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Sandwich electrochemical immunoassay for the detection of Staphylococcal enterotoxin B based on immobilized thiolated antibodies

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Abstract

A new approach for the sensitive detection of Staphylococcal enterotoxin B (SEB) is presented based upon an electrochemical enzymatic immunoassay that utilizes thiolated antibodies immobilized on a gold surface. This method relies on the use of amine-or sulfhydryl-reactive heterobifunctional cross-linkers for the introduction of 2-pyridyl-disulfide groups to the antibody. The disulfide-containing linkages are subsequently cleaved with a suitable reducing agent, such as dithiothreitol (DTT), and the thiolated antibody–gold bond is covalently formed on a gold working electrode. Various cross-linking agents for immobilization of the capture antibody onto the gold electrode were investigated and compared. Factors influencing the thiolation and immobilization were investigated and optimized. The feasibility of such antibody immobilization and the subsequent sandwich enzyme immunoassay is demonstrated for the sensitive detection of SEB. The detection limit estimated from a representative dose-response curve is 1 ng/mL, corresponding to 5 pg in a 5- μ L sample. Coupling the specificity of immunoassays with the sensitivity and low detection limits of electrochemical detection shows real promise for future sensing technology in enabling the development of single-use disposable devices.

Keywords

Staphylococcal enterotoxin B; sandwich electrochemical immunoassay; thiolated antibody

1. Introduction

The threat of biological warfare agents, defined as living organisms that include viruses and bacteria, and toxins arising from living organisms, is an ever increasing concern in the battle against terrorism. Rapid, accurate, and reliable detection of such agents is important for the implementation of suitable measures to protect public health. Additionally, foodborne pathogens present serious risk to food safety and the nation's food supply chain, threatening

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deep economic impact (Ligler, 2000; Mead et al., 1999). *Staphylococcus* food poisoning is one of the most common forms of foodborne illness, resulting from the ingestion of Staphylococcal enterotoxins formed in foods by enterotoxinogenic strains of *Staphylococcus aureus*. The enterotoxins of *S. aureus* form a group of 12 distinct proteins. Based on their antigenic properties, these enterotoxins are classified into three subfamilies of SEA, SEB, and SEI (Dinges et al., 2000). SEB is classified as an exotoxin and is most commonly associated with food poisoning, although genuine concern exists with regards to its application as a biological warfare agent. Exposure to relatively small amounts ($<1\text{ }\mu\text{g}$) can produce toxic shock (Barg and Harris, 1997; Aitichou et al., 2004) and the current toxic dose for aerosol form is $0.02\text{ }\mu\text{g/kg}$ of body weight (Sapsford et al., 2005). Dairy products are frequently involved in food poisonings at a level of enterotoxin as low as 0.5 ng/mL (Bergdoll, 1991).

Desirable biosensor characteristics for the identification of SEB include high sensitivity, fast response time, the capability for unbiased detection of low concentrations of SEB in complex samples that include various potentially interfering background materials, and overall system portability. Numerous biosensor approaches have been applied to the detection of SEB, including enzyme-linked immunosorbent assays (ELISA) (Morissette et al., 1991), a microchip displacement immunoassay (Haes et al., 2006), surface plasmon resonance (Rasooly, 2001), and fiber optic array biosensor (Sapsford et al., 2005). We are unaware of any electrochemical biosensors being applied to the detection of SEB, which is surprising considering the analytical attributes of electrochemical techniques: robust, economical to mass produce, easily quantifiable, and capable of achieving excellent detection limits in small volumes (Nyholm, 2005). Furthermore, the required instrumentation is relatively simple and can be miniaturized with low power requirements. Electrochemical detection is not affected by the sample components that might interfere with spectroscopic detection, such as particles, chromophores, and fluorophores. Measurements have been made on whole blood without interference from red blood cells, hemoglobin, or bilirubin, for example (Yao et al., 1995). Electrochemical enzyme immunoassays that capitalize on the selectivity of antigen-antibody reactions have evolved dramatically over the past two decades (Heineman and Halsal, 1985; Halsal et al., 1988; Purushottama et al., 2001). Excellent (attomole-septomole) detection limits have been reported earlier (Bauer et al., 1996).

Antibody immobilization is vital to the successful development of an electrochemical based immunosensor for SEB. Various immobilization schemes have previously been developed based on direct physical adsorption, silanized layer, polymer membrane, e.g., polyethyleneamine (PEI), crosslinking with bovine serum albumin (BSA), gold-protein A complex formation, silanization with 3-aminopropyltriethoxysilane (3-APTES), cross-linking through glutaraldehyde, biotin-avidin complex, and self-assembled monolayer (Storri et al., 1998). Although applicability has been demonstrated for the detection of various analytes, such immobilization methods pose certain challenges during the assay, in addition to those related to limited specificity and stability of the immobilized layers. Additional issues include, extended immobilization times necessary to achieve good loading (Wilson and Nie, 2006), limited surface loading of antibodies (Storri et al., 1998), non-specific binding or handling of reagents (Nyholm, 2005). A novel immobilization method

exploiting a direct covalent bond formation between a gold electrode surface and thiolated antibodies has previously been demonstrated (Park and Kim, 1998) for the QCM-based detection of *Salmonella*. This approach has a number of advantages. Immobilization based on covalent bond formation results in a clean and efficient immobilization with uniform surface coverage. The gold substrate serves as both the electrode and a heterogeneous solid phase. Also, the thiol–gold chemistry is very well established and can be used to efficiently control the antibody immobilization. Antibody arrays could readily be developed in the future as well using this approach.

In this paper, we present a method of antibody immobilization with application to an electrochemical enzyme immunoassay for the detection of SEB (Figure 1). This protocol relies on attaching thiol functionality to a primary (capture) antibody. Amine- or sulphydryl-reactive heterobifunctional cross-linkers are covalently linked to the antibody, thereby, introducing 2-pyridyl-disulfide groups to the antibody. The resulting disulfide-containing linkages are subsequently cleaved with reducing agent, such as dithiothreitol (DTT), to give a thiolated antibody that can covalently attach to a gold electrode. The resulting immobilization offers the advantages of a simplified immobilization procedure for sandwich immunoassay when compared to other immobilization schemes. The feasibility of such antibody immobilization and the subsequent sandwich electrochemical enzyme immunoassay is demonstrated successfully for the sensitive detection of Staphylococcal enterotoxin B.

2. Experimental

2.1. Apparatus.

Square-wave voltammetric measurements were performed using the μ Autolab Type II (Eco Chemie, The Netherlands) with General Purpose Electrochemical System (GPES) software. The detection was carried out in a 1.5-mL electrochemical cell containing the thiolated antibody immobilized working electrode (3-mm diameter), a Ag/AgCl reference electrode and a platinum wire counter electrode in a standard three-electrode configuration. All three electrodes and the cell holder to house the electrode assembly were procured from CH Instruments (Austin, TX).

2.2. Reagents and Buffers

All chemicals were of reagent grade and used as received. Aqueous solutions were prepared with Milli-Q water (Millipore, Bedford, MA). Reagent grade dithiothreitol (DTT), *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP), sulfosuccinimidyl 6-[3-(2-pyridyldithio)-propionamido]hexanoate (sulfo-LC-SPDP), and 3,3'-dithiobis[sulfosuccinimidylpropionate] (DTSSP) were obtained from Pierce (Rockford, IL). Potassium phosphate (mono and di-basic), sodium chloride, sodium acetate, tris (hydroxymethyl)aminoethane (Tris)-HCl, bovine serum albumin (BSA), α -naphthyl phosphate (α -NPP), NaOH, acetic acid, dimethyl sulfoxide (DMSO), and HCl were obtained from Sigma (St. Louis, MO). Staphylococcal enterotoxins (A, B, C, and D, 1 mg each) were purchased from Toxin Technology (Sarasota, FL) and dissolved in Millipore purified water to give a final concentration of 100 mg/mL.

The detection buffer (DB) consisted of 100 mM Tris and 100 mM NaCl at pH 8.0; the antigen/antibody dilution buffer consisted of 20 mM phosphate buffer with 150 mM NaCl (PBS, pH 7.4); the washing buffer (WB) consisted of 100 mM PBS (pH 7.4); and, the immobilization buffer (IB) consisted of 100 mM PBS containing 0.03% BSA (pH 7.4). The detection buffer of 100 mM Tris containing 100 mM NaCl (pH 8.0) was chosen because of the enhanced activity of the alkaline phosphatase (ALP) enzyme tag (Simopoulos et al., 1994; Plocke et al., 1962). A pH of 7.4 was utilized for the antibody immobilization and antigen recognition experiments according to a previously optimized report (Park and Kim, 1998). No further pH optimization studies were carried out. BSA was added to the incubation buffer to reduce non-specific adsorption effects. Standard solutions of enterotoxins (10 mg/mL) were prepared in 20 mM phosphate buffer (with 100 mM NaCl, pH 7.4), and further dilutions were prepared daily using the same buffer. Anti-SEB (2.1 mg/mL) and alkaline phosphatase (ALP)-tagged anti-SEB (6.1 mg/mL) were obtained from US Biologicals (Swampscott, MA), and further dilutions of these stock solutions were prepared daily in 20 mM phosphate buffer with 100 mM NaCl, pH 7.4. Cross-linker solutions of sulfo-LC-SPDP and DTSSP (20 mM) were prepared in water, while SPDP was dissolved in DMSO. The stock solutions of cross-linkers were prepared fresh immediately prior to use and then discarded due to hydrolysis of the *N*-hydroxysuccinimide (NHS)-ester moiety in the cross-linkers that renders it non-reactive (Pierce application note 0279.2). A working solution of α -NPP (100 mM) was prepared daily in the detection buffer.

The disulfide bond resulting from the thiolation procedure may be cleaved by reduction with a reducing agent, DTT (150 mM), prepared in 100 mM acetate buffer containing 100 mM NaCl. A pH of 4.5 was chosen to protect native protein disulfide bonds (Carlsson et al., 1978).

2.3. Thiolation Procedure

Thiolation (cross-linking) agent was dissolved in the appropriate amount of solvent to give a final concentration of 20 mM. A 2- μ L quantity of 2.1 mg/mL anti-SEB was added to 4 μ L of PBS buffer solution. To this, a 1- μ L quantity of 20 mM cross-linking solution was added and allowed to react for 60 min, unless otherwise mentioned. Subsequently, 3.5- μ L of 150 mM DTT solution was added and allowed to react for 30 min for the reduction to take place. Finally, the reaction product was placed on a gold disk electrode and incubated for 60 min (unless otherwise mentioned) at room temperature.

2.4. Antibody Immobilization

The cleanliness of the gold surface is the key to the self-deposition of thiolated antibodies. Hence, before modification, the electrode was polished successively for 2 min with 0.3 and 0.05 μ m alumina slurries and thoroughly rinsed with copious amounts of water and sonicated in water for 2 min. The electrode surface was further conditioned according to a previously described procedure (Davies and Leary, 1989). Briefly, the electrode was placed in a 1.2 M solution of NaOH for 10 min and 1.2 M hydrochloric acid for 5 min, and, finally, a drop of concentrated hydrochloric acid was placed on the electrode surface for 30 s. The electrode was thoroughly washed with water between each successive step.

2.5. Sandwich Immunoassay

The thiolated antibody was placed on a gold disk electrode and allowed to incubate for 60 min at room temperature. After covalent attachment of the thiol–gold complex, the electrode surface was washed carefully with 100 mM PBS (pH 7.4) containing 100 mM NaCl. A small quantity (5- μ L) of SEB solution of an appropriate concentration was placed on the antibody immobilized electrode and allowed to incubate for 30 min, resulting in selective antigen recognition. After carefully washing the electrode to remove excessive or unbound antigen (SEB) solution, another drop (5- μ L) of secondary antibody (ALP-tagged anti-SEB) was placed on the electrode surface and incubated for 30 min. The secondary antibody binds to the secondary recognition sites on the antigen of the capture antibody–antigen complex, resulting in a sandwich being formed. The immunosensor was washed thoroughly with the wash buffer, and then placed in an electrochemical cell containing α -NPP substrate for a 20 min incubation period. The availability of enzyme (ALP) tag determines the biocatalytic conversion of non-electroactive substance, α -NPP, into the electroactive product, α -NP.

Control experiments were performed utilizing the basic procedure described above, except that the SEB was replaced with SEA, SEC, or SED. To determine the extent of nonspecific binding, additional control experiments were performed by carrying out the entire sandwich immunoassay in the absence of SEB.

2.6. General Electrochemical Assay Procedure

The assay procedure consisted of placing the immunosensor in an electrochemical cell containing 0.9 mL detection buffer (100 mM Tris, 100 mM NaCl, pH 8.0). A 100- μ L quantity of substrate (100 mM α -NPP in detection buffer) was added, and the modified electrode surface was allowed to incubate for 20 min in order to generate sufficient product prior to electrochemical detection of the enzymatically liberated α -naphthol.

Square-wave voltammetric measurements of α -NP were performed in an electrochemical cell using a 2-min accumulation time at a deposition potential of -0.1 V in a stirred Tris buffer solution (100 mM with 100 mM NaCl, pH 8.0). Once the stirring was stopped, the solution was equilibrated for 5 s, and the potential was scanned from 0 to 0.45 V using the following parameters: frequency, 10 Hz; step potential, 4 mV; amplitude, 25 mV. The α -naphthol anodic peak was baseline corrected using the moving average mode of the GPES (Autolab) software, and was recorded for quantitative purposes as the analytical signal. Batch electrochemical measurements were carried out in a 1.5-mL voltammetric cell at room temperature. All potentials are given with regards to the Ag/AgCl reference electrode (CH Instruments, Austin, TX).

2.7. Safety Considerations

Staphylococcal enterotoxins and anti-SEB are hazardous and care should be taken while handling.

3. Results and Discussion

The overall scheme for generating a thiolated antibody for modifying the gold disk electrode consists of first reacting the primary (capture) antibody with a cross-linker, in order to introduce 2-pyridyl-disulfide bonds. The disulfide bonds, cross-linked using thiolation reagents, can be cleaved by reduction with dithiothreitol (DTT) to introduce sulfhydryl groups. Subsequently, the thiolated antibody is spread on a gold disk electrode, resulting in antibody immobilization.

The individual steps involved in performing the sandwich electrochemical enzyme immunoassay for the detection of SEB are summarized in Figure 1. The antibody-antigen complex is formed following exposure and incubation of the antibody immobilized electrode with a solution of antigen (SEB). A sandwich is created by further exposing the immunosensor to (ALP)-labeled secondary (detection) antibody. To generate an electroactive product, an ALP appropriate substrate (α -naphthyl phosphate) is added and the solution incubated. Alkaline phosphatase that is tagged to the secondary antibody hydrolyzes α -NPP, thereby, generating α -naphthol, an electroactive compound. Subsequent electrochemical detection of enzymatically liberated α -naphthol produces a quantitative signal that is proportional to the target analyte concentration.

Successful development of this type of electrochemical sandwich immunoassay is greatly dependent on an immobilization scheme for the capture antibody that is efficient, reproducible, and leads to a more sensitive antigen response. More efficient orientation of thiolated antibodies will lead to better recognition of the target antigen, SEB. Therefore, we investigated several cross-linkers for successful thiolation and the results are depicted in Figure 2. Of these thiolation agents, both sulfo-LC-SPDP (Figure 2d) and SPDP (Figure 2c) are thiol-cleavable heterobifunctional cross-linkers, while DTSSP (Figure 2b) is a homobifunctional cross-linker. Experimental optimization was achieved by performing the complete sandwich immunoassay for SEB while varying the cross-linker used during the initial thiolation step. Sulfo-LC-SPDP produced the most sensitive response, followed by SPDP and DTSSP. In contrast to the heterobifunctional cross-linkers (sulfo-LC-SPDP and SPDP), the DTSSP-modified thiolated antibody is a homobifunctional cross-linker that is only amine-reactive, and, hence, has more limited thiolation capability and lower sensitivity. The lower activity observed may be due to either conformational changes after conjugation, or the limited availability of lysine groups (after thiolation) responsible for binding SEB. Between the two heterobifunctional cross-linkers, thiolation based on the SPDP reagent resulted in lower sensitivity when compared to the sulfo-LC-SPDP reagent. This may be attributed to a shorter spacer arm ($6.8 \text{ \AA}$ compared to $15.6 \text{ \AA}$ in sulfo-LC-SPDP) that introduces steric hindrance, thereby, reducing the number of active binding sites with SEB. These results are in agreement with earlier observations (Park and Kim, 1998). Reduced steric hindrance, as in the case of sulfo-LC-SPDP, will introduce site-directed and more efficient immobilization, providing a higher yield of reactive antigen-binding sites that are more likely to face away from the gold surface. All further experiments were performed using sulfo-LC-SPDP as the cross-linker for thiolation of the primary anti-SEB. In the absence of any cross-linking agent (data not shown), we did not observe any noticeable difference from the control experiment. For these short immobilization times, the

immobilization of thiolated antibodies provides better antibody loading than simple adsorption methods (due to the absence of any thiolation agent). In a separate study, it was observed, for example, that antibody immobilization via adsorption requires at least 18 h at 4 °C to generate similar antibody loading to silane-mediated covalent attachment (Wilson and Nie, 2006).

Optimization of the immobilization conditions was investigated as a function of various parameters, including the amount of capture antibody, thiolation reaction time, thiolated antibody immobilization time, and secondary (enzyme-tagged) antibody concentration. The impact of each of these variables is summarized in Figure 3. Efficient antibody immobilization is important for enabling sensitive antigen detection, and is highly dependent on the amount of thiolated antibody and its ability to be immobilized on the electrode surface. While keeping the amount of thiolation cross-linking agent constant at 2.85 mM, the primary (capture) antibody concentration was varied from 0 to 1 mg/mL for the thiolation reaction (Figure 3A). As expected, increasing the concentration of capture antibody concentration resulted in a more sensitive electrochemical response, indicating the increased availability of antibody recognition sites on the electrode surface. The signal levels off between 0.6 and 0.8 mg/mL, and, in fact, further increases in the capture antibody concentration in the thiolation reaction mixture resulted in a small decrease in the signal, presumably due to saturation of the electrode surface that results in blocking some of the antigen recognition sites. A similar decrease in signal was reported in the literature in connection with an immunofiltration unit and amperometric enzyme immunosensor (Carnes and Wilkins, 2005). The primary anti-SEB concentration was, therefore, fixed at 0.6 mg/mL. The thiolation reaction was carried out using the addition of 20 mM sulfo-LC-SPDP reagent, as recommended by the supplier. The addition of higher concentrations of either sulfo-LC-SPDP reagent or capture antibody resulted in precipitation of the antibody during the reaction, possibly due to denaturation as observed in a separate study (Singh and Sairam, 1989).

Figures 3B and 3C depict the effect of thiolation reaction time and thiolated antibody immobilization time on the overall analytical signal for 10 ng/mL of SEB antigen. The sulfo-LC-SPDP cross-linking agent was added to the antibody solution and incubated for increasing lengths of time, followed by completion of the sandwich immunoassay to assess the impact of thiolation reaction time (Figure 3B). Increasing the reaction time from 15 min. to 60 min. resulted in an increase in the electrochemical signal due to an improved thiolation yield and greater antibody immobilization on the electrode surface. Further increases in thiolation reaction time, however, did not increase the signal, indicating completeness of the reaction under the experimental conditions used in this study. The impact of the subsequent thiolated antibody immobilization time (on a gold disk electrode) is shown in Figure 3C. Increasing the immobilization time from 15 min to 60 min improved the antibody binding, as indicated by the increase in electrochemical signal observed when completing the sandwich immunoassay. Although incremental increases in response were observed at incubation times above 1 h, the signal gain was relatively small, so an incubation time of 1 h was chosen to be optimal.

The concentration of ALP-tagged secondary antibody was also optimized as a function of the overall sensitivity of the sandwich immunoassay (Figure 3D). Increasing the concentration of secondary antibody from 0.5 to 5 $\mu\text{g/mL}$ results in a nearly linear increase in the electrochemical signal, and leveling off, thereafter. A concentration of 3 $\mu\text{g/mL}$ ALP-tagged secondary antibody was chosen as a compromise between sensitivity and overall background signal generated during substrate incubation. Increasing levels of ALP-tagged secondary antibody lead to higher background signals, necessitating the use of lower than optimal substrate concentrations that might require longer incubation times. To increase the sensitivity, we typically utilized a 20-min substrate incubation step (at 10 mM concentration) prior to measurement. This step acts to increase the concentration of enzymatically liberated electroactive product.

Utilizing the derived optimal sandwich immunoassay parameters, the dynamic range and overall sensitivity of the SEB assay was determined. As can be seen from Figure 4, increasing levels of SEB result in well-defined square-wave voltammetric signal for the α -naphthol product. The α -naphthol peak increases linearly with the increase in SEB concentration in the 1 – 20 ng/mL range, exhibiting nonlinearity for higher concentrations. The corresponding calibration plot is shown as an inset. It is not surprising to observe such a limited dynamic range, considering the longer incubation times employed in this study. The sensor sensitivity, measured as a slope, was $10.8 \text{ nA} \cdot \text{ng}^{-1} \cdot \text{mL}^{-1}$ ($R = 0.999$). The detection limit estimated from this dose-response curve was 1 ng/mL. Such detection limits correspond to 5 pg in a 5- μL sample solution. The background signal observed from the control experiment performed without any antigen, as shown in Fig. 2 (a), indicates minimal nonspecific binding effects and is commensurate with an SEB concentration of 0.25 ng/mL. Such detection limits are expected to be adequate for real life applications. The detection limits realized using the protocol described here represent almost an order of magnitude improvement over the commercial product developed by Tetracore, Inc (Cat. No. S-1004). Lower detection limits are expected to be achieved either through biometallization (Hwang et al., 2005) or through signal amplification using multilayer enzyme (Munge et al., 2005) schemes to yield highly sensitive detection.

The selectivity of the assay depends on the immobilized primary antibody's ability to selectively bind the antigen of interest. A set of interference tests were performed, wherein the SEB was replaced by a large excess (1 $\mu\text{g/mL}$ compared to 1 ng/mL of SEB) of SEA, SEC, or SED, respectively. The results are presented in Figure 5. The voltammetric signals obtained from these other forms of Staphylococcal enterotoxins are nearly the same as that of the control experiment performed to determine the extent of non-specific binding (Figure 5a). Assay conditions were the same as those used in the SEB assay, indicating that the overall immunosensor performance is excellent.

The reproducibility of the sandwich immunoassay from electrode to electrode was tested on six different gold electrodes for the target analyte (1 ng/mL of SEB, data not shown). The mean analytical signal obtained was $10.0 \pm 0.45 \text{ nA}$, yielding a RSD of ~7%. To test the reusability of the immunosensor when using the same electrode, the immobilized electrodes were washed with 0.2 M glycine/HCl (pH 2.5) at least three times between assays. As shown in Figure 6, approximately 80% of the original response was retained after 2 assays,

whereupon the voltammetric response of the sensor became unstable and the signal decreased by about 35% during the third assay. The stability of the modified electrode was found to be poor when placed in aqueous solutions for extended periods of time, a result which was attributed to hydrolysis of the gold-thiol bond in water, as observed previously (Liu et al., 2000). Note also that regeneration of thiolated surfaces is generally poor (averaging 36%) (Storri et al., 1998).

4. Conclusions

In conclusion, we have demonstrated a new approach for electrochemical enzyme immunosensors based on the immobilization of thiolated antibodies on gold substrates. To our knowledge, this is the first report demonstrating the use of thiolated antibodies in electrochemical enzyme immunoassays. Different crosslinking agents were tested for efficient antibody thiolation, and the sensitivity of the electrochemical response was compared. Applicability was demonstrated for the analysis of Staphylococcal enterotoxin B (SEB) and a detection limit of 1 ng/mL in a 5- μ L sample, corresponding to 5-pg, was obtained. The sensor selectivity for SEB was excellent in the presence of other Staphylococcal enterotoxins. The approach presented here is general and can be readily applied to a variety of antigen/antibodies of importance in the fields of clinical, pharmaceutical or environmental immunoassays. Future work will involve improvements in lowering the limits of detection via signal amplification schemes. The direct immobilization of thiolated antibodies on gold electrodes provides a very promising approach. The electrochemical detection will be extremely useful in creating miniaturized devices for use in field assays. We believe that the work presented here will provide an important foundation for the future development of multianalyte single-use disposable devices for decentralized testing. Work is underway in our laboratory in performing multiple quantitative immunoassays of important biowarfare agents based on microelectrode arrays.

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Figure 1:
Electrochemical sandwich immunoassay based on immobilization of thiolated antibodies.

Figure 2:

Comparison of various thiolation cross-linkers by performing the complete sandwich assay while using a different cross-linker during thiolation: sulfo-LC-SPDP (d); SPDP (c); and DTSSP (b). The control experiment (a) was performed using sulfo-LC-SPDP in the absence of any antigen (SEB). Operating conditions: anti-SEB, 0.6mg/mL; SEB, 5ng/mL; thiolation cross-linking agent, 2.85mM; DTT concentration, 50 mM; SEB-ALP, 3 μ g/mL; thiolation reaction time, 60 min.; thiolated antibody immobilization time, 60 min. Substrate (10 mM α -naphthol) was added and incubated for 20 min prior to square-wave voltammetric measurements. Other conditions: accumulation time, 2 min.; quiet time, 5 s.; deposition potential – 0.1V; scanned potential range, 0 – 0.45 V; frequency, 10 Hz; step potential, 4 mV; amplitude 25 mV.

Figure 3: Optimization of (A) primary (capture) antibody concentration, RSD 7% (B) thiolation reaction time, RSD 3% (C) thiolated antibody immobilization time, RSD 11%, and (D) secondary (enzyme-tagged) antibody concentration, RSD 10%. Other conditions, as in Figure 2(d).

Figure 4: Concentration dependence for SEB. (a) 1, (b) 2.5, (c) 5, (d) 10, and (e) 20 ng/mL. Also shown as inset is the calibration data. Other conditions, as in Figure 2(d).

Figure 5: Selectivity experiments. (a) control experiment (without any SEB added). (b) SEA, (c) SEC, (d) SED, and (e) SEE. (f) SEB. Concentrations, SEB 1 ng/mL, and all others were 1 μ g/mL. Other conditions, as in Figure 2(d).

Figure 6:
Reproducibility data for a single electrode assay of SEB following regeneration attempts via washing three times in 0.2 M glycine/HCl (pH 2.5) between each subsequent assay (1–5). Other conditions, as in Figure 2(d).