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An ultrasensitive chemiluminescence biosensor for cholera toxin based on ganglioside-functionalized supported lipid membrane and liposome

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ABSTRACT

A novel chemiluminescence biosensor based on a supported lipid layer incorporated with ganglioside GM1 was developed for the detection of cholera toxin. The planar supported lipid membrane was prepared as biosensing interface via spontaneous spread of ganglioside-incorporated phospholipid vesicles on the octadecanethiol-coated gold surface. The specific interaction of multivalent CT by ganglioside GM1 molecules enables the biosensor to be implemented via a sandwiched format using a liposome probe functionalized with GM1 and horseradish peroxidase (HRP). Then, the presence of the target CT could be determined via the HRP-catalyzed enhanced chemiluminescence reaction. The developed strategy offers several unique advantages over conventional biosensors in that it allows for an easy construction and renewal of the sensing interface, a small background signal due to low non-specific adsorption of serum constituents on the lipid membrane, and effective immobilization of multiple biocatalytic amplifiers and recognition components via common phospholipid reagents. The developed biosensor was shown to give chemiluminescence signal in linear correlation to CT concentration within the range from 1 pg mL^{-1} to 1 ng mL^{-1} with readily achievable detection limit of 0.8 pg mL^{-1}.

1. Introduction

Cholera toxin (CT) is a protein enterotoxin secreted by the bacterium *Vibrio cholerae* that can cause an epidemic disease leading to rapid dehydration, acidosis, and even death in several hours without appropriate treatment. Most cholera cases are reported in many underdeveloped countries, and it is estimated that cholera causes approximately 120,000 deaths annually. As a typical biomarker of *V. cholerae*, CT has become a very important target in biological detection, and there has been increasing interest in the development of rapid and sensitive methods for the determination of CT (Spangler and Tyler, 1999; Stine and Pishko, 2004; Williams and Jenkins, 2008).

Specific determination of CT can be implemented either using the antibody to CT or ganglioside GM1, a natural sensitive receptor for cholera toxin. Antibody-based immunosensors for CT were developed using impedance measurements on ion-sensitive field-effect transistor devices together with surface plasmon resonance (SPR) (Zayats et al., 2002) and fluorescence microarray technology (Taitt et al., 2002). However, a majority of immunoassays for CT could be benefited from the use of ganglioside GM1. Ganglioside GM1 has advantages over antibody in two aspects: (1) GM1 can bind to the biological toxin with strong affinity comparable to that of antibody, and the binding process for GM1 is much more rapid than that of antibody, which makes GM1 especially useful in rapid determination cases; (2) GM1 can be readily incorporated into the phospholipid bilayer structure to construct specific capture or detection probe for CT, while the immobilization of antibody frequently requires several high cost chemical steps. The binding affinity and specificity of CT with GM1 have been investigated by surface plasmon resonance and impedance spectroscopy (Terrettaz et al., 1993). Therefore, ganglioside GM1 and analogues have found widespread use in cholera toxin detection. Based on the sensing surface constructed by biological membrane-like lipid films with incorporated GM1 analogues, a reagentless optical biosensor was developed for rapid assay of protein toxins using the resonant mirror instrument and SPR (Phillips et al., 2006; Kuziemko et al., 1996; Fisher and Tjärnhage, 2000). Direct homogenous detection of CT was reported based on the color changes (Schofield et al., 2007; Pan and Charych, 1997) and fluorescence quenching (Song et al., 1998; Song and Swanson, 1999) or resonant energy transfer (Song et al., 2000; Ma and Cheng, 2006) using liposomes incorporated with GM1 or its analogue. However, these single-step techniques used to show relatively low detection sensitivity, which hindered the proliferation of these methods to realistic applications. To improve the detection sensitivity, sandwiched immunoassay techniques for CT was then reported through the combination of capture antibody with GM1-incorporated liposome probe labeled with fluorescent

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reagents (Singh et al., 2000; Ho et al., 2007; Rowe-Taitt et al., 2000; Edwards and March, 2007), colorimetric markers (Ahn-Yoon et al., 2003), enzymes (Alfonta et al., 2001a,b) and electroactive species (Viswanathan et al., 2006). The strategies, though offering sensitivity enhancement, might suffer from the difficulty in regenerating the sensor interface, since the immobilized antibody would unavoidably lose some activity during the elution treatment.

In the present study, a novel chemiluminescence biosensor was developed for the detection of CT based on a supported lipid membrane as sensing surface and the HRP/GM1-functionalized liposome as detection probe. The biosensing surface could be prepared with ease and high reproducibility via spontaneous spread of ganglioside-incorporated phospholipid vesicles on the octadecanethiol-coated gold surface (Radler et al., 1995; Reviakine and Brisson, 2000). The implementation of liposome-based detection probe allowed for an efficient incorporation of the GM1 receptor and covalent conjugation of multiple biocatalytic amplifier HRP through common phospholipid components. Also, 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 (Chen et al., 2007; Lee et al., 2005; Puu et al., 1995; Vamvakaki et al., 2005). Moreover, the application of enhanced chemiluminescence reaction in the detection of HRP-bearing liposome afforded a further signal amplification and background alleviation. In addition, the supported lipid-based biosensing surface allowed the surface to be renewed easily and rapidly, implying that our technique would hold considerable potential in the development of sensitive, simple and renewable biosensors for clinical and public health areas.

2. Experimental methods

2.1. Reagents and apparatus

1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), phosphoethanolamine (PE), monosialoganglioside (GM1), Sephadex G-100, glycine and octadecanethiol were purchased from Sigma Chemical Co. Glutaraldehyde was from Fluka. Cholera toxin B subunit (CT) was kindly supplied by Shanghai United Cell Biotechnology Co. Ltd. Bovine serum albumin (BSA), horseradish peroxidase (HRP), IgG, HSA, IgE and thrombin were obtained from Beijing Dingguo Biotechnology Company (Beijing, China). Phosphate buffer saline (PBS, pH 7.4) was prepared using 0.067 M Na_2HPO_4 and 0.067 M KH_2PO_4. The glycine–NaOH solution (pH 7.4) was prepared using 3 M glycine and 3 M NaOH. CT solution of varying concentrations were prepared in 0.067 M PBS and stored at 4 °C. Stocking DMSO solution of *p*-iodophenol was prepared as 10 mM. Luminol solution was prepared as 150 mM using 0.5 M NaOH. 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.

The chemiluminescence spectra were recorded on a fluorescence spectrophotometer (Jobin Yvon Fluorolog3, France). The quartz crystal analyzer (QCA 922) was purchased from Serkoeg & G Co. Ltd. (Japan). Piezoelectric quartz crystals (9 MHz, gold electrodes) were supplied from Chenxing Radio Equipments (Beijing, China).

2.2. Preparation of the GM1-functionalized liposomes

The GM1-functionalized liposomes were prepared according to a documented procedure (Singh et al., 1995) with slight modifications described as follows: Stock solutions of DPPC, PE and GM1 were prepared in a chloroform/methanol mixture (6:1, v/v). The lipid mixture (1 mg in total) of DPPC, PE and GM1 was prepared in the molar ratio of 17:5:1 (DPPC/PE/GM1) in a 5-mL round-bottomed flask. After the mixture was sonicated for 5 min, the organic solvent was removed by rotary evaporation under reduced pressure (0.09 MPa), leaving a thin lipid film on the inside wall of the flask. To the dried lipids, 2 mL of PBS was added. The lipids were hydrated in a water bath at 50 °C 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 liposome suspension was centrifuged at 3000 rpm (644 g) for 15 min to remove residual multilamellar vesicles and aggregated lipids. The liposome solution was stored at 4 °C until further use. The diameter of the resulting liposome was measured to be 115 ± 15 nm using a dynamic light scattering system (Zeta Plus, Brookhaven Instruments Corp.). The amount of GM1 in the resulting liposomes was measured by the dimethyl methylene blue dye method (De Lucca et al., 1995), and it was estimated that 2200 molecules of GM1 were on the outer surface of each liposome. Liposome concentration and the number of HRP per liposome were determined to be 2.3×10^{12} liposomes mL^{-1} and 46, respectively as described elsewhere (Singh et al., 1996; Alfonta et al., 2001a,b).

2.3. Coupling of HRP to the GM1-functionalized liposomes

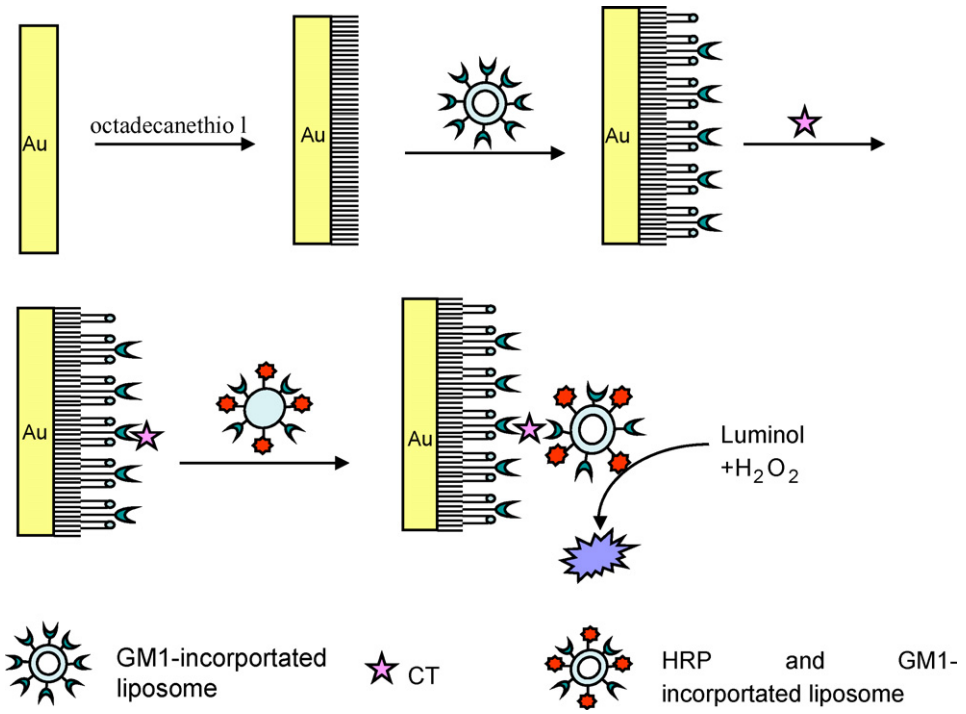
Liposomal conjugates with HRP were prepared via glutaraldehyde coupling according to a documented method (Nakano et al., 2001) as previously described by us (Chen et al., 2007): To 0.5 mL of 2.5% glutaraldehyde solution, 5 mL of liposomes (0.5 mg lipid mL^{-1}) were added in drops under gently stirring for 1 h at 25 °C. Excess glutaraldehyde solution was disposed by dialysis overnight in PBS. Then, 0.5 mL of 5 mg mL^{-1} HRP in PBS was added under gently stirring for 1 h at 25 °C. In order to block excess aldehyde groups on the liposome surface, 1 mL of 3 M glycine–NaOH buffer was added followed by incubation overnight at 4 °C. The liposome-coupled and -uncoupled HRP in the resulting solution were separated by Sephadex G-100 column and the HRP- and GM1-incorporated liposome were kept at 4 °C until use.

2.4. Supported lipid membrane formation on electrode

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 2 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.

2.5. Analytical protocol for chemiluminescence determination of CT

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, the biosensing surface was immersed in HRP- and GM1-functionalized liposomes for 15 min. The resulting sensing surface was rinsed with PBS thoroughly. The biosensor was placed in a cuvette containing 0.8 mL PBS solution of 1.65 mM *p*-iodophenol and 16.5 mM luminol. After luminescence background stabilized, 80 μL 100 mM H_2O_2 solution was injected immediately into the



Scheme 1. Schematic diagram of the developed chemiluminescence biosensor for CT.

cuvette. The real-time output of luminescence intensity was monitored.

3. Results and discussion

3.1. Analytical principle of the chemiluminescence biosensor for CT

A schematic representation of the detection principle of this chemiluminescence biosensor is shown in Scheme 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 liposome probe with HRP and GM1. The presence of HRP is signaled through an enhanced chemiluminescence reaction involving HRP-catalyzed oxidation of luminol by H_2O_2.

The developed biosensor offered several unique advantages over conventional sandwiched immunoassay techniques in CT detection. First, a GM1-incorporated supported lipid membrane-based sensing surface was constructed through self-assembly technique, which enabled the surface to be prepared and renewed with ease and high reproducibility, avoiding the activity loss of antibody in the immobilization. Second, GM1 was reported to bind rapidly to CT with strong affinity and specificity. This allowed the biosensor to exhibit a fast kinetics in detection and become especially useful in rapid determination cases. Third, the implementation of liposome-based detection probe allowed for an efficient incorporation of the GM1 receptor and covalent conjugation of tens of HRP molecules through PE, affording much greater signal amplification than conventional HRP–antibody conjugate probe. Also, 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. Additionally, *p*-iodophenol was introduced as a signal enhancer in the chemiluminescence-based HRP detection system, resulting in a further signal amplification and background alleviation.

3.2. Optimization of molar fraction of GM1 in lipid membrane and HRP coverage on liposome surface

It was reported that the surface density of GM1 receptor in lipid membrane would influence the orientation of CT protein at the membrane (Terrettaz et al., 1993; Shi et al., 2007). To enable the analyte protein accessible by both the sensor surface and the liposome, the concentration of GM1 should be optimized in the lipid membrane. Under controlled conditions in preparing the liposome, the concentration of GM1 in the lipid membrane was determined by the amount of GM1 in the lipid solution. Fig. 1(A) depicts the chemiluminescence response of the biosensor as a function of varying GM1 fraction in the lipid solution. It can be seen from Fig. 1(A) that the maximal chemiluminescence response is obtained with the lipid solution with 17:5:1 in molar ratio for PC:PE:GM1.

In the developed strategy the concentration of CT was determined via the HRP-catalyzed chemiluminescence reaction. The detection sensitivity was then strongly dependent upon the number of HRP molecules that could specifically bind to one analyte protein, thereby determined by the HRP coverage on the liposome surface. The coverage of HRP on the liposome surface could be controlled by the concentration of PE. With varying PE fraction, the coverage of amino reactive groups, on the liposome surface for covalent conjugation of HRP could be modulated, thus adjusting the coverage of HRP on the liposome surface. Therefore, the HRP coverage on the liposome surface could be represented by the PE fraction in the liposome composition. Fig. 1(B) shows the influence of the HRP coverage on the liposome surface upon the chemiluminescence response of the biosensor. As observed in Fig. 1(B), the largest luminescence intensity appears when the

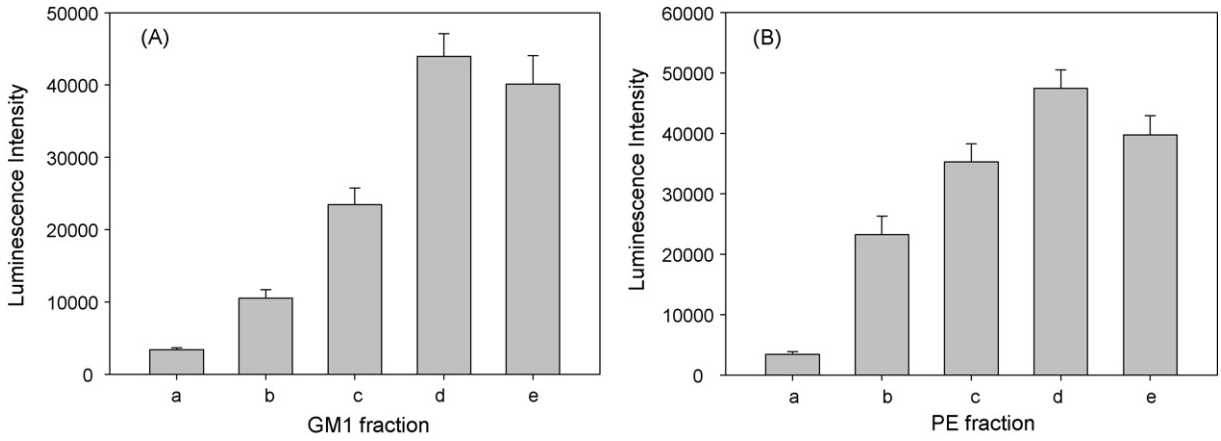


Fig. 1. (A) The chemiluminescence intensity of the developed biosensor obtained from the supported lipid membrane-modified sensing surfaces with different GM1 fraction when the concentration of CT was 50 pg mL^{-1}. The lipid vesicle for the supported lipid membrane formation with molar ratio of PC:PE:GM1: (a) 17:5:0, (b) 17:5:0.425, (c) 17:5:0.567, (d) 17:5:1, and (e) 17:5:1.7. (B) The chemiluminescence intensity of the developed biosensor obtained from the HRP/GM1-incorporated liposomes with different PE fraction when the concentration of CT was 50 pg mL^{-1}. The lipid vesicle prepared for the HRP/GM1-incorporated liposomes with molar ratio of PC:PE:GM1: (a) 22:0:1, (b) 20:2:1, (c) 18:4:1, (d) 17:5:1, and (e) 15:7:1.

molar ratio for PE:PC:GM1 is 5:17:1. With more PE fraction, the liposome, though possessing more amino groups, might induce spatial hindrance in covalent coupling of HRP and unstability of the liposomes.

3.3. Optimization of chemiluminescence assay medium

To obtain maximized sensitivity enhancement in chemiluminescence detection, we investigated the conditions of chemiluminescence assay medium for HRP-catalyzed oxidation of luminol by H_2O_2 with *p*-iodophenol as the enhancer. In the absence of enhancers, HRP produces relatively low chemiluminescence. The *p*-iodophenol has been reported to enhance the chemiluminescence associated with the HRP-catalyzed oxidation of luminol (Easton et al., 2002; Kapeluich et al., 1997). Fig. 2 depicts the chemiluminescence response of the biosensor as functions of varying concentrations of luminol, H_2O_2 and *p*-iodophenol in the assay medium. As shown in Fig. 2, the maximum chemiluminescence signal is achieved when the concentrations of luminol, H_2O_2 and *p*-iodophenol are 15, 9 and 1.5 mM, respectively, in the reaction medium. According to the experimental data in Fig. 2c, HRP catalyze the luminol oxidation in the presence of *p*-iodophenol, and this compound results in up to 225-fold increase in luminescence intensity over the unenhanced reaction.

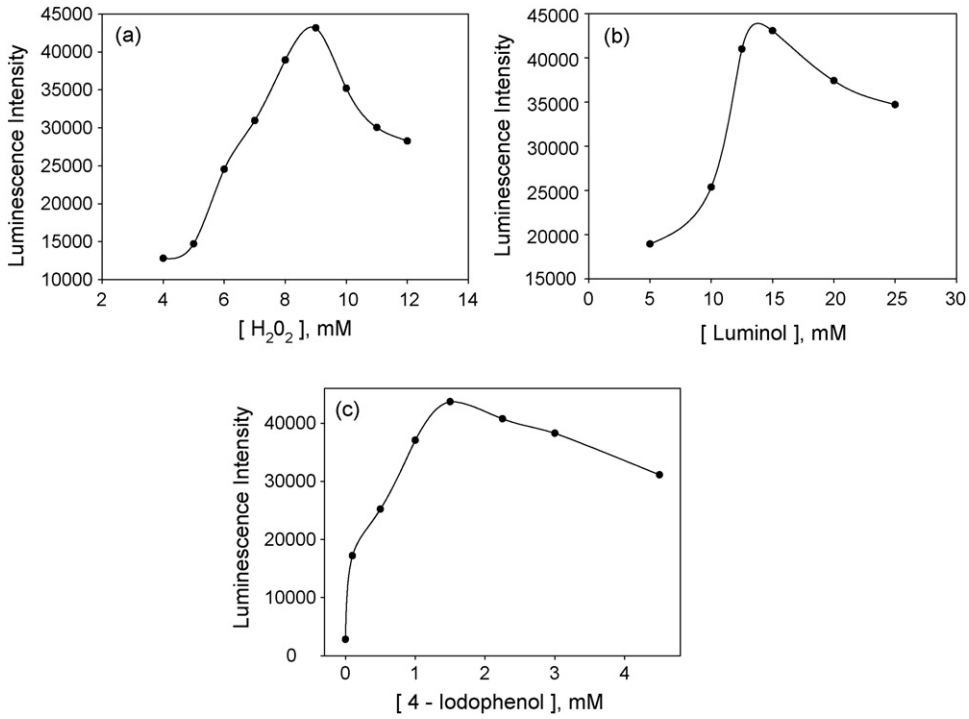


Fig. 2. The chemiluminescence intensity of the developed biosensor obtained from the optimization of the assay medium: (a) varying luminol concentration with the concentrations of H_2O_2 and *p*-iodophenol being 9 and 1.5 mM, respectively in the assay medium; (b) different H_2O_2 concentration with the concentrations of luminol and *p*-iodophenol being 15 and 1.5 mM, respectively in the assay medium; (c) varying *p*-iodophenol concentration with the concentrations of luminol and H_2O_2 being 15 and 9 mM, respectively in the assay medium.

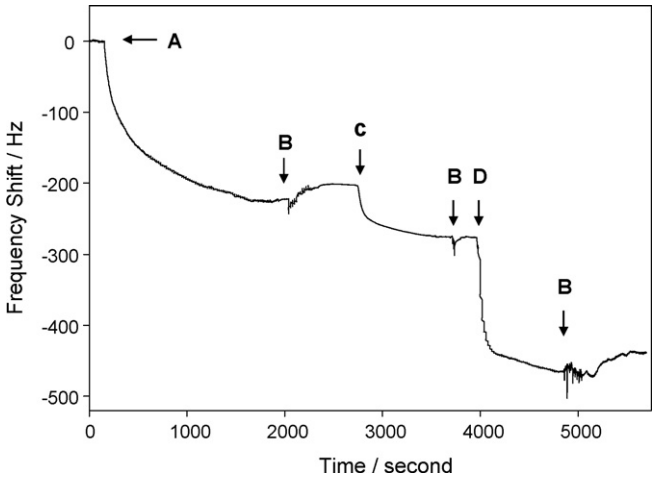


Fig. 3. The real-time interfacial interaction processes of the sensing surface in the determination of CT as monitored using microgravimetric QCM analysis. Arrow A denotes the addition of GM1-incorporated liposome to form the supported lipid membrane covered sensor surface, arrows B represent wash with buffer, arrow C indicates the addition of 1 μg mL^{-1} CT and arrow D represents the addition of HRP/GM1-incorporated liposome.

3.4. Piezoelectric measurements of the interfacial processes

Fig. 3 illustrates the interfacial interaction processes of the sensing surface in the determination of CT as monitored using microgravimetric QCM analysis. The frequency of the piezoelectric quartz crystal in the liquid phase would vary with the change in the adsorbed mass on the sensing surface. As one sees from Fig. 3, the introduction of liposome on the octadecanethiol-coated gold surface induces a frequency decrease of 244 Hz, 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 (1 μg mL^{-1}) was added to reaction cell. Binding of CT on the GM1-incorporated supported lipid membrane results in a further frequency decrease of 73 Hz. After rinsing with PBS, the addition of the liposome probe with GM1 and HRP again produces a frequency decreases up to 175 Hz, implying that the sandwiched complex was formed on the biosensor surface. These results gave evidence that the expected interfacial processes were performed, confirming that the analytical protocol was feasible for the determination of CT.

3.5. Analytical performance of the developed biosensor

Fig. 4 depicts the dependency of the peak intensity of chemiluminescence profiles in response of different CT concentrations. The inset curves show the chemiluminescence signal of the developed biosensor in response of different CT concentrations. It is seen that with increasing CT concentration, the chemiluminescence profiles exhibit stronger peaks. The results depict that peak intensity increases with CT concentration increased from 1 pg mL^{-1} to 1 ng mL^{-1}. A linear response range is obtained in the range from 1 pg mL^{-1} to 1 ng mL^{-1} with a good linear relationship (R^2 = 0.993). The detection limit was calculated in terms of the 3σ rule (three times standard deviation over the blank).

The chemiluminescence responses of the developed biosensor were measured to common serum proteins. The results show that the chemiluminescent signals to BSA (50 μg mL^{-1}), HSA (50 μg mL^{-1}), human IgG (50 μg mL^{-1}), human IgE (50 μg mL^{-1}) and thrombin (50 μg mL^{-1}) are 3414 (R.S.D. = 7.8%), 3516 (R.S.D. = 8.5%), 3856 (R.S.D. = 6.9%), 3259 (R.S.D. = 7.1%), 3782 (R.S.D. = 8.9%), respectively. The responses for these interference proteins are smaller than that obtained with 50 pg mL^{-1} CT (43,095, R.S.D. = 7.3%), 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.

In order to investigate the feasibility of the developed technique to be implemented for clinical analysis, the human serum sample

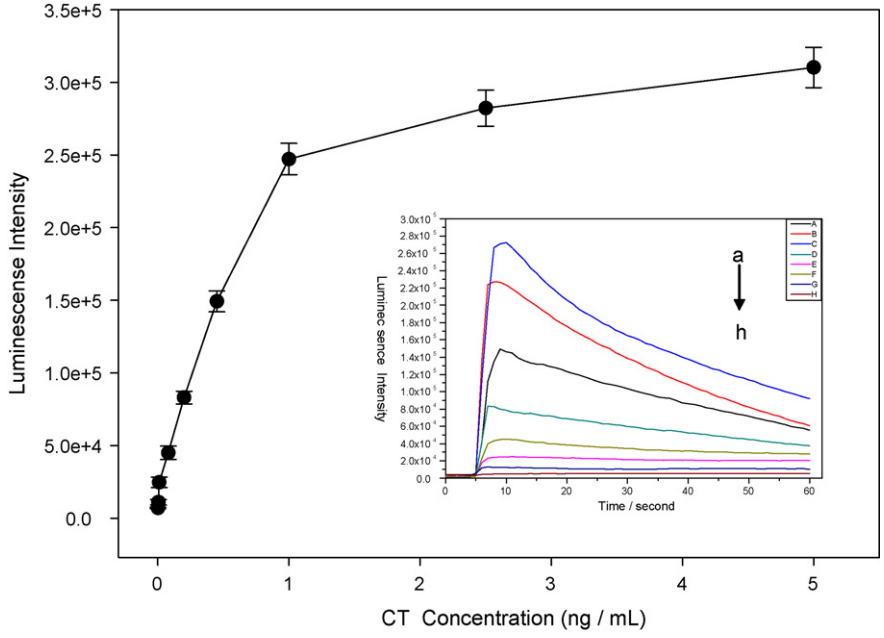


Fig. 4. The calibration curve describing the relationship between the chemiluminescence intensity and varying concentrations of CT by the developed biosensor. Inset: The chemiluminescent signals for different concentrations of CT by the developed biosensor: (a) 2.5 ng mL^{-1}, (b) 1 ng mL^{-1}, (c) 0.3 ng mL^{-1}, (d) 0.1 ng mL^{-1}, (e) 0.05 ng mL^{-1}, (f) 0.01 ng mL^{-1}, (g) 0.005 ng mL^{-1}, and (h) 0.001 ng mL^{-1}.

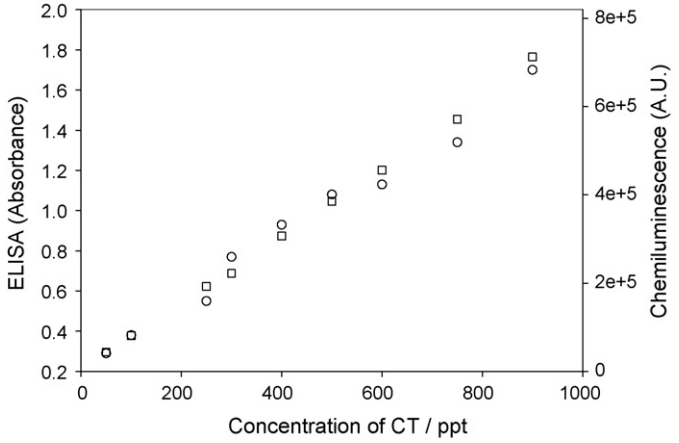


Fig. 5. Correlation between the chemiluminescence results (circles) from the developed method and ELISA (squares) results.

with different CT content added were determined by the developed biosensor and the ELISA method. The data from both the new method (circles) and ELISA (squares) were shown in Fig. 5. The results indicate that the as-developed biosensor offers analytical results in good consistency with ELISA.

The developed biosensor was prepared using the supported lipid membrane technology. This biologically compatible membrane not only alleviated of non-specific adsorption of serum proteins, but also allowed a ready regeneration of the sensor interface. Indeed, the external phospholipid membrane could be removed via rinsing with ethanol, which could renew the octadecanethiol-assembled electrode surface for re-spreading the liposome and regenerating the sensor immediately. As a matter of fact, it was observed that the biosensor could be generated via immersing the octadecanethiol-assembled electrode in a suspension of GM1-functionalized liposome for 30 min. Moreover, the octadecanethiol-assembled electrode surface could be stored in air for a long period (more than 3 months), and the resulting biosensor could be used throughout this period for CT detection without significant loss of sensitivity.

4. Conclusion

The present study developed a novel biosensor for CT detection using the supported lipid membrane-based sensing surface and the HRP/GM1-functionalized liposome-based detection probe. The biosensing surface could be prepared and renewed with ease and high reproducibility. Also, 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. Furthermore, the implementation of liposome-based detection probe allowed for efficient conjugation of multiple biocatalytic amplifier HRP followed by the enhanced chemiluminescence detection, thereby affording enormous signal amplification and background alleviation. The results demonstrated that the developed biosensor could allow sensitive, selective and reusable detection of CT with highly resistance to non-specific adsorption. It was 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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