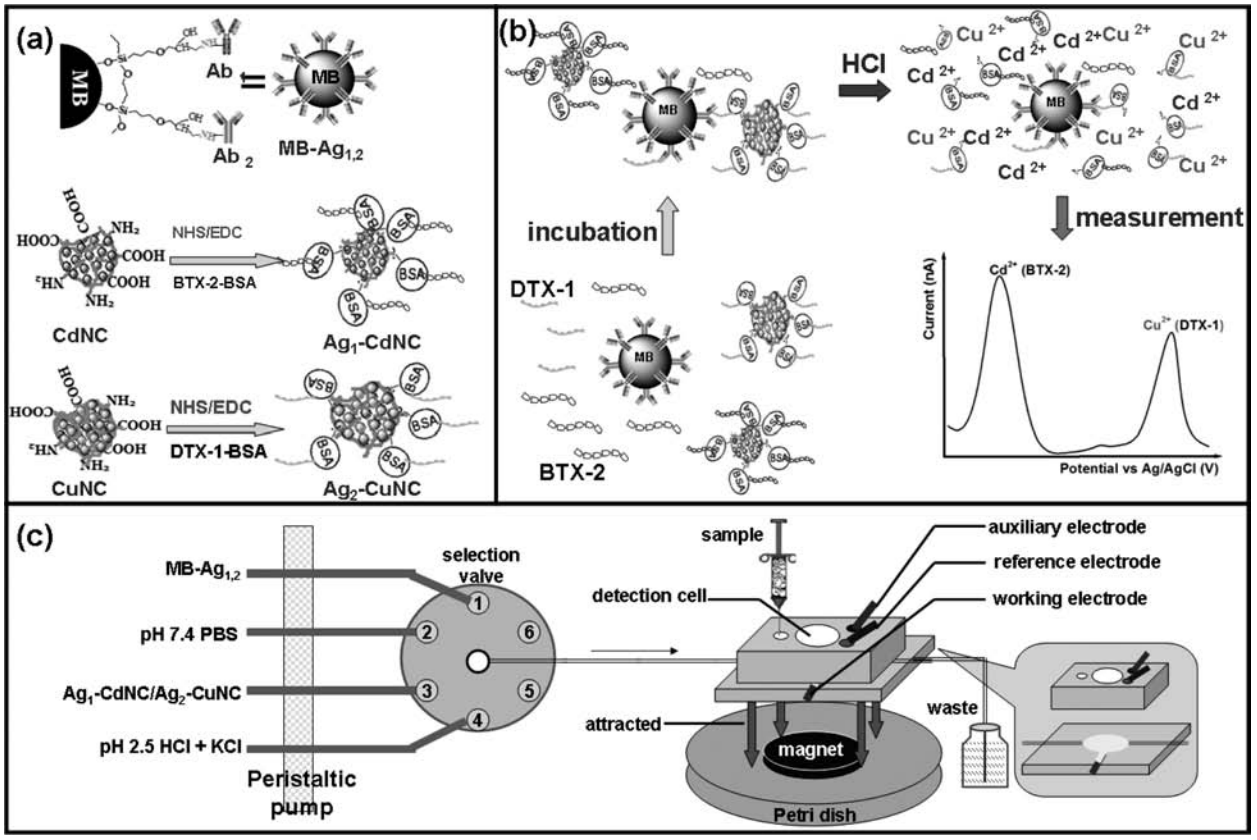
Brevetoxin B (BTX-2) is a neurotoxin produced by algae called *Ptychodiscus brevis* Davis or *Gymnodinium breve* Davis. It can cause intoxication and even mortality through consumption of brevetoxin-contaminated shellfish and affect respiratory irritation through aerosol exposure at coastal areas.[28] Dinophysistoxin-1 (DTX-1) is produced by the dinoflagellates, *Dinophysis* spp. and *Prorocentrum* spp. DTX-1 strongly inhibits protein phosphatases, especially type 2A, leading to inflammation in the intestinal tract, diarrhea, and most importantly, tumor promotion.[29] Herein, we design a new flow-through electrochemical immunoassay for simultaneous monitoring of BTX-2 and DTX-1 using bifunctionalized MBs as immunosensing probes and distinguishable metal nanocluster-labeled molecular tags as traces. To prepare the distinguishable molecular tags, cadmium and copper metal nanoclusters are initially prepared using the artificial peptides with amino acid sequences CCCYYY and then the prepared metal nanoclusters are used for the label of bovine serum albumin−BTX-2 and bovine serum albumin−DTX-1 conjugates. On the basis of the competitive-type immunoassay format, the formed magnetic immunocomplex with metal nanoclusters is collected onto a homemade magnetic detection cell. The electrochemical signals are simultaneously achieved at various peak potentials after metal ions are stripped from the corresponding metal nanoclusters. The corresponding currents depend upon the concentration of two analytes.

EXPERIMENTAL PROCEDURES

Materials and Reagents. Individual standard stock samples of BTX-2 (purity ≥ 98% by HPLC) and DTX-1 (purity ≥ 98% by HPLC) were purchased from Express Technology Co., Ltd. (Beijing, China). Monoclonal mouse anti-BTX-2 (designated as mAb_1) antibodies, monoclonal mouse anti-DTX-1 (designated as mAb_2) antibodies, BTX-2−bovine serum albumin (BTX-2−BSA) conjugates, and DTX-1−bovine serum albumin (DTX-1−BSA) conjugates were obtained from Dingguo Biotechnology Co., Ltd. (Beijing, China). Fe_3O_4 MBs (particle size ∼ 100 nm) in an aqueous suspension with a concentration of 25 mg mL^{-1} were obtained from Chemicell GmbH (Berlin, Germany). BSA (96−99%), (3-glycidyloxypropyl) trimethoxysilane ($C_9H_{20}O_5Si$, GOPS), *N*-hydroxysuccinimide (NHS), and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) were supplied by Sigma-Aldrich. The peptide H_2N−CCCYYY−COOH (C and Y stand for L-cysteine and tyrosine, respectively) was purchased from Dingguo Biotechnology Co., Ltd. (Beijing, China). All other reagents were of analytical grade and used without further purification. Ultrapure water obtained from a Millipore water purification system (≥18 MΩ, Milli-Q, Millipore, Billerica, MA) was used in all runs. A 0.01 M solution of hydrochloric acid (pH 2.5) containing KCl (0.01 M) served as the supporting electrolyte. Phosphate-buffered saline (PBS, 0.1 M, pH 7.4) was prepared by adding 12.2 g of K_2HPO_4, 1.36 g of KH_2PO_4, and 8.5 g of NaCl in 1000 mL of deionized water.

Preparation of mAb_1- and mAb_2-Functionalized MBs (MB−$mAb_{1,2}$). To obtain bifunctionalized immunosensing probes, mAb_1 and mAb_2 antibodies were co-immobilized onto the MBs using a covalent conjugation method, as described in our recent paper.[18] Briefly, 20 mg of MBs was initially dried at 100 °C for 1 h and then incubated with 5 mL of GOPS/dry toluene (1:20, v/v) for 12 h at room temperature (RT) under gentle stirring. During this process, GOPS molecules were conjugated onto the MBs through the reaction between −OH groups on the MB and $-OCH_3$ groups on the GOPS. The GOPS-functionalized MBs were separated by an external magnet and washed thoroughly with toluene and ethanol to remove the physically adsorbed GOPS. After that, the obtained GOPS−MBs were heated for 1 h at 100 °C under N_2 to achieve active epoxy groups on the surface and then dispersed into 2 mL of PBS at pH 7.4 ($C_{[MB]} \approx$ 10 mg mL^{-1}). Afterward, 50 μL of mAb_1 antibodies (1.0 mg mL^{-1}) and 50 μL of mAb_2 antibodies (1.0 mg mL^{-1}) were simultaneously added to the 2 mL of GOPS−MB suspension and incubated for 12 h at 4 °C under gentle stirring. The excess biomolecules were removed by magnetic separation. Finally, the as-prepared bifunctionalized MBs

Scheme 1. (a) Schematic Illustration of Immunosensing Probes and Molecular Tags, (b) Measurement Principle of the Multiplexed Electrochemical Immunoassay, and (c) Construction of the Flow-Through Detection Cell



(designated as MB−mAb$_{1,2}$) were stored in 2 mL of pH 7.4 PBS at 4 °C when not in use ($C_{[MB]} \approx 10$ mg mL^{-1}).

Preparation of Distinguishable Metal Nanocluster-Labeled Molecular Tags. In this work, the distinguishable metal nanoclusters, including cadmium and copper metal nanoclusters, were prepared by coupling the corresponding metal ions with the artificial peptides with amino acid sequence CCCYYY, consulting the literature, with some modification.[30,31] Typically, 100 μL of $Cd(NO_3)_2$ aqueous solution (250 mM) was initially dropwised into 800 μL of CCCYYY solution (1.5 mM) under vigorous stirring. After the mixture was adjusted to pH ∼12 using NaOH, the suspension was sealed into the vial and stored at 37 °C for 8 h without disturbance. During this process, Cd^{2+} ions could be reduced into cadmium nanoclusters through the phenolic group of tyrosine (Y) under alkaline conditions and captured by the −SH group of L-cysteine (C). After that, the CCCYYY-based cadmium nanoclusters (designated as CdNCs) were collected by centrifugation for 20 min at 15000*g*. Finally, the obtained CdNCs were redispersed into 500 μL of distilled water ($C_{[Cd]} \approx 50$ mM).

Next, the as-prepared CdNCs were used for the label of BTX-2−BSA conjugates through carbodiimide coupling because of the presence of H_2N−CCCYYY−COOH. Briefly, 2.2 mg of NHS and 3.0 mg of EDC were dissolved into 500 μL of CdNC suspension ($C_{[Cd]} \approx 50$ mM), followed by continuous stirring for 45 min at RT. Afterward, 500 μL of BTX-2−BSA (1.0 mg mL^{-1}) was added drop by drop into the mixture under continuous stirring at 150 rpm and left at RT for 20 h. After completion of the incubation, the conjugates were centrifuged for 10 min at 15000*g* to remove the precipitates. Finally, the obtained conjugates were dialyzed in a dialysis bag against 0.1 M pH 7.4 PBS at RT for 24 h by changing the buffer every 6 h to remove non-conjugated BTX-2−BSA. The as-prepared BTX-2−BSA-conjugated CdNCs (designated as Ag$_1$−CdNC) were dispersed into 500 μL of PBS (0.1 M, pH 7.4) ($C_{[Cd]} \approx 50$ mM). By the same token, the CCCYYY-based copper nanoclusters (designated as CuNC) were synthesized and functionalized with DTX-1−BSA conjugates (designated as Ag$_2$−CuNC). The fabrication procedure of these two molecular tags is schematically illustrated in Scheme 1.

Competitive-Type Multiplexed Assay Protocol and Electrochemical Measurement. The competitive-type multiplexed electrochemical immunoassay was performed in a sequential injection mode by coupling the CHI 630D electrochemical analyzer (CH Instruments, Inc., Shanghai, China) with a homemade flow-through detection cell. More details were described in the Supporting Information. Scheme 1 shows the fabrication process and measurement principle of the multiplexed electrochemical immunoassays. Initially, 100 μL of MB−mAb$_{1,2}$ ($C_{[MB]} \approx 10$ mg mL^{-1}) flowed into the detection cell and collected on the indium tin oxide (ITO) surface with an external magnet. After that, 100 μL of molecular tags comprising Ag$_1$−CdNCs and Ag$_2$−CuNCs ($M_{[Cd]}/M_{[Cu]}$ = 1:1) and 100 μL of standards/samples including various concentrations of BTX-2 and DTX-1 were mixed and injected into the detection cell. The mixture was incubated for 25 min at RT without the external magnet. During this process, the analytes in the sample and the haptens in the molecular tags competed with the corresponding antibodies immobilized on the MB−mAb$_{1,2}$. Afterward, the unbound analytes and molecular tags were removed by the washing buffer with the aid of the external magnet. Finally, the assay solution (pH 2.5) flowed into the detection cell, and the electrochemical signal was collected using square wave anodic stripping voltammetry (SWASV). Captured CdNC and CuNC were initially dissolved with pH 2.5 HCl, and the resulting solution was deposited at the potential of −1.0 V for 120 s by the reduction of Cd^{2+} and Cu^{2+} ions. The anodic stripping (reoxidation of metal to metal ions) of electrodeposited metal was performed in the potential from 600 to −1200 mV with a potential step of 4 mV, a frequency of 25 Hz, and an amplitude of 25 mV. All electrochemical measurements were performed in an unstirred electrochemical cell at 25 ± 1.0 °C. Note that the *Sinonovacula constricta* supernatant was used for sample matrix throughout, except when specifically stated.

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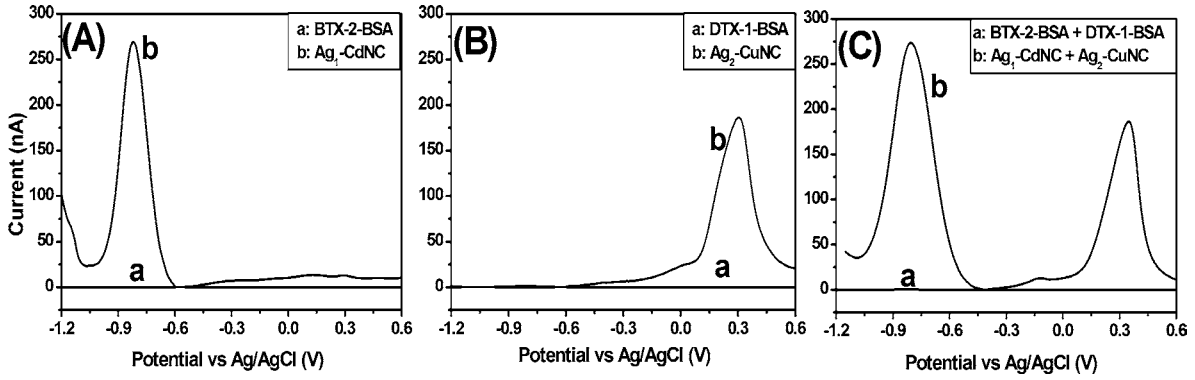

Figure 1. SWASV responses of the as-prepared MB−$Ab_{1,2}$ in pH 2.5 HCl solution containing 0.01 M KCl after incubation with (A) excess BTX-2−BSA and $Ag_{1,2}$−CdNC, (B) excess DTX-1−BSA and Ag_2−CuNC, and (C) excess BTX-2−BSA + DTX-1−BSA and Ag_1−CdNC + Ag_2−CuNC for 25 min at RT (a) without and (b) with nanocluster labels.

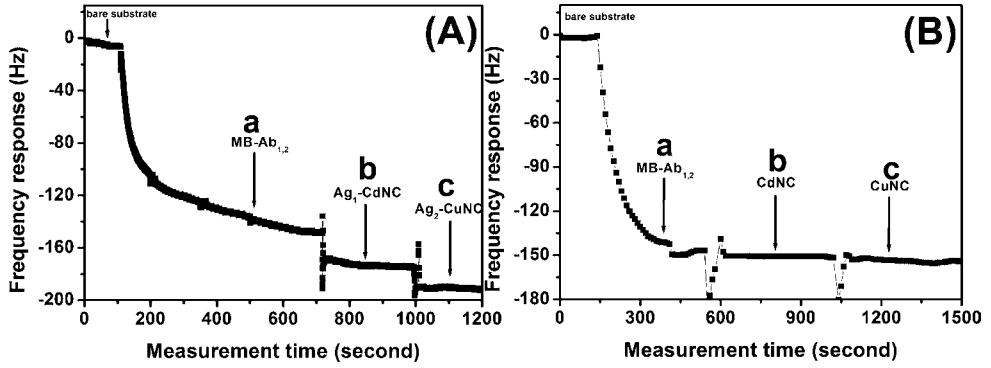

Figure 2. (A) QCM response of (a) 100 μL of MB−$Ab_{1,2}$ ($C_{[MB]} \approx 10$ mg mL^{-1}) immunosensing probe, (b) probe "a" after incubation with 100 μL of 50 mM Ag_1−CdNC, and (c) probe "b" after incubation with 100 μL of 50 mM Ag_2−CuNC. (B) QCM response of (a) 100 μL of MB−$Ab_{1,2}$ ($C_{[MB]} \approx 10$ mg mL^{-1}) immunosensing probe, (b) probe "a" after incubation with 100 μL of 50 mM CdNC, and (c) 100 μL of 50 mM CuNC (note that during the process, the gold substrate was used for the replacement of the ITO electrode).

Samples and Preparation Procedure. Mollusk standards were prepared as follows: Seafood samples including *Musculista senhousia*, *S. constricta*, and *Tegillarca granosa* were purchased from the local Carrefour supermarket (Fuzhou, China). A total of 5 g of minced sample tissue was initially placed in a centrifuge tube; then 20 mL of dimethyl sulfoxide (50%, w/v) was added; and then the mixture was centrifuged for 10 min at 2500*g*. The resulting supernatant was passed sequentially through a 100 nm nylon mesh, number 1 filter and a GF/B filter (Whatman) in turn (note that the extract color was slightly opalescent yellow). After that, BTX-2 and DTX-1 standards with various levels were spiked into the supernatant, respectively.

Statistical Analysis. All measurements were carried out in triplicate. A statistical data analysis was performed using Statistics Analysis System (SAS), version 9.0, and Statistical Program for Social Sciences (SPSS), version 9.0, software packages. Comparisons between dependent variables were determined using analysis of variance (ANOVA), Duncan's multiple range test, correlation analysis, and multiple regression analysis. Results are expressed as the mean value ± standard deviation (SD) of three determinations, and statistical significance was defined at $p \leq 0.05$.

RESULTS AND DISCUSSION

Characteristics of Immunosensing Probes and Molecular Tags. For the immunosensing probes, the biomolecules were immobilized onto the surface of MBs, exploiting the epoxy−amino reaction. In comparison to the protocol reported by our group previously, i.e., synthesis of biofunctionalized magnetic graphene nanosheets,[19] two antibodies have been in the work directly conjugated onto the MBs. Because of the decreased mass of the resulting bifunctionalized nanoparticles, their homogeneous dispersion in solution has been facilitated. In this work, antibodies, including mAb_1 and mAb_2, were conjugated onto the surface of MBs through the epoxy−amino reaction, as shown in our previous paper.[18] During this process, MBs not only acted as a substrate for conjugation of biomolecules but also enabled the rapid separation and purification of functional nanostructures after synthesis. For the synthesis of molecular tags, the peptides (CCCYYY) containing two domains were designed for the preparation of metal nanoclusters. Domains one and two are CCC and YYY, respectively. Under the alkaline condition, the YYY domain can reduce metal ions to the corresponding zero-value metals by the phenolic OH group of tyrosine.[30] Meanwhile, the zero-value metals are captured via the −SH group of cysteine to form the metal nanoclusters.[30] The H_2N−CCCYYY−COOH molecules are penetrated into or enclosed the metal nanoclusters. The $-NH_2$ or −COOH residues can be used for the conjugation of biomolecules through the carbodiimide coupling method. To verify the formation of metal nanoclusters, the as-synthesized metal nanoclusters were initially characterized using transmission electron microscopy (TEM) (see Figure S1 of the Supporting Information). Panels a and b of Figure S1 of the Supporting Information show typical TEM images of CdNC and CuNC at the low magnitude, respectively. Their topological structures were similar. Favorably, the nanoclusters could be observed at the high magnitude (see panels c and d of Figure S1 of the Supporting Information).

Electrochemical Characteristics and Specific Reaction of Multiplexed Immunoassays Using Various Molecular Tags. To investigate the effect of metal nanoclusters on the electrochemical characteristics of the developed immunoassay, two types of molecular tags with and without metal nanoclusters (i.e., BTX-2−BSA/DTX-1−BSA and Ag_1−CdNC/Ag_2−CuNC) were used for the determination of zero analyte on the MB−$Ab_{1,2}$ with the same assay mode. As shown from curves "a" in panels A−C of Figure 1, no voltammetric peaks were observed within the applied potential, regardless of using BTX-2−BSA/DTX-1−BSA alone or together. The results indicated that BTX-2−BSA and DTX-1−BSA could not produce the electrochemical signal. When using Ag_1−CdNC/Ag_2−CuNC as molecular tags, however, two strong voltammetric peaks (i.e., −0.8 V for BTX-2 and 0.35 V for DTX-1) were detected on the MB−$Ab_{1,2}$ (curves "b" in panels A−C of Figure 1). More significantly, the voltammetric peaks did not interfere with each other compared to curves "b" in panels A and B of Figure 1. These results revealed that the signal of electrochemical immunoassays mainly derived from the labeled metal nanoclusters. Because of the difference of peak potentials of Cd^{2+} and Cu^{2+} ions ($\Delta E \approx 115$ mV), the distinguishable metal nanocluster-labeled molecular tags could be employed for simultaneous detection of BTX-2 and DTX-1 in a single run.

Accompanying that, a new concern to be produced is whether the electrochemical signal originated from the nonspecific adsorption of metal nanoclusters with the MB−$Ab_{1,2}$. To tackle this issue, the synthesized MB−$Ab_{1,2}$ were used for direct interaction with metal nanoclusters (i.e., CdNC and CuNC), and the signal was recorded using the quartz crystal microbalance (QCM) method. For comparison, the MB−$Ab_{1,2}$ was also used for direct interaction with the molecular tags (i.e., Ag_1−CdNC and Ag_2−CuNC). The results are shown in Figure 2. In the QCM assay, the frequency difference (ΔF_x) was related to the mass accumulation (ΔM) on the quartz crystal electrode surface according to the Sauerbrey equation.[32] If the as-prepared MB−$Ab_{1,2}$ non-specifically adsorbed the metal nanoclusters, the frequency would be changed after injection of CdNC and CuNC. As indicated from Figure 2A, the frequency of the MB−$Ab_{1,2}$-modified probe increased upon the addition of Ag_1−CdNC and Ag_2−CuNC. The reason might be the fact that the haptens on the nanoclusters reacted with the antibodies on the MBs. After the unmodified CdNC and CuNC were injected into the detection cell, however, the frequency of the QCM probe was almost unchanged (Figure 2B). The results revealed that the measurement signal derived from the specific interaction with the biomolecules on the Ag_1−CdNC/Ag_2−CuNC.

Optimization of Experimental Conditions. To ensure the optimal analytical properties of the developed immunoassay, some experimental parameters, including incubation time for the antigen−antibody reaction, pH of the assay solution, and dissolution time of metal nanoclusters, should be investigated. As indicated from Figure S2 of the Supporting Information, the optimal conditions were (i) 25 min for the incubation between antibodies and antigens, (ii) pH 2.5 HCl solution containing 0.01 M KCl as the detection solution, and (iii) 20 min for dissolution time of metal nanoclusters.

Evaluation of Cross-Talk and Cross-Reactivity. To investigate the cross-talk of the multiplexed electrochemical immunoassay, several control tests comprising single analyte (i.e., BTX-2 or DTX-1), multianalyte comprising BTX-2 and DTX-1, and unspiked sample (i.e., blank seafood specimen) were analyzed using the MB−$Ab_{1,2}$ as immunosensing probes and Ag_1−CdNC/Ag_2−CuNC as molecular tags. As seen from Table S1 of the Supporting Information, the single analyte could only cause the change of one corresponding peak current. Only in the simultaneous presence of BTX-2 and DTX-1, the peak currents at two peak potentials could be changed. Hence, the cross-talk on the MB−$Ab_{1,2}$ was acceptable. Further, when blank seafood samples were analyzed using the multiplexed immunoassay as the control tests, all probes showed a substantially low signal shift (Δi < 5 nA; see Table S1 of the Supporting Information), in contrast to the results obtained when spiked specimens were assayed. These results revealed that the multiplexed electrochemical immunoassay exhibited low cross-talk and cross-reactivity between both analytes.

To further investigate the specificity of the multiplexed electrochemical immunoassay for the determination of BTX-2 and DTX-1, we challenged the system with their analogues and other marine toxins, including BTX-1, BTX-3, DTX-2, DTX-3, okadaic acid (OA), pectenotoxin-6 (PTX-6), and yessotoxin (YTX). As seen from Figure 3, the multiplexed immunoassay

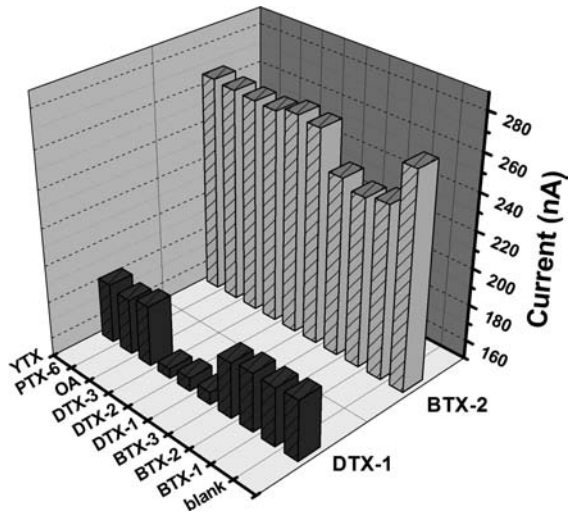

Figure 3. Specificity of the multiplexed immunoassay toward other marine toxins and analogues. These standards were prepared by spiking them into the blank *S. constricta* supernatant. The evaluation was made by comparison to the shift of the multiplexed immunoassay in the SWASV current relative to zero analyte.

exhibited a high cross-reactivity (CR) of >95% for BTX-1, BTX-3, DTX-2, and DTX-3, while no false compliant results were obtained for OA, PTX-6, and YTX. The false-positive results for BTX-1 and BTX-3 might be the fact that the used anti-BTX-2 antibodies have a CR of 97.45 and 120.63% for BTX-1 and BTX-3, respectively,[33] while DTX-1 has a similar structure with DTX-2 and DTX-3. More inspiringly, BTX and DTX did not interfere with each other. Therefore, the specificity of the multiplexed electrochemical immunoassay was acceptable.

Calibration Curves of the Multiplexed Immunoassay. To monitor the sensitivity and dynamic range of the electrochemical immunoassay, a SWASV measurement with a competitive-type immunoassay format was applied to detect BTX-2 and DTX-1 standards in pH 2.5 HCl + KCl, using MB−$Ab_{1,2}$ as immunosensing probes and Ag_1−CdNC/Ag_2−CuNC as molecular tags. As shown in Figure 4a, the peak currents of the multiplexed immunoassay decreased with the increase of

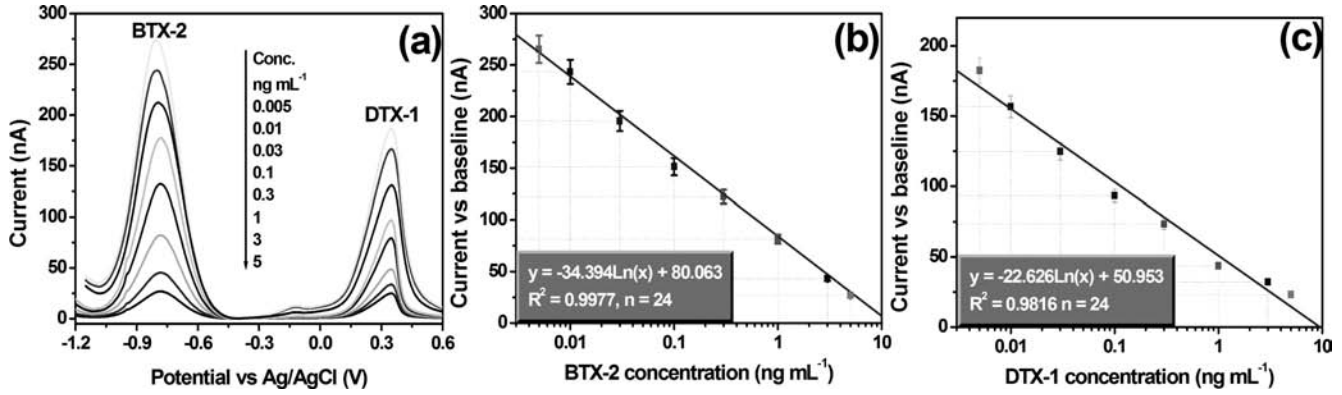

Figure 4. (a) SWASV responses and (b and c) calibration curves of the flow-through multiplexed electrochemical immunoassay toward BTX-2 and DTX-1 standards using MB−$Ab_{1,2}$ as immunosensing probes and Ag_1−CdNC/Ag_2−CuNC as molecular tags in pH 2.5 HCl solution containing 0.01 M KCl.

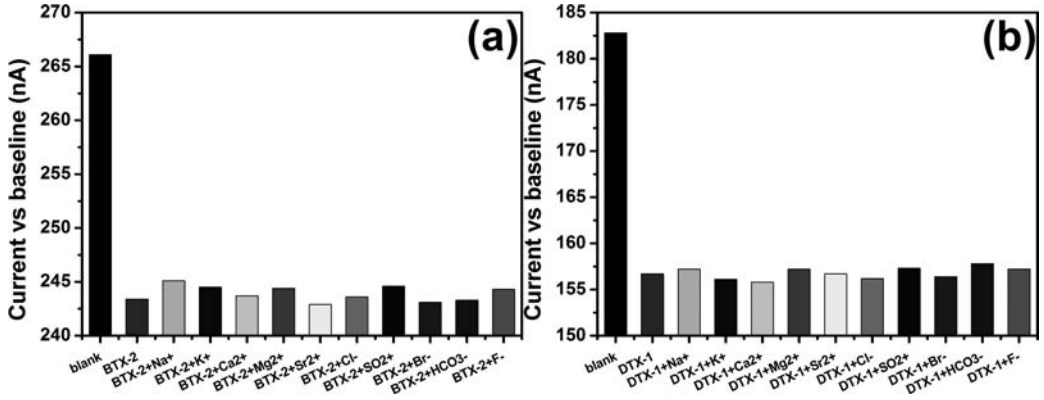

Figure 5. Effect of various sample matrices on the current of the electrochemical multiplexed immunoassay. The evaluation was made by comparison to the electrochemical signal of the multiplexed immunoassay before and after the addition of interfering components with high concentration (0.1 M used in this case) into the low-concentration (a) BTX-2 and (b) DTX-1 standards (0.01 ng mL^{-1} used as an example).

BTX-2 and DTX-1 levels. Both calibration plots displayed a good linear relationship between the peak currents and the logarithm of the analyte concentration in the ranges of 0.005−5.0 ng mL^{-1} for both analytes (panels b and c of Figure 4). The correlation coefficients were 0.998 and 0.991 for BTX-2 and DTX-1 (n = 24), respectively. The sensitivity of the developed immunoassay was determined on the basis of the values obtained for detection and quantification limits. The limit of detection (LOD) is defined as the lowest concentration of analyte that can be detected with an acceptable accuracy, whereas the limit of quantification (LOQ) represents the lowest amount of analyte that can be reliably quantified. LOD and LOQ were calculated from the parameters obtained from the regression curve, using LOD = $3S_b/s$ and LOQ = $10S_b/s$, where "S_b" is the standard deviation of the y intercept and "s" is the slope.[34] Under the given conditions, the calculated LODs and LOQs were found to be 1.8 and 6.0 pg mL^{-1} for BTX-2 and 2.2 and 7.3 pg mL^{-1} for DTX-1, respectively. The obtained LODs were partially lower than those of competitive-type ELISA (0.6 ng $well^{-1}$),[35] immunochromatographic assay (10 ng mL^{-1}),[33] electrochemical immunoassay (10 pg mL^{-1}),[36] and electrochemiluminescenc immunoassay (0.05 ng mL^{-1})[37] for BTX-2, liquid chromatographic method (1.2 pg mL^{-1}),[38] HPLC−FL (0.5 pg mL^{-1} and 2.0 ng mL^{-1}),[39,40] and LC−MS (10 pg mL^{-1})[41] for DTX-1. The results indicated that the electrochemical immunoassay enabled a wide linear range and low LOD. When the levels of BTX-2/DTX-1 in the sample are higher than 5.0 ng mL^{-1}, an appropriate dilution is preferable.

Precision, Sample Matrix Effect, and Stability. The precision of the multiplexed immunoassay was assessed by estimating the relative standard deviations (RSDs; n = 5) of intra- and interassays. Three different concentrations of both marine toxins were repeatedly assayed, using identical batches of MB−$Ab_{1,2}$ and molecular tags throughout. Experimental results indicated that the RSDs of the intra-assay between five runs were 5.3, 6.2, and 4.8% for 0.01, 1.0, and 5.0 ng mL^{-1} BTX-2 and 3.9, 5.7, and 4.5% for 0.01, 1.0, and 5.0 ng mL^{-1} DTX-1, respectively, whereas the RSDs of the interassay with various batches were 6.5, 7.9, and 6.8% for BTX-2 and 8.8, 7.4, and 9.3% for DTX-1 toward the above-mentioned analyte. The low RSDs indicated that the multiplexed electrochemical immunoassay could be regenerated and used repeatedly and further verified the possibility of batch preparation.

To investigate the interfering effects of sample matrix components on the analytical properties of the multiplexed immunoassay, several possible components in the seawater, including Na^+, K^+, Ca^{2+}, Mg^{2+}, Sr^{2+}, Cl^-, SO_4^{2+}, Br^-, HCO_3^-, and F^-, were added to the *S. constricta* supernatant (matrix) containing BTX-2 and DTX-1 standards, respectively. As shown in Figure 5, the large shifts in the current were obtained in the presence of the target analytes compared to the blank *S. constricta* matrix. More substantially, these coexisting sample matrix components did not obviously affect the electrochemical

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Table 1. Comparison of the Assayed Results for Real Seafood Samples Using the Developed Electrochemical Immunoassay and the Referenced ELISA Method

	multiplexed immunoassay (mean ± SD, ng mL^{-1}, n = 3)[a]		ELISA (mean ± SD, ng mL^{-1}, n = 3)[a]		t_{exp}[b]	
sample[c]	BTX-2	DTX-1	BTX-2	DTX-1	BTX-2	DTX-1
1	–[d]	–	–	–	–	–
2	0.041 ± 0.003	0.036 ± 0.001	0.042 ± 0.002	0.033 ± 0.004	0.48	1.26
3	0.83 ± 0.09	1.22 ± 0.07	0.81 ± 0.05	1.07 ± 0.08	0.34	2.44
4	3.58 ± 0.17	4.39 ± 0.21	3.31 ± 0.18	3.86 ± 0.43	1.89	1.92
5	–	–	–	–	–	–
6	0.037 ± 0.002	0.021 ± 0.003	0.041 ± 0.003	0.016 ± 0.002	1.92	2.40
7	1.23 ± 0.07	0.97 ± 0.09	1.12 ± 0.09	114 ± 0.08	1.67	2.45
8	4.58 ± 0.32	3.12 ± 0.27	4.74 ± 0.43	2.75 ± 0.17	0.52	2.01
9	–	–	–	–	–	–
10	0.067 ± 0.005	0.092 ± 0.004	0.059 ± 0.003	0.096 ± 0.002	2.38	1.55
11	1.46 ± 0.07	1.78 ± 0.1	1.53 ± 0.09	1.59 ± 0.08	1.06	2.57
12	3.49 ± 0.92	4.87 ± 1.03	3.16 ± 0.48	4.18 ± 0.74	0.55	0.94

[a]The mean value of three assays (n = 3). [b]The calculated method of the t_{exp} value was described in our previous paper.[42] [c]Samples 1–4, 5–8, and 9–12 were used as *S. constricta*, *M. senhousia*, and *T. granosa* supernatants as matrices, respectively. Samples 1, 5, and 9 were the unspiked specimens. These samples were made available as the extract supernatant using the sample preparation described in the Experimental Procedures. [d]The symbol "–" suggested that the sample could be assayed by the corresponding method.

signals of the multiplexed immunoassay. The high specificity and anti-interference of the multiplexed immunoassay were most likely a consequence of the specific antigen–antibody reaction.

Additionally, the stability of MB–$Ab_{1,2}$ and molecular tags was investigated over a 30 day period. When MB–$Ab_{1,2}$ and molecular tags were stored at 4 °C and measured intermittently (every 2–3 days), they retained 96.2, 94.1, and 91.6% of their initial signals after a storage period of 10, 20, and 30 days, respectively. We speculate that the slow decrease of the signals was mainly attributed to the following two issues: (i) the gradual deactivation of the immobilized biomolecules on the nanostructures and (ii) the leakage of minor amounts of zero-value metal nanoparticles from the CCCYYY-based nanoclusters.

Analysis of Real Seafood Samples and Evaluation of Method Trueness. The measurement trueness and applicability of the developed immunoassay for testing real seafood samples, namely, 12 spiked seafood specimens with different levels of BTX-2 and DTX-1, such as *S. constricta*, *M. senhousia*, and *T. granosa*, were assessed using the multiplexed electrochemical immunoassay and commercialized available ELISA (ultraviolet detector) as a reference method. The results are summarized in Table 1. Statistical comparison of the experimental results of the multiplexed electrochemical immunoassay to those of ELISA was performed using a t test for comparison of means preceded by the application of a F test. No significant differences were encountered between the two methods at the 0.05 significance level (Table 1) because the t_{exp} values were in all cases below the t_{crit} value ($t_{crit[4, 0.05]}$ = 2.77). The results revealed a good agreement between both analytical methods. More importantly, when the unspiked (zero analyte) samples (i.e., samples 1, 5, and 9) were analyzed using the multiplexed electrochemical immunoassay as the control tests, all of the MB–$Ab_{1,2}$-associated probes showed substantially very low signal shifts, in contrast to those for other positive (spiked) samples. Therefore, the multiplexed electrochemical immunoassay could be considered as an optional scheme for simultaneous detection of BTX-2 and DTX-1 in seafood.

In summary, we report the proof of concept of a new multiplexed electrochemical immunoassay strategy for simultaneous determination of BTX-2 and DTX-1 in seafood using bifunctionalized MBs as capture probes and distinguishable metal nanocluster-labeled biomolecules as traces. In contrast to conventional electrochemical labels that are immediately converted into electroactive species for signal readouts, the metal nanocluster-based labels are controlled to conjugate on the bifunctionalized MBs that can produce the corresponding electroactive behaviors by the anodic stripping voltammetric method, thus creating a very sensitive and specific tactic for signal transduction. Also, the bifunctionalized MBs could be used for simultaneous capture of two analytes, could decrease the used amount of immunosensing probes, and thus make them homogeneously disperse into the microfluidic device. Relative to the conventional enzyme-labeled methods, use of metal nanoclusters is simple and feasible. Importantly, the supporting electrolyte of the developed electrochemical immunoassay was simple and universal without the need of an enzyme substrate, e.g., hydrogen peroxide, and reduced the contamination toward the analyte. To fully assess the application potential and added value of the multiplexed immunoassay, future research should focus on other target analytes and sample types.

■ ASSOCIATED CONTENT

Supporting Information

Competitive-type multiplexed assay protocol, TEM images of CdNC and CuNC nanoclusters (Figure S1), effect of the incubation time for the antigen–antibody interaction, pH of the assay solution, and dissolution time of nanoclusters on the current of the multiplexed immunoassay (Figure S2), and interference degree or cross-talk level (Table S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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