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A Novel Piezoelectric Biosensor for Cholera Toxin Detection Based on Ganglioside GM1 Incorporated Supported Lipid Membrane and Biocatalyzed Deposition Amplification

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A novel piezoelectric method for the detection of cholera toxin based on the supported lipid layer incorporated with ganglioside GM1 and the biocatalyzed deposition amplification has been developed. The planar supported lipid membrane was prepared as biosensing interface *via* spontaneous spread of ganglioside GM1-incorporated phospholipid vesicles on the octadecanethiol-coated gold surface. The specific interaction of multivalent cholera toxin by ganglioside GM1 molecules enables the biosensor to be implemented *via* a sandwiched format using anti cholera toxin antibody (CT antibody). After the "sandwich" immunoreaction, the horseradish peroxidase (HRP) labeled goat antirabbit IgG antibody (HRP-G-anti RIgG) was introduced into the detection cell to combine with the anti cholera toxin antibody on the sensor surface. The enzyme labeled HRP-G-anti RIgG act as a biocatalyst which could accelerate the oxidation of 4-chloro-1-naphthol by H_2O_2 to yield the insoluble product benzo-4-chlorohexadienone on the surface of quartz crystal microbalance, resulting in a mass increase that was reflected by a decrease in the resonance frequency of the quartz crystal microbalance. The proposed approach could allow for the determination of cholera toxin in the concentration range of 0.1-500.0 ng/mL. The interference experiment results demonstrated that the developed biosensor performed with highly resistance to nonspecific adsorption.

Key Words: Cholera toxin, Supported lipid membrane, Biocatalyzed deposition amplification, Enzyme, Quartz crystal microbalance.

INTRODUCTION

Cholera toxin (CT) is a protein enterotoxin produced by vibrio cholerae. Most cholera cases are reported in Southeast Asia, Africa and South America. It is estimated that cholera affects over 120 000 persons annually[1] and can be fatal in 50 % of cases where facilities for treatment are not available[2]. It does not only affect impoverished areas but also poses a serious threat as a potential tool for bioterrorism[3,4]. Cholera toxin is the major virulent factor of toxigenic strains of *V. cholerae*[5]. It has a common hetero-hexameric structure, consisting of a single, enzymatically active A subunit (CT-A,27 kDa), non-covalently linked to a pentameric core of five identical receptor-binding B subunits (CT-B, 58 kDa)[6]. The biological action of cholera toxin is initiated by the binding of CT-B to the ganglioside GM1 receptor on the intestinal cell membrane followed by internalization of CT-A into the cell where it activates adenylatecyclase[7].

Despite the understanding of its molecular mechanism, cholera remains a major concern throughout the world and there has been increasing interest in the development of rapid and sensitive methods for the determination of cholera toxin. Fluorescence microarray technology[8]; colour change[9]; fluoroimmunoassay[10-13]; flow cytometry[14]; resonant mirror[15]; surface plasmon resonance (SPR)[16] and surface plasmon resonance in combination with an ion selective field effect transistor device[17]. These methods are well-proven and widely accepted. However, they are often viewed as laborious and time-consuming, requiring a significant investment in equipment, materials and maintenance. Therefore, searching for rapid, sensitive methods with real-time output and low cost is still of considerable significance.

The quartz crystal microbalance (QCM) technique, which offers some advantages including high sensitivity, real-time output, cost effectiveness and experimental simplicity, has attracted research interest in recent years[18-21]. The QCM is a mass-sensitive transducer device, in view of the dependence of its oscillation frequency on the total mass of the oscillating transducer[22]. Since Shons *et al.*[23] first reported the method using the QCM for cow serum IgG antibody detection in 1972, the development of the QCM immunoassay has been the subject of a series of investigations[24,25]. To enhance the assay sensitivity, Ebersole and Ward[26] described an amplified mass immunosorbent assay (AMISA) with a QCM. The results demonstrated

that the amplification routes *via* catalytic processes, such as enzymatic reactions, could significantly extend the detection limits of piezoelectric assay. Since then, various enzymes which could stimulate the biocatalytic precipitation of insoluble products have been utilized for the mass amplification[27,28].

In this study, a novel piezoelectric method based on ganglioside GM1 functionalized supported lipid membrane and biocatalyzed deposition had been developed for the detection of cholera toxin.

The biosensing surface could be prepared with ease *via* spontaneous spread of ganglioside GM1-incorporated phospholipid vesicles on the octadecanethiol-coated gold surface[21,29]. The use of ganglioside GM1 as recognition receptors in a biosensor have many advantages over antibodies[29] ganglioside GM1 molecules are very small in comparison to antibodies and, therefore, a higher surface concentration of receptors is possible. This will increase the degree of interaction with the analyte, giving lower detection limits. Also, ganglioside GM1 molecules are generally more stable than antibodies, giving an increased usable lifetime of the biosensor surface.

The biocatalyzed precipitation of an insoluble product produced on electrode supports mediated by the Horseradish peroxidase labeled goat antirabbit IgG antibody (HRP-G-anti RIgG) was utilized as a signal amplification route for biosensing. In brief, the quartz crystal surface was coated with ganglioside GM1 functionalized supported lipid membrane. The specific interaction of multivalent CT by ganglioside GM1 molecules enables the biosensor to be implemented *via* sandwiched format using CT antibody. And then the HRP-G-anti RIgG bound to the CT antibody was used as a biocatalyst for the oxidative precipitation of 4-chloro-1-naphthol by H_2O_2 to yield the insoluble product benzo-4-chlorohexadienone on the QCM surface, resulting in an obvious frequency change that corresponded to the level of CT analyte. The proposed approach was successfully applied to the determination of CT in buffer or sample solutions, implying that our technique would hold considerable potential in the development of sensitive, simple biosensors for clinical and public health areas.

EXPERIMENTAL

1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), ganglioside GM1 (GM1) from bovine brain, cholera toxin from vibrio cholerae, anticholera toxin antibody produced in rabbit (CT antibody), 4-chloro-1-naphthol and octadecanethiol were purchased from Sigma-Aldrich (Shanghai, China). Horseradish peroxidase labeled goat anti-rabbit IgG antibody (HRP-G-Anti-RIgG), casein, ovalbumin, human serum albumin (HSA) and bovine serum albumin (BSA) were obtained from Beijing Dingguo Biotechnology Company (Beijing, China). Phosphate buffer saline (PBS) solution (pH 7.4) was prepared by using 0.1 M Na_2HPO_4 and 0.1 M KH_2PO_4. Cholera toxin solution of varying concentration were prepared in PBS (pH 7.4) and stored at 4 ºC. The 'piranha' solution was a mixture of 98 % H_2SO_4 and 30 % H_2O_2 in a volume ratio of 7:3. Doubly distilled water was used throughout the experiments. All other chemicals used were of analytical grade.

The PQC (AT-cut, 9MHz, gold electrodes) was obtained from Chen Xing Radio Equipments (Beijing, China). To keep a steady oscillation in the solution, the PQC was sealed on one side with an O-ring of silicone rubber covered by a plastic plate that formed an air chamber isolated from aqueous solution. The detection was performed by setting the piezoelectric sensor in a laboratory-made reaction cell. The resonance frequency was monitored with a quartz crystal analyzer (QCA922, Seiko EG & G Co. Ltd., Japan) and recorded by a computer in Windows XP professional operating system. The experimental temperature was controlled with a thermostat (model CSS501, Chongqing Experimental Equipments, China).

Preparation of the GM1-functionalized liposomes: Unilamellar liposomes were prepared as described elsewhere[21]. Stock solutions of lipids, gangliosides were prepared in 6:1 chloroform/methanol (v/v) mixture. The total lipids were mixed (1 mg in total weight) in the mole ratio of 20:1 (DPPC/GM1) in a 5 mL round-bottom flask. After the sonication of the mixture for 15 min, the organic solvent was removed by rotary evaporation under reduced pressure (0.09MPa), leaving a thin lipid film on the inside wall of the flask. To the dried lipids, 2 mL of PBS (pH 7.4), was added. The lipids were hydrated in a water bath at 50 ºC and rotated vigorously to form multilamellar vesicles (MLVs). The MLV suspension was sonicated using a probe-type sonicator to reduce the average size of the liposomes. The resulting unilamellar liposomes were centrifuged at 3000 rpm for 20 min to remove residual multilamellar vesicles and aggregated lipids. The liposome solution was stored at 4 ºC until further use.

Quartz crystal microbalance surface modification: The gold electrode (5 mm diameter) was treated with "piranha" solution, then washed thoroughly with a copious amount of doubly distilled water and finally dried at room temperature. The bare gold surface was immersed in a freshly made 10 mM octadecanethiol in ethanol overnight to allow the thiols to form a hydrophobic monolayer on the gold surface. The sensor surface was then rinsed with ethanol thoroughly and air-dried. Exposure of the hydrophobic sensor surface to an aqueous suspension of GM1-incorporated liposomes resulted in spontaneous spread of supported planar lipid monolayer. The GM1-incorporated supported lipid membrane modified sensor was kept in PBS for further use.

Detection method: The supported lipid membrane-modified sensor was reacted with 50 µL sample solution containing varying concentrations of CT for 15 min. After thoroughly rinsing with PBS, 50 µL of anti CT antibody (1:1000 dilution ratio) was injected into the solution, leading to the immunoreaction between the CT, captured by GM1 on probe surface and the CT antibody. About 0.5 h later, the QCM was rinsed with PBS and distilled water after removing the solution. Next, a portion of 40 µL PBS and 40 µL HRP-G-anti RIgG (1:400 dilution ratios) were added and incubated in the cell for 0.5 h. Thereafter, the cell was drained and rinsed as described above. Finally, the biocatalytic deposition solution and PBS were injected into the cell, the probe cell was contain 200 µL solution, consisting of 1×10^{-3} M of 4-chloro-1-naphthol and 1.5×10^{-4} M H_2O_2. The resonance frequency of the QCM was recorded by QCA922 connected to a computer as the immunoreaction proceeded after each addition of analyte until equilibrium was reached. The control test and inteference study were acted in

a similar way. All experiments were performed with triplicate measurements and the experimental temperature was controlled at 25 ºC.

RESULTS AND DISCUSSION

A schematic representation of the detection principle of this biocatalyzed deposition biosensor is shown in Fig. 1. The gold surface is modified by a self-assembled monolayer of octadecanethiol, producing a hydrophobic surface. The GM1-functionalized liposomes are spread over the hydrophobic surface and formed a biosensor surface of supported lipid membrane with incorporated GM1. Due to the specific interaction of CT with up to five GM1 receptors, the toxin is captured on the biosensor surface on interacting with the CT sample solution and then sandwiched by the CT antibody and HRP-G-anti RIgG. The presence of HRP is signaled through an enhanced biocatalyzed reaction involving HRP-catalyzed oxidation of 4-chloro-1-naphthol in the presence of H_2O_2. The precipitation of the insoluble product on the QCM surface results in a mass increase that is reflected by a decrease in the resonance frequency of the QCM. The QCM signal is proportional to the amount of analyte (CT) in buffer or sample solutions.

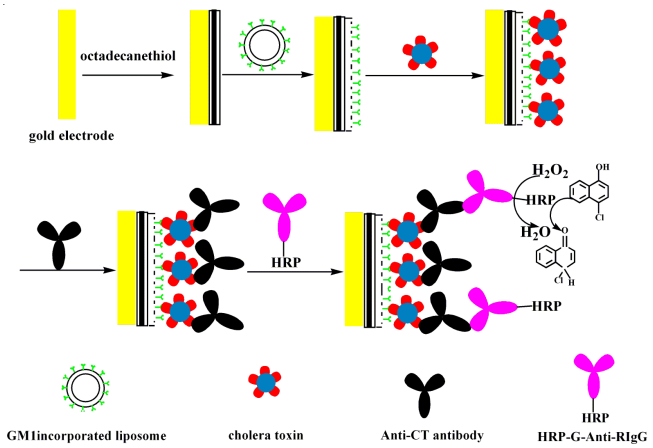

Fig. 1. Schematic diagram of the developed biocatalyzed deposition biosensor for cholera toxin

Characteristics of frequency response for the biosensor: Fig. 2 illustrates real time interaction processes of the sensing surface in the determination of CT as monitored using microgravimetric QCM analysis. A denotes the addition of GM1-incorporated liposome to form the bilayer lipid membrane covered sensor surface, B represent wash with PBS, C inidcates the the addition of 2 µg /mLCT to the probe cell, D represents the addition of anti CT antibody, E indicates the addition of HRP-G-anti RIgG, F represents the deposition of the precipitation on the sensor surface. Fig. 2 showed that the introduction of liposome on the octadecanethiol-coated gold surface induces a frequency decrease, indicating that the supported lipid membrane forms *via* spontaneous spread of ganglioside-incorporated phospholipid vesicles on the hydrophobic surface. After rinsing with PBS, the analyte CT (2 µg/mL) was added to reaction cell. Binding of CT on the GM1-incorporated supported lipid membrane results in a further frequency decrease. After rinsing with PBS, the addition of

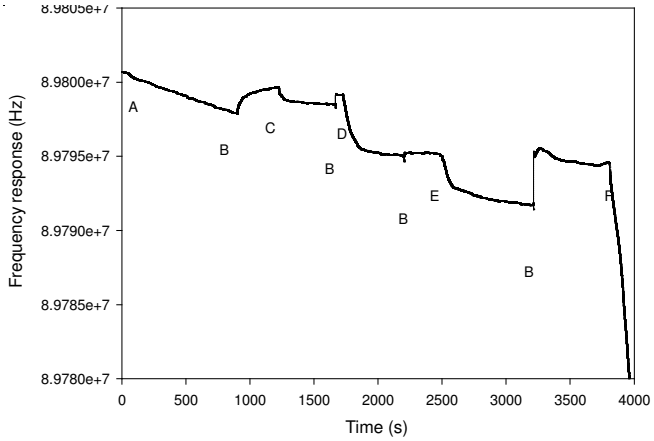

Fig. 2. Real-time interaction processes of the sensing surface in the determination of CT as monitored using microgravimetric QCM analysis

the anti CT antibody and HRP-G-anti RIgG again produces a frequency decreases, implying that the sandwiched complex was formed on the biosensor surface. The subsequent biocatalyzed precipitation of benzo-4-chlorohexadienone results in a significant decrease in the resonance frequency of QCM, indicating that an amplified detection of the CT is observed. These results gave evidence that the expected interfacial processes were performed, confirming that the analytical protocol was feasible for the determination of CT.

Optimization of the experimental conditions

Molar fraction of GM1 in lipid membrane and incubation period of liposome on sensor surface: The surface density of GM1 receptor in lipid membrane would influence the sensitivity of the sensor[30]. To enable the CT be recognized by the GM1 receptors on the sensor surface, the GM1 incorporated liposome was prepared according to a documented procedure[21] with slight modifications as described before. The incubation time of liposome on the sensor surface to form the lipid membrane was investigated by monitoring the decrease of frequency resonance of the sensor after the liposome was added to the probe cell. As shown in Fig. 3, the frequency resonance decrease reach a plateau when the incubation period was beyond 0.5 h, indicating the probe surface was fully coverd with GM1 tethered lipid membrane. Therefore, a incubation time of 0.5 h was selected in the subsequent study.

Effect of the anti CT antibody concentration: To this sanwiched immunoassay, the CT antibody served as the bridge to connect the CT and HRP-G-anti RigG whose quantity is of great importance. The CT antibody concentration was optimized by comparing dose-response curves (Fig. 4). To our surprise, the frequency response (ΔF) to the immunoreaction peaks at dilution ratio of 1:1000 without a plateau level, *i.e.*, "saturation" as expected. Increasing the concentration of anti CT antibody over the peaking value decreases the frequency response. There are two possible explanations for the results above: the first might relate to a decreased formation of sandwiched immunocomplex for the ratio between antigen and antibody at too low anti CT antibody concentrations. The second may be attributed to that high anti CT antibody might result in an increased steric hindrance. The diution ratio of 1:1000 was accepted in the following experiment.

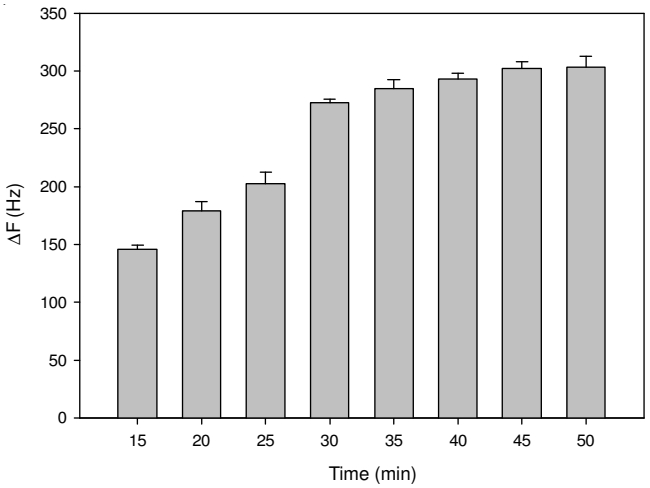

Fig. 3. Time dependence of the probe under different incubate periods of the liposome

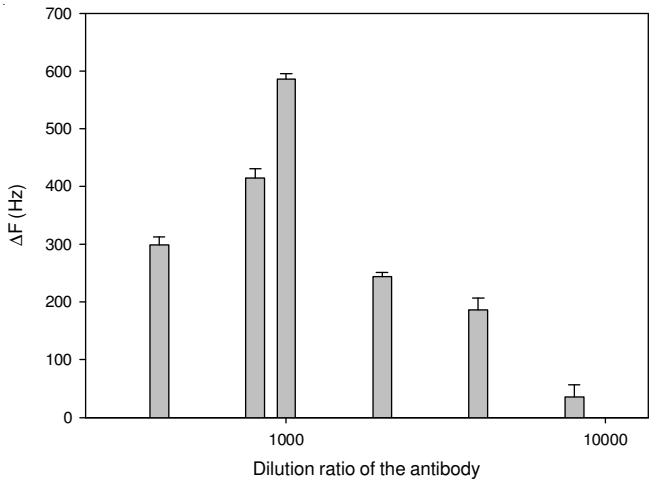

Fig. 4. Frequency responses of vary anti CT antibody dilution ratios in the assay medium (the sensor surfaces were incubated with GM1 tethered lipid membrane and had reacted with 2.5 μg/mL CT)

Effect of the HRP-G-anti RIgG concentration: The HRP-G-anti RIgG concentration is another important factor for the amplified approach. Thus, the effect of the HRP-G-anti RIgG concentration was investigated. As shown in Fig. 5, the frequency response gradually decreases with the HRP-G-anti RIgG dilution ratio up to 1:400, after that it begins to level off. To "saturate" the biting sets of anti CT antibodies on sensor surface and save the reagents, a HRP-G-anti RIgG dilution ration of 1:400 was adopted in the following experiment.

Effect of the biocatalytic deposition time: A phosphate buffer containing 1×10^{-3} M 4-chloro-1-naphthol and 1.5×10^{-4} M H_2O_2 was adopted as the biocatalytic deposition solution[20]. When the concentration of biocatalytic deposition solution was set, the biocatalytic deposition time should also be optimized. As can be seen from Fig. 6, the frequency response ascends with the increase of the biocatalytic deposition time before 15 min and then to be stable. Therefore, a biocatalytic deposition time of 15 min was selected in the subsequent study.

Analytical performance: To obtain the calibration curve, several samples with various concentrations of CT were measured

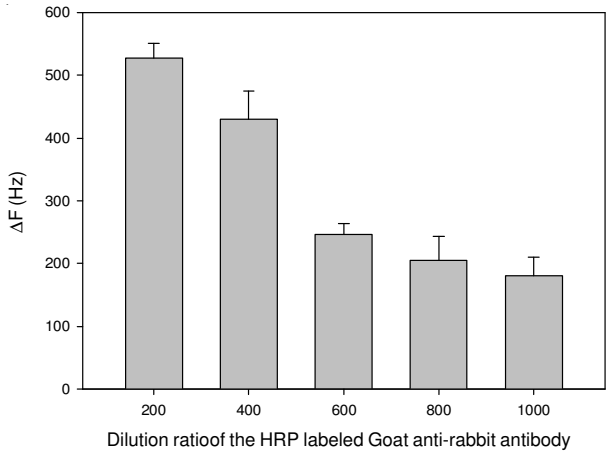

Fig. 5. Different HRP-G-anti RIgG concentration with the dilution ratio of anti CT antibody and incubation time being 1:1000 and 0.5 h, respectively in the assay medium

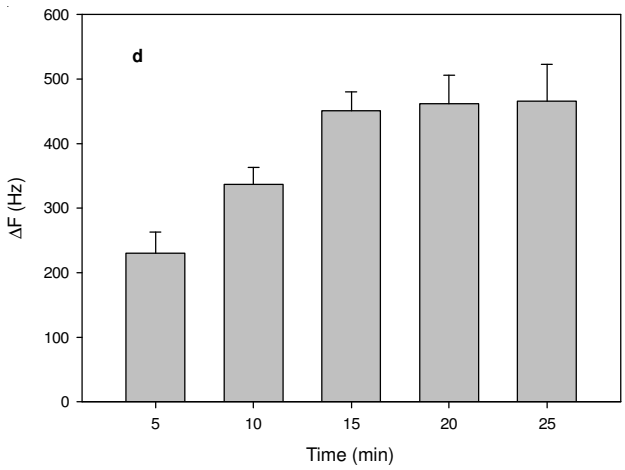

Fig. 6. Influence of different time intervals for the catalyze reaction

under optimal experimental conditions. As can be seen from Fig. 7, the frequency response has a good linear relationship with the logarithm of CT concentration in the range of 0.1-500.0 ng/mL. The linear regression equation is y = 259.62 + 116.9 log x with a correlation coefficient of 0.9910 and the detection limit is 0.1 ng/mL.

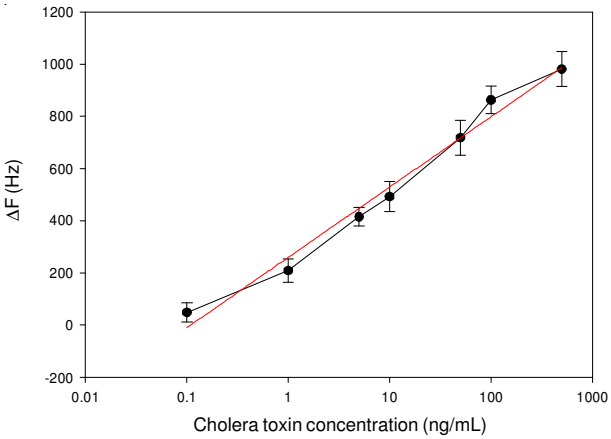

Fig. 7. Calibration curves of the relationship between the frequencies response and the concentration of cholera toxin. The red line describes the linear relationship between frequency responses (ΔF) *vs*. the logarithm of CT concentration

Inteference study: The frequency responses of the developed biosensor were measured to common serum proteins. Fig. 8 depicts the frequency signals to HSA (10 mg/mL), Casein (saturated solution in PBS), ovalbumin (saturated solution in PBS), BSA (10 mg/mL). The responses for these interference proteins are smaller than that obtained with 100 ng/mL CT, indicating that the lipid membrane-modified interface is inherently resistant to non-specific adsorption of serum constituents and the developed sensor demonstrates excellent selectivity for CT detection. Therefore, the developed strategy was expected to hold promise for the detection of CT in complicated serum specimens.

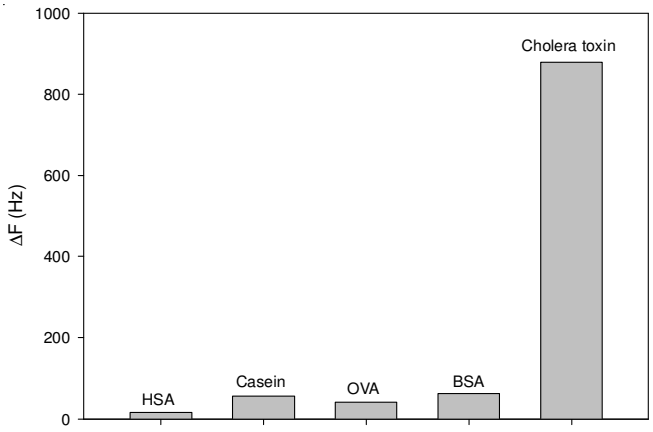

Fig. 8. Frequency of the developed biosensor to different proteins: HSA (10 mg/mL), casein (saturated solution in PBS), ovalbumin (saturated solution in PBS), BSA (10 mg/mL) and CT (100 ng/mL)

Conclusion

The present study developed a novel biosensor for cholera toxin detection using the supported lipid membrane-based sensing surface and the amplification method of biocatalyzed precipitation on the quartz crystal microbalance surface for cholera toxin detection by microgravimetric analysis. The lipid membrane-based interface at the biosensing surface and the liposome probe provided a biocompatible environment that was beneficial for the resistance of non-specific adsorption of serum constituents, thus ensuring a low background signal in the assay. The biocatalyzed precipitation of an insoluble product on the quartz crystal microbalance surface enable the amplification of the sensing event. The proposed approach has proved to be capable of detecting cholera toxin at levels down to 0.1 ng/mL. The interference experiment results demonstrated that the developed biosensor could allow sensitive, selective and detection of cholera toxin with highly resistance to nonspecific adsorption. It is expected that the developed strategy might furnish an ideal protocol for sensitive detection of various protein targets in clinic diagnosis and medical researches.

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