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Magneto-controlled electrochemical immunoassay of brevetoxin B in seafood based on guanine-functionalized graphene nanoribbons

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ABSTRACT

A facile and feasible magneto-controlled immunosensing platform was designed for sensitive electrochemical immunoassay of brevetoxin B (BTX-2) in seafood by using guanine-assembled graphene nanoribbons (GGNRs) as molecular tags on a home-made magnetic carbon paste electrode. Initially, monoclonal mouse anti-BTX-2 antibodies were covalently immobilized on the surface of magnetic beads, which were used as the immunosensing probes for the capture of BTX-2. The recognition elements were prepared by chemical modification of bovine serum albumin-BTX-2 conjugates (BTX-2-BSA) with the GGNRs. Based on a competitive-type immunoassay format, the formed magnetic immunocomplex was integrated on the electrode with an external magnet, followed by determination in pH 6.5 phosphate-buffered solution containing 2 μM $Ru(bpy)_3Cl_2$. Under optimal conditions, the electrochemical signals decreased with the increasing BTX-2 concentrations in the sample. The dynamic concentration range spanned from 1.0 pg mL^{-1} to 10 ng mL^{-1} with a detection limit of 1.0 pg mL^{-1} BTX-2. Inter- and intra-batch assay precisions were substantially improved by resorting to the GGNR manifold. The method featured unbiased identification of negative (blank) and positive samples. No significant differences at the 95% confidence level were encountered in the analysis of 12 spiked samples including *S. constricta, M. senhousia and T. granosa* between the electrochemical immunoassay and commercially available enzyme-linked immunosorbent assay (ELISA) for determination of BTX-2.

1. Introduction

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 (Wang and Ramsdell, 2011). Recently, various analytical methods and detection techniques have been reported for determination of BTX, such as liquid charomatography/mass spectrometry (Wang and Ramsdell, 2011; Wang et al., 2004a, 2004b), electrochemical immunoassay (Tang et al., 2011b), ELISA (Tsumuraya et al., 2010; Zhou et al., 2010; Tsumuraya et al., 2006), radioimmunoassay (Bottein et al., 2010), and immunochromatographic assay (Zhou et al., 2009). Despite many advances in this field, there is still the quest for new schemes and strategies for improvement of the assay sensitivity and simplicity.

Electrochemical immunoassay, based on highly specific molecular recognition of antigens by antibodies, holds great potential as the next-generation detection strategy (Nie et al., 2009; Zhang et al., 2011). The method measures the formation of the antigen-antibody complexes on the immunosensing probes, and detects them via an indicator system (Mello and Kubota, 2002). Typically, bioactive enzymes including horseradish peroxidase (HRP) and alkaline phosphatase (AP) are usually utilized as electron donors and acceptors (Wang, 2005; Singh, 2011). Recently, we designed an electrochemical immunoassay of BTX-2 using HRP-labeled anti-BTX-2 antibodies as traces (Tang et al., 2011b). During this process, HRP molecules were covalently conjugated onto the antibodies. One of the problems commonly associated with the covalent binding is the bioactive decrease when enzyme molecules are exposed to reactive groups and harsh reaction conditions (Tang et al., 2006). More adversely, the bioactivity of the enzymes is easily affected by the external factors including pH of the assay solution and temperature. A preferable strategy is to use

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the electroactive species as the indicators, e.g., thionine (Liu et al., 2011), guanine/adenine nucleobases (Wang et al., 2004a, 2004b), and metal ions (Cu^{2+}, Cd^{2+}, Ag^{+}, or Zn^{2+}) (Zhang et al., 2012). Guanine is an organic compound belonging with the purine group and one of the four standard bases which form pairs to make up the DNA helix. It is also one of the most easily oxidized nitrogenous bases, and has been commonly used for label-free DNA analysis and protein assay (Diculescu et al., 2005; Ming-Hung, 2008). Although nucleobases undergo reduction at mercury electrode and oxidation at various solid electrodes such as carbon, gold, silver, platinum, and copper, the use of these processes as analytical signals is limited (Huang et al., 2006). Moreover, the voltammetric peak of pure guanines is poorly developed with very high overpotential that the peaks merge into the background current (Huang et al., 2006). The rapidly emerging research field of nanotechnology, and the processes used to generate, manipulate and deploy nanomaterials, provides excitingly new possibilities for advanced development of new analytical tools and instrumentation for bioanalytical and biotechnological applications (Knopp et al., 2009).

Graphene nanoribbon (GNR), thin elongated strips of sp^2 bonded carbon atoms, has captured worldwide interest (Zhu and Tour, 2011; Jiao et al., 2009; Yang et al., 2008). Largely, the electronic states of the GNR depend on their edge structures. The zigzag edge of the GNR provides the edge localized state with non-bonding molecular orbitals near the Fermi energy, resulting in the large changes in optical and electronic properties from quantization (Han et al., 2010; Cai et al., 2010; Kosynkin et al., 2009). It has been reported that graphene sheets can strong bind the single-stranded DNA, such as aptamers and nucleobases, as a result of the hydrophobic and π-stacking interactions between the nucleobases and graphene (Tang et al., 2011a). Alike, a large number of guanine nucleobases can be conjugated onto the GNRs, thereby enhance the immobilized amount of guanines for improvement of the electrochemical signal. Another major issue for development of electrochemical immunoassay is to achieve a high sensitivity (Tang et al., 2011c). Ropp and Thorp found that the nucleobase 8-oxo-guanine could be selectively oxidized by the redox catalyst $Ru(bpy)_3^{3+/2+}$ (bpy=2,2′-bipyridine) with current enhancement in the presence of native guanine (Ropp and Thorp, 1999). Proposed mechanisms involve the interaction of guanine radicals with Ru^{3+} to generate the $Ru^{\bullet 2+}$ excited state and the reduction of Ru^{2+} to Ru^{+} by the guanine radicals, followed by annihilation of Ru^{3+} and Ru^{+} (Richter, 2004). Guanine radicals can be formed upon one-electron oxidation, either via electrode processes or by reaction with Ru^{3+}.

Herein, we report the proof-of-concept of a new magneto-controlled electrochemical immunoassay of brevetoxin B (BTX-2) in seafood using the guanine-functionalized GNR conjugated with BTX-2-BSA conjugates (BGGNRs) as molecular tags, and monoclonal mouse anti-BTX-2 antibody-functionalized magnetic beads (Ab-MBs) as immunosensing probes. Based on a competitive-type immunoassay mode, the analyte (BTX-2) and the immobilized BTX-2-BSA on the BGGNRs compete with the anti-BTX-2 antibody on the Ab-MBs. The aim of this work is to construct a novel magneto-controlled electrochemical immunoassay of BTX-2 in seafood by coupling guanines with graphene nanoribbons for signal amplification.

2. Experimental

2.1. Chemicals and reagents

Individual standard stock samples of brevetoxin B (BTX-2, purity ≥ 98% by HPLC) was purchased from Express Technol. Co., Ltd. (Beijing, China). Monoclonal mouse anti-BTX-2 antibodies (anti-BTX-2, 2.3 mg mL^{-1}, CR ≥ 90% for BTX-1 and BTX-3,) and BTX-2-bovine serum albumin conjugates (BTX-2-BSA, 2.0 mg mL^{-1}, purity ≥ 96%) were obtained from Jilin University (China). Magnetic Fe_3O_4 beads (MBs, particle size: ~100 nm) in an aqueous suspension with a concentration of 25 mg mL^{-1} were obtained from Chemicell GmbH (Berlin, Germany). Bovine serum albumin (BSA, 96%–99%), and (3-glycidyloxypropyl) trimethoxysilane ($C_9H_{20}O_5Si$, GOPS) were obtained from Sigma–Aldrich. Multiwalled carbon nanotubes (MWCNT, CVD method, purity 98%, diameter 80–100 nm, and length 1–2 μm) were supplied by Shenzhen Nanoport Co. Ltd. (China). *N*-Hydroxysuccinimide (NHS) and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) were supplied by Sigma–Aldrich. Guanine nucleobases were purchased from Dingguo Biotechnol. 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) was used in all runs. Phosphate-buffered saline (PBS, 0.1 M) with various pHs was prepared by mixing the stock solutions of 0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4, and 0.1 M KCl was added as the supporting electrolyte.

2.2. Preparation of graphene nanoribbons (GNRs)

Graphene nanoribbons (GNRs) were prepared according to the literature with a little modification (Kosynkin et al., 2009). First, the MWCNTs were pretreated by continuously sonicating the MWCNTs in a mixture containing H_2SO_4 and HNO_3 (3: 1, v/v) for 3 h. The resulting suspension was filtered, washed with water, and dried. Following that, 15 mg of MWCNTs and 75 mg of $KMnO_4$ were slowly added into 15 mL of 18 M H_2SO_4 in turn (*Caution*!). After gentle stirring for 60 min at room temperature (RT), the mixture was heated to 55–70 °C in an oil bath for further reaction until the color of the mixture went from black to dark brown. Afterward, the mixture was removed from the heat source, cooled to RT and poured onto 400 mL of ice containing 5 mL of 30 wt% H_2O_2 (*Note*: The aim of this step is to prevent precipitation of insoluble MnO_2). The resulting suspension was centrifuged for 10 min at 5000 g to remove the large-sized nanosheets and nanostructures. Consequently, the upper suspension was filtered and washed with acidic water followed by ethanol and ether. Finally, the as-prepared graphene nanoribbons (GNRs) were dried in vacuum at 60 °C for further use.

2.3. Synthesis and bio-labels of guanine-functionalized graphene nanoribbons

First, guanine-functionalized graphene nanoribbons (GGNRs) were prepared as follows: 1.0 mg of GNRs was dispersed into 1.0 mL of distilled water, and then 100 μL of 10 mM guanines were added into the mixture. After gentle stirring for 48 h at RT, the resulting suspension was centrifuged (10,000 g) for 15 min at 4 °C. The as-prepared GGNRs were re-dispersed into 1.0 mL of distilled water ($C_{[GNR]} \approx 1.0$ mg mL^{-1}).

Next, the as-prepared GGNRs were used for the label of BTX-2-BSA conjugates through carbodiimide coupling, as shown in Scheme 1a. In brief, 2.2 mg of NHS and 3.0 mg of EDC were dissolved into 1.0 mL of 1.0 mg mL^{-1} GGNR suspension followed by continuous stirring for 45 min. Afterward, 300 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 leaved at RT for 20 h. After completion of the incubation, the conjugates were centrifuged for 10 min at 5000 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 GGNRs (designated as BGGNRs) were dispersed into 1.0 mL