Anal Bioanal Chem (2009) 393:1539–1544
DOI 10.1007/s00216-008-2559-x

ORIGINAL PAPER

A capacitive biosensor for detection of staphylococcal enterotoxin B

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Received: 19 August 2008 / Revised: 6 November 2008 / Accepted: 1 December 2008 / Published online: 19 December 2008

Abstract A sensitive method for the detection of staphylococcal enterotoxin B (SEB) using a flow-injection capacitive biosensor is presented. SEB was purified from a crude culture filtrate of *Staphylococcus aureus* through three chromatographic steps. The first two steps were based on ion-exchange chromatography, and the last step was carried out on a gel filtration column. The SEB recovery values after the purification stages were 88%, 74%, and 12%, respectively. A horseradish peroxidase labeled anti-staphylococcal enterotoxin B was prepared by the periodate method and was further employed in a sandwich-enzyme-linked immunosorbent assay (ELISA) for the determination of SEB concentrations in different samples obtained during the processing of the crude filtrate. The capacitive biosensor could detect SEB concentrations as low as 0.3 pg ml^{-1} with a linearity ranging from 2.8 pg ml^{-1} to 2.8 ng ml^{-1} under optimized conditions. The response time was about 10 min. A good agreement was achieved between the developed capacitive biosensor system and ELISA as a reference method for detection of SEB levels in different purification samples. The newly developed sensor has the benefits of simplicity, high sensitivity, and multiple use capability.

Keywords Staphylococcal enterotoxin B · Capacitive biosensor · Self-assembled monolayers · ELISA · Horseradish peroxidase

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Introduction

Staphylococcal enterotoxin B (SEB), a single-chain polypeptide (molecular mass, 28.336 kDa) consisting of 239 amino acid residues, is a water-soluble pyrogenic exotoxin produced by strains of *Staphylococcus aureus* [1]. It is one of at least five antigenically distinct enterotoxins that have been identified (SEA, SEB, SEC, SEE, TSS T-1) [2, 3]. SEB is the most potent toxin in this group and has a remarkable heat stability and resistance to inactivation by proteases in the gastrointestinal tract. It is also stable against pH changes (pH 4–10) and has an isoelectric point (p*I*) of 8.6 [4–6]. In addition to its effect as potent gastrointestinal tract toxin, it also functions as a superantigen that simultaneously binds to class II major histocompatibility complex molecules on antigen-presenting cells without being processed by interacting with a specific variable region of the β subunit of T-cell receptor, and consequently, it stimulates a nonspecific proliferation of such T cells, release of cytokines, and cytotoxic activity [7]. There is a high correlation between its activity as potent gastrointestinal toxin and its function as a superantigen, although these two functions are localized on separate domains of the protein; this was evidenced by a concomitant loss of its enterotoxic activity with loss of superantigenic activity [8, 9]. Due to its potent immunostimulatory effect, high morbidity rate, inherent stability, and ease of dissemination, it can be employed as a biological aerosol weapon [10–12].

The lethal dose of SEB is relatively low (LD_{50} ~20 ng/kg), whereas it can cause incapacitating effects at lower concentrations (effective dose, ED_{50} ~0.4 ng/kg). For these reasons, methods that selectively and sensitively detect extremely low levels of the toxin are highly desirable [13]. Several methods have been employed to detect SEB. Some

of these methods include piezoelectric crystal immunosensor with a limit of detection (LOD) ~2.5 μg/ml [14]; capillary electrophoresis, LOD ~85 ng/ml [15]; surface plasmon resonance, LOD ~10 ng/ml [16]; fiber-optic-sandwich immunoassay, LOD ~5 ng/ml [17]; liquid chromatography-mass spectrometry, LOD ~5 ng/ml [18]; fluorescence-based sensors, LOD ~4 ng/ml [19]; surface plasmon resonance-mass spectrometry, LOD ~1 ng/ml [20,21]; bidiffractive grating biosensor, LOD ~1 ng/ml [22]; latex agglutination, LOD ~500 pg/ml [23]; magnetoelastic immunosensor, LOD ~500 pg/ml [24]; fluorescent latex microparticle immunoassay, LOD ~125 pg/ml [25]; enzyme-linked immunosorbent assay (ELISA), LOD ~100 pg/ml [10]; fluorescence arrays, LOD ~100 pg/ml [26–28]; immuno-magnetic separation-flow cytometry, LOD ~10 pg/ml [29,30]; time-resolved fluorescence assay, LOD ~10 pg/ml [31]; piezoelectric-excited millimeter-sized cantilever sensors (PEMC), LOD ~2.5 fg/ml [32]; and indirect double sandwich fluorogenic enzyme-linked immunoassay, LOD ~0.1 fg/ml [33].

Over the last years, capacitive biosensors have established a niche for analysis of proteins due to its high sensitivity, high selectivity, reusability, and capability to quantify trace amounts of the analyte in a short time frame [34–39]. The ability of these sensors to measure trace concentrations at the picogram level has applications in forensics, in diagnostics, and in a manufacturing setting. In this paper, we discuss the development of a flow-injection biosensor system with a capacitive transducer to measure the SEB content in different samples obtained along a multi-step purification of SEB from *S. aureus* fermentation filtrate. The accuracy of the system was assessed through a comparison to an ELISA test employed for parallel detection of SEB concentrations.

Materials and methods

Chemicals

Enterotoxin B from *S. aureus* (SEB), 1-fluoro-2,4-dinitrobenzene (FDNB), and polystyrene multiwell plates were purchased from Sigma (St. Louis, MO, USA). Polyclonal antistaphylococcal enterotoxin B antibodies developed in rabbit (Anti-SEB), horseradish peroxidase type VIa, α lipoic acid, bicinchoninic acid solution, dry acetonitrile 99.8%, 0.05 M carbonate–bicarbonate buffer capsules (pH 9.6), and *o*-phenylenediamine dihydrochloride (Sigma FAST OPD peroxidase substrate tablet set) were all from Sigma-Aldrich Chemie (Steinheim, Germany). *N*-(3-Dimethylaminopropyl)-*N*′-ethyl-carbodiimide hydrochloride (EDC) and sodium borohydride were purchased from Fluka, Sigma-Aldrich Gmbh, Germany. Bovine serum albumin and Tween 20 were purchased from ICN Biomedicals, Malmö, Sweden. 1-Dodecanethiol was obtained from Aldrich (Deisenhofen, Germany). Ethylene glycol was a product of Riedel-de-Haën, Sigma-Aldrich Gmbh, Germany. Sodium periodate was from Janssen Chemica, Belgium. Hydrogen peroxide (30% *w*/*v*) was a product of Merck. Alumina slurries with particle diameter of 0.1 μm were obtained from Struers, Denmark. The buffer salts were of analytical quality. Chromatographic columns were from Pharmacia (Uppsala, Sweden) and matrices were from Amersham Biosciences AB (Uppsala, Sweden). Concentrated culture filtrate from *S. aureus* fermentation was a generous gift from Novozymes Bio-pharma AB (Lund, Sweden) and was used as a starting material for the SEB purification. The crude culture filtrate had a protein content of 13.3 mg ml^{-1} and 292 ng ml^{-1} of SEB.

Chromatographic steps

The first chromatographic step was performed on a XK-50 column (50×130 mm) packed with 580 ml of Sepharose Sulphopropyl (SP) Fast Flow matrix. The matrix was equilibrated with two column volumes of 30 mM sodium acetate buffer, pH 5.5. The crude culture filtrate (4 l) was diluted three times with water, acidified with glacial acetic acid to pH 5.5, then loaded into the column at a flow rate of 5 ml min^{-1}. After washing the column with three column volumes of equilibration buffer, the bound proteins were eluted with 30 mM sodium phosphate buffer (PB), pH 7.5, at a reversed flow rate of 5 ml min^{-1}. The protein content of the effluent was monitored at 280 nm and 50-ml fractions were collected.

The second chromatographic step was carried out on a XK-26 column (26×550 mm) packed with 150 ml of Sepharose Sulphopropyl (SP) High-Resolution matrix. The column was equilibrated with 10 mM PB, pH 6.5, and the pooled fractions from the previous step were adjusted to pH 6.5 with 1 M acetic acid, then a 350-ml sample was loaded to the column at a flow rate of 1 ml min^{-1}. After washing with three column volumes of equilibration buffer, the bound proteins were eluted with an exponential gradient (0–140 mM NaCl) in equilibration buffer at a flow rate of 1 ml min^{-1}. Ten-milliliter fractions were collected, and the SEB content was determined by ELISA. This step was repeated in order to process all the material obtained in the previous step.

The third and final purification step involved gel filtration on a XK-10 column (10×810 mm) packed with 70 ml of Sephadex G-75 superfine. The column was equilibrated with 0.1 M ammonium carbonate buffer, pH 8, and a 5-ml sample of the ELISA positive fractions, pooled from the previous step, was applied to the column at a flow rate of 0.5 ml min^{-1}. One-milliliter fractions were

collected, and the SEB content was determined by ELISA as described in "Determination of SEB content at different purification steps using ELISA".

Determination of the protein content at different purification steps

The total protein concentrations were measured in a microtiter plate format using bicinchoninic acid (BCA) method [40].

Preparation of horseradish peroxidase conjugated anti-SEB

Five milligrams of horseradish peroxidase type VIa were dissolved in 1 ml freshly prepared 0.3 M sodium bicarbonate, pH 8.1, then 0.1 ml of 1% FDNB in absolute ethanol was added and mixed gently for 1 h at room temperature. One milliliter of 0.08 M sodium *m*-periodate was added to the previous mixture and mixed gently for 30 min at room temperature. The reaction was then stopped by mixing with 1 ml 0.16 M ethylene glycol in distilled water for 60 min at room temperature. The solution was dialyzed against three 1-l changes of 0.01 M sodium carbonate buffer, pH 9.5 at 4 °C. Five milligrams of anti-SEB was added to the previous solution and mixed gently for 3 h at room temperature. Finally, 5 mg of sodium borohydride was added and left at 4 °C for 3 h, and the solution was dialyzed against phosphate-buffered saline (PBS), pH 7.2 at 4 °C. The dialyzed solution was applied to a XK-10 column (10×810 mm) packed with 70 ml of Sephadex G-75 superfine equilibrated with PBS at a flow rate of 0.5 ml min^{-1}. One-milliliter fractions were collected, and their absorbances at 403 and 280 nm were measured, respectively. Fractions were tested for peroxidase activity, and positive fractions were pooled and stored at −20 °C [41].

Determination of SEB content at different purification steps using ELISA

Anti-SEB was diluted in 0.05 M carbonate–bicarbonate buffer, pH 9.6 to a final concentration of 10 μg ml^{-1}, then 0.1 ml was incubated in microtiter wells overnight at 4 °C. All wells were washed 12 times with 0.25 ml PBS-T (10 mM phosphate buffer pH 7.4, 150 mM NaCl, 0.05% Tween 20). A standard SEB concentration of 100 ng ml^{-1} in PBS-T was added to the first well and diluted 1:1 till 0.05 ng ml^{-1}. A 0.1-ml sample from each of the three purification steps as well as the initial culture filtrate was applied to microtiter wells and diluted in the same manner before the plate was incubated for 1 h at 37 °C. After washing, 0.1 ml of the prepared peroxidase conjugated anti-SEB (diluted 1:100 in PBS-T) was added to each well and incubated for 1 h at 37 °C. Finally, the wells were washed again, and 0.2 ml of the substrate solution (*o*-phenylenediamine dihydrochloride) was added to each well, then the plate was incubated in the dark for 30 min at room temperature. After incubation, the plate was read at 405 nm on a microtiter plate reader.

Preparation of the modified gold electrode

A gold rod electrode (∅3 mm, 99.99% purity) was employed as a transducer in the capacitive biosensor. The electrode was cleaned with Piranha solution ((H_2O_2/H_2SO_4, 1:3) for 3 min then washed thoroughly with distilled water. Polishing of the electrode was carried out on two steps; the first was done using 1% alumina suspension in distilled water for 3 min, and in the second step, the same solution containing 0.5 ml of 0.1% CrO_3 in HCl was used for 2 min. The electrode was rinsed thoroughly with water, placed in a Teflon holder, and plasma-cleaned for 15 min. The plasma-cleaned electrode was immersed in 1% α lipoic acid in absolute ethanol overnight, then washed with dry acetonitrile, and incubated with 1% EDC solution in dry acetonitrile for 5 h. Thereafter, the electrode was rinsed with 10 mM phosphate buffer, pH 7.4 and dried with nitrogen gas. The electrode was then incubated with 3.64 mg ml^{-1} anti-SEB in the same buffer overnight at 4 °C. Finally, the electrode surface was blocked using 10 mM 1-dodecanethiol in ethanol and placed in the flow cell.

Determination of SEB content at different purification steps using a capacitive biosensor

The basic set-up of the flow-injection based capacitive biosensor was previously described [34]. The mobile phase (10 mM PB, pH 7.4), filtered through a 0.22 μm filter and degassed prior to use, was pumped with a peristaltic pump (M-312 Gilson, France) at a flow rate of 0.1 ml min^{-1}. At 1-min intervals during the binding event between the SEB and the anti-SEB, 50 mV potential pulses were applied to the working electrode, yielding current response signals and changes in capacitance (ΔC) that were registered. A stock solution of 2.8 μg ml^{-1} SEB in the immobilization buffer was prepared then serially diluted to obtain nine solutions with SEB concentrations from 0.03 pg ml^{-1} to 2.8 μg ml^{-1}. Aliquots were injected using a 0.25-ml loop, then ΔC at each concentration was calculated, and finally, a calibration curve was plotted. Samples withdrawn from the initial culture filtrate and different purification steps were diluted, as dictated by the linear range of the calibration curve, then loaded to the biosensor and ΔC was determined. A regeneration step with 25 mM glycine/HCl buffer, pH 2.4 was performed after each sample injection.

Results and discussion

Purification of SEB from *S. aureus* culture filtrate

The crude culture filtrate had a total protein content of 13.3 mg ml^{-1} and 292 ng ml^{-1} of SEB. After the first purification step, 14 fractions of 700 ml as a total volume (Abs. ≥0.5) were selected and applied to the second column. The total protein concentration was reduced to 0.26 mg ml^{-1} with 88% recovery of SEB. In the second purification step, the partially purified filtrate was divided into two aliquots and loaded separately into the column, and the collected fractions were tested by ELISA. Forty-four fractions of 440 ml gave positive ELISA results and were further processed on the gel filtration column. This step showed a reduction in the protein concentration to 0.04 mg ml^{-1} and a 74% recovery of SEB. In the last step, a 5-ml sample of the pooled fractions from the second step was applied to the gel filtration column, 17 fractions of 17 ml were proved to contain SEB, and a recovery of 12% of purified SEB was achieved, whereas the protein concentration was below the detection limit of the BCA method (Table 1).

Detection of SEB by ELISA technique

A calibration curve obtained by ELISA at different concentrations of SEB has shown a linearity range between 1.6 and 25 ng ml^{-1} with the regression equation of $y = 0.0121x + 0.1285 (R^2 = 0.9989)$, where y is the absorbance at 405 nm and x is the concentration of SEB in ng ml^{-1}. The relative standard deviation (RSD) values were 2.9%, 2.8%, 4.8%, 3.1%, and 2.8% for triplicate measurements at the SEB concentrations of 1.6, 3.1, 6.3, 12.5, and 25 ng ml^{-1}, respectively. The concentrations of SEB in different samples obtained among different steps of purification were determined and presented in Table 1.

Capacitive biosensor for detection of SEB

The principal of capacitance measurements is based on the theory of the electrical double layer. Measurements were made by applying a potential pulse 50 mV every minute and recording the current transients evoked by the potential step according to Eq. 1.

$$i(t) = u/\mathrm{Rs}\ \exp\left(-t/\mathrm{Rs}C\right) \quad (1)$$

where $i(t)$ is the current at time t, u is the applied pulse potential, Rs is the resistance of recognition layer, t is the time elapsed after the potential pulse was applied, and C is the total capacitance measured at the working electrode/solution interface. Taking the logarithm of Eq. 1, the values of Rs and C can be easily calculated by the computer software (PotStat, Pascal 7.0), employing the linear relationship between ln(i) and t. The total capacitance at the working electrode/solution interface was continuously monitored in the capacitive biosensor program. The regeneration solution (0.25 ml) was loaded to the flow cell prior to sample analysis then after 15 min, a stable base line was achieved. Thereafter, an aliquot of 0.25 ml of the SEB sample was then injected, and after 10 min, the data were collected and manually processed. The total capacitance was plotted as a function of time, then an average of capacitance readings at the last 5 min before loading of the sample was calculated and assumed as the base line C_1. Another average of capacitance readings at the last 5 min after sample injection was calculated and considered as the capacitance due to affinity binding between SEB molecules and the immobilized anti-SEB on the gold electrode C_2. Finally, the change in capacitance (ΔC) was calculated as $C_1 - C_2$ and recorded for each concentration of standard SEB as shown in Fig. 1. A calibration curve of SEB obtained by plotting the logarithm of standard SEB concentration versus the calculated ΔC at each concentration was employed for determination of SEB levels in different purification samples and the results are presented in Table 1. A linear relationship was established between ΔC and the logarithm of SEB concentration within the dynamic sensing range from 2.8 pg ml^{-1} to 2.8 ng ml^{-1} and the limit of detection was 0.3 pg ml^{-1}. The linear regression equation was ΔC (nanofarads)=0.2648×log (SEB concentration in picograms per milliliter)+0.7657, with a correlation coefficient of 0.9565 as shown in Fig. 2. The RSD was between 3.4% and 12.5% at the tested SEB concentrations.

Table 1 Purification scheme of staphylococcal enterotoxin B

Step	Start volume (ml)	Collected volume (ml)	Total protein (mg ml^{-1})	SEB (ng ml^{-1}) by ELISA	SEB (ng ml^{-1}) by CB	Recovery (%)
Crude filtrate	4,000		13.31	292	350	100
1st purification step	4,000	700	0.26	1,472	1,582	88
2nd purification step	700	440	0.04	1,840	1,930	74
Last purification step	5	17	Not detected	95	97	12

CB capacitive biosensor

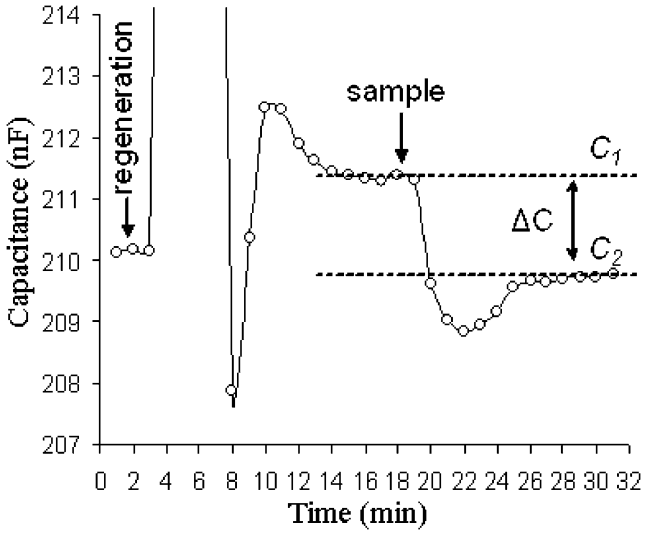

Fig. 1 Capacitance changes after electrode regeneration with 25 mM glycine/HCl buffer, pH 2.4 and injection of 283 pg ml^{-1} SEB, where C_1 and C_2 represent the baselines before and after the SEB injection, respectively. ΔC represents the decrease in capacitance due to the binding between SEB and the immobilized anti-SEB, and it equals $C_1 - C_2$

Further increase in the concentration of SEB was accompanied by a constant response value, indicating a saturated binding by the immobilized anti-SEB of SEB. The system was regenerated after each sample injection without a significant decrease in the measured capacitance after regeneration.

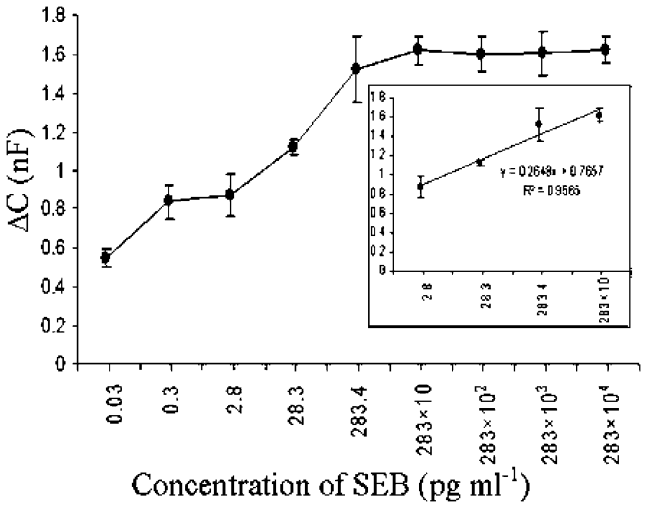

Fig. 2 Calibration curve for SEB detection measured with the capacitive biosensor in 10 mM PB (pH 7.4). The curve shows the relationship between the capacitance change and logarithm of SEB concentration from 0.03 to 283×10^4 pg ml^{-1}. The *inset* shows the linear range of capacitance changes versus the logarithm of SEB concentration from 2.8 to 283×10 pg ml^{-1}. *Symbols* denote mean values of triplicate measurements of capacitance. The capacitance is presented by mean values and standard deviations of all measurements

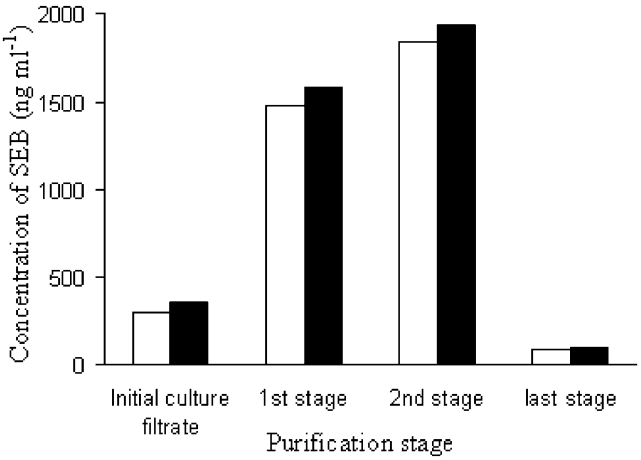

Fig. 3 Comparison between SEB levels (nanograms per milliliter) at different purification steps, assayed by ELISA (*empty bars*) and capacitive biosensor (*filled bars*)

Assessment of the capacitive biosensor

The newly developed analytical tool was assessed by setting a comparison between the concentrations of SEB as determined by it and those determined by ELISA technique as a reference method. A good agreement was achieved between both methods indicating that the capacitive biosensor could be a potentially useful analytical tool for direct assay of trace amounts of SEB (Figs. 3 and 4). The concentration of SEB determined by the capacitive biosensor was slightly higher than that assayed by ELISA, probably due to a higher degree of nonspecific binding of various proteins to the immobilized antibodies on the gold surface. On the other hand, the harsh washing (12 times) employed in ELISA technique has prevented such nonspecific binding.

Conclusions

The flow-injection capacitive biosensor system is potentially useful for detection of staphylococcal enterotoxin B

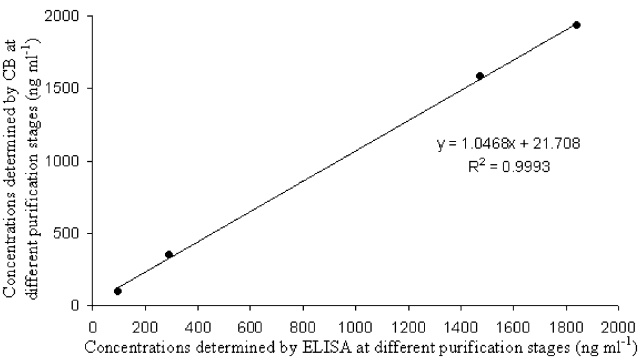

Fig. 4 Assessment of the capacitive biosensor: correlation between concentrations of SEB assayed by ELISA and capacitive biosensor

in buffer with good linearity at concentrations from 2.8 pg ml^{-1} to 2.8 ng ml^{-1} and a detection limit of 0.3 pg ml^{-1}. The technique is also suitable for monitoring of trace amounts of the toxin in crude *S. aureus* fermentation fluid. At the present time, SEB is determined by several methods previously reviewed in the introduction section. The technique described here represents a more sensitive approach for detection of SEB compared to these methods except the PEMC with a reported LOD value of 2.5 fg ml^{-1} [32] and the indirect double sandwich fluorogenic enzyme-linked immunoassay with an LOD value of 0.1 fg ml^{-1} [33]. The merit of the technique described here lies in the fact that it is a label-free technique that can measure low levels of SEB with simplicity, high sensitivity, and multiple use capability. Unlike discrete sampling methods, the capacitive biosensor system can run continuously for hours until it encounters the target analyte and does not consume antibody reagents in a direct detection mode. The effective regeneration of the sensing surface will allow the online measurement of SEB in processes handling fermentation broth. This will improve the possibility to control the level of the toxin in the waste streams from the downstream processing. Furthermore, the ability of the capacitive biosensor to detect SEB at subtoxic concentrations allows the technique to be explored in many applications such as in forensics and contamination recovery studies where detection of very low concentrations of the residual toxin is extremely valuable.

Acknowledgment Ministry of Higher Education, Egypt is gratefully acknowledged for financial support to Mahmoud Labib during his study at the Department of Biotechnology, Lund University.

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