Chapter 6

Ultra-sensitive Capacitive Biosensor Developed for the Monitoring of Endotoxins in Fermentation liquid

6.1 Introduction

Endotoxins are high-molecular weight complexes of lipopolysaccharides (LPS), which constitute the major element of the outer cell wall of Gram-negative bacteria (McCuskey *et al.*, 1996). Three distinct regions build up the endotoxin molecule: lipid A, core oligosaccharide and the O-specific antigen (Raetz, 1990), where lipid A is responsible for many of the patho-physiological effects associated with gram-negative bacterial infection. Effects of endotoxin exposure on humans include fever, diarrhea, vomiting, septic shock, pyrogenicity, and disseminated intravascular coagulation (Anderson *et al.*, 2002; Morrison and Ulevitch, 1978; Karima and *et al.*, 1999). Despite the advances in medical therapy, sepsis carries a mortality rate of 10-15% in children and up to 40% in adults (Carcillo and Cunnion, 1997).

Endotoxins are a significant contaminant in food, water and process fluids from the fermentation industry. As many bioprocesses are used for production of pharmaceutical proteins, endotoxins becomes an important issue in manufacturing processes. Hence, as endotoxins should be completely removed in the final product, determination of trace amounts of remaining endotoxin is of great interest.

Detection of endotoxin is generally involved the use of lectins, a group of proteins known for their specific affinity towards sugar molecules (*e.g.*, oligosaccharides on cell surfaces, glycoprotein) (Franz and Ziska, 1980). Endotoxin neutralizing protein (ENP) originally isolated from American horseshoe crab (*Limulus polyphemus*) is a lectin with specific affinity towards the endotoxin molecule together with a few other types of LPSs (Aketagawa, *et al.*, 1986). As of today, the available methods for endotoxin detection are all based on a lectin derived from American horseshoe crab (*Limulus polyphemus*). Although the *Limulus Ambeocyt Lysate* (LAL)

assays have been proven to be sensitive (*i.e.* 0.1 pM) and reliable methods for endotoxin monitoring, various potential interfering substances can be identified, e.g. coagulation reagents in biological fluids (Kang and Luo, 1999; Petsch and Anspach, 2000). Moreover, LAL assays are expensive and non-functional for the detection of endotoxins in ultra-trace amounts. Therefore, development of new, less labor-intense methods with high sensitivity and specificity for the direct detection of endotoxins is highly desirable.

This project reports the development of a biosensor system using a capacitaive transducer to detect the affinity binding of endotoxin by immobilized lectin for the direct detection of trace amounts of bacterial endotoxin in fermentation fluids from *E.coli*.

6.2 Materials

Lectin (endotoxin neutralizing protein (ENP) from *Limulus polyphemus*) and Endotoxin standards were obtained from Sigma (St. Louis, USA), 1-dodacanthiol was obtained from Aldrich (Deisenhofen, Germany). All other materials and the preparation of buffer were as in section 5.2.

6.3 Methods

6.3.1 Preparation of endotoxins from *E.coli*

Endotoxins are part of the outer membrane of the cell wall of Gram-negative bacteria (e.g. *E.coli).* Generally, after *E.coli* cultivation, endotoxin molecules are relatively easy to extract from the cell suspension by treatment with, e.g. phenol-water mixtures. To start the preparation, the retrieved *E.coli* cell paste (7.9 g) was divided into two equal portions and suspended in 50 mM Tris-HCl, pH 7.40. For enzymatic cell disruption, 0.15 mg of lysozyme was added to one of the portions with subsequent shaking for 30 min (30 rpm). Both sample types (*i.e.* cell suspensions with and without lysozyme) were then disrupted using an ultrasonicator (UP 400S/2, Dr. Hielscher GmbH, Stahndorf, Germany) with a 0.5 cm probe that was set to 0.5-second-on/0.5-second-off pulse cycles, 60 % amplitude of maximum sonic intensity (105 W cm^{-2}). The probe was placed in the sample six times, one minute at a time with one minute interruption. After disruption, the cell debris was removed by

centrifugation at 21800×g for 10 min and the supernatant containing endotoxin was stored at -20 °C.

6.3.2 Endotoxin extraction with phenol-water

One ml of the supernatant containing endotoxin was suspended in 20 ml of water with 20 ml of 90 % (w/v) phenol, and the mixture was kept for 15 min at 65 °C. After cooling the vessel to 10 °C on ice, the emulsion was centrifuged at 1560×g for 45 min. Three layers; a water layer, a phenol layer, and a layer containing insoluble residues were observed. The water layer was removed and 20 ml of water was added to the remaining phenol layer and the insoluble residues and the mixture was treated at 65 °C for 15 min in a water bath. The combined water extract mixture was dialyzed for 4 days (cellulose membrane, molecular weight cut off 3 kDa) against distilled water to remove phenol and small amounts of low-molecular-weight bacterial substances (Westphal and Jann, 1965). The extractant containing endotoxin inside the dialysis tube was concentrated at 38 °C under reduced pressure to a volume of 5 ml and thereafter freeze-dried giving a white-yellowish powder. The dry endotoxin preparation was reconstituted in 500 µl of 10 mM phosphate buffer, pH 7.20 and stored at 4°C.

6.3.3 Immobilization of Lectin

Preparation of gold rod electrode, SAM of thioctic acid on gold and the subsequent activation by EDA followed the same procedure as described in section 5.3.3.1 and 5.3.3.2. The electrode was then immersed in 0.5 mg ml^{-1} lectin solution in 100 mM phosphate buffer pH 7.20 overnight at 4°C. Finally, before being placed in the biosensor flow-cell, the electrode was reacted in a 10 mM 1-dodecanethiol ethanolic solution for 20 min to block any pinholes or bare spots on the electrode surface.

6.3.4 Capacitance measurement

The basic experimental set-up of the flow-injection based capacitive biosensor system for endotoxin was the same as the one described in section 5.3.4. (Figures 5.7 and 5.8).

6.3.5 Optimization of the capacitive biosensor

The operating conditions of the capacitive biosensor in the flow-injection system were optimized for type, pH and concentration of the regeneration solution, sample volume, flow rate and type and pH of running buffer. The running buffer used throughout the experiments was a 10 mM phosphate buffer, pH 7.20 (except when the effects of buffer types were tested). The effects of type, pH and concentration of regenerate solution were tested with conditions; 250 μl min^{-1} flow rate of buffer, 250 μl sample volume. The effect of the sample volume was tested at a flow rate of 100 μl min^{-1}.

6.3.6 Determination of the amount of endotoxin in real samples

The amount of endotoxin in the samples was analysed by the chromogenic LAL test (Kang and Luo, 1999) (the result obtained by Novozymes Biopharma AB, Lund, Sweden) and the flow injection capacitive biosensor system. In the case of the capacitive biosensor, the concentration of endotoxin in the samples were calculated from the calibration equation obtained using four different concentrations of standard endotoxin (Sigma) under optimum conditions; 50 μl min^{-1} flow rate of running buffer (10 mM phosphate pH 7.20) and 100 μl sample volume.

6.3.7 Comparison between the results obtained from the capacitive biosensor system and LAL-test

The flow injection capacitive biosensor system was validated by comparing the results to those obtained using the chromogenic LAL test. In making such a comparison, the principle interest will be whether the proposed method gives results that are significantly higher or lower than the established method. So, the analysis using the Wilcoxon signed rank test (Triola, 1998) was used in this work. This is one type of statistical tests uses to handle data which may not be normally distributed. The comparison was done by comparing each pair of data. Then these values were arranged in numerical order without regard to sign. The numbers were then ranked; in this process they keep their signs but are assigned number indicating their order. The positive and negative ranks were summed individually. The lower of

these two figures was taken as the test statistic. If this value is less than the one given in Table 6.1 the two populations varied significantly ($P<0.05$).

Table 6.1 Critical values for the Wilcoxon signed rank test; statistic at $P < 0.05$ for n = 6 to 37 where n is the number of data pair (Triola, 1998). The null hypothesis can be rejected when the test statistic is ≤ the tabulated value.

n	Two-tailed test	n	Two-tailed test
6	1	22	66
7	2	23	73
8	4	24	81
9	6	25	90
10	8	26	98
11	11	27	107
12	14	28	117
13	17	29	127
14	21	30	137
15	25	31	148
16	30	32	159
17	35	33	171
18	40	34	183
19	46	35	195
20	52	36	208
21	59	37	222

6.4 Results and discussion

6.4.1 Electrochemical performance of the immobilization process

In the capacitive biosensor system the insulating property of the self-assembled monolayer on the electrode surface is of vital importance. The degree of insulation was examined using cyclic voltammetry with a permeable redox couple (*i.e.* $K_3[Fe(CN)_6]$) in the electrolyte solution as shown in Figure 6.1. At the clean gold surface the redox couple was oxidized and reduced according to curve a. in Figure 6.1. The redox peaks decreased when thioctic acid was self-assembled on the clean gold surface (curve b. Figure 6.1). With the lectin immobilized on the electrode, the insulating property of the electrode surface was further increased (curve c. Figure 6.1). A final capping of the electrode surface was achieved by the treatment with 1-dodecanethiol, as can be seen from the disappearance of the redox peaks in curve d. Figure 6.1.

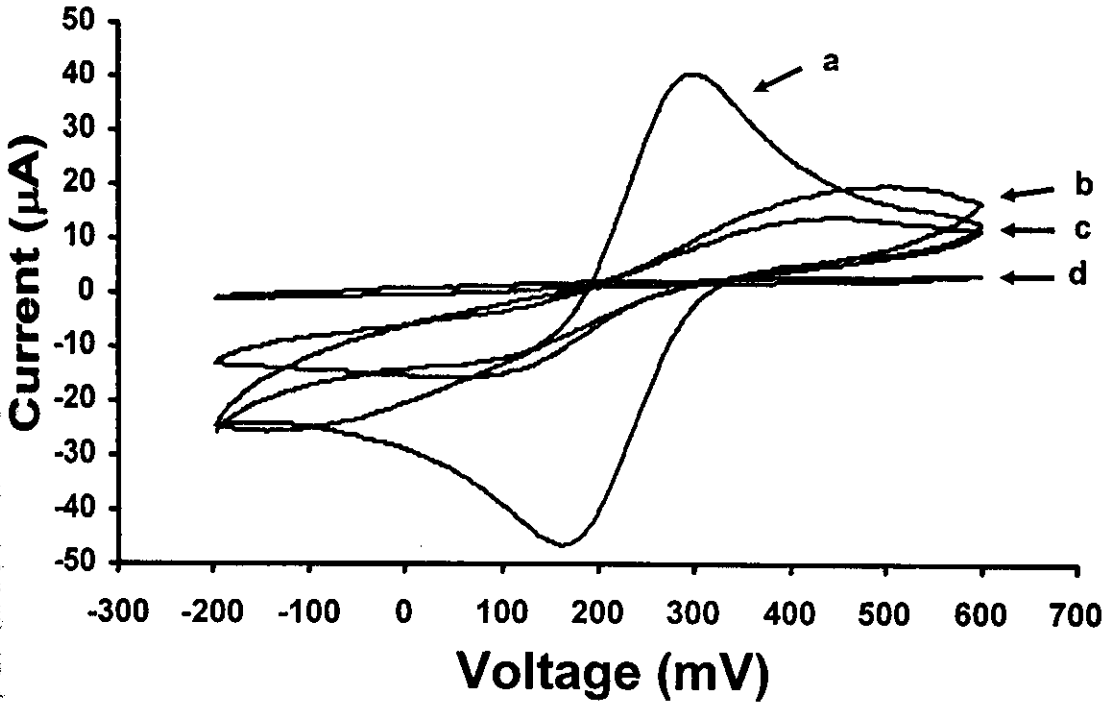

Figure 6.1 Cyclic voltammograms of a gold electrode obtained in a 5 mM $K_3[Fe(CN)_6]$ containing 0.1 M KCl solution at scan rate of 50 mV s^{-1}. All potentials are given *vs* SCE. (a) clean gold, (b) thioctic acid covered gold, (c) lectin modified thioctic acid couple gold, and (d) as in (c) but after 1-dodecanethiol treatment.

6.4.2 Optimization of the flow injection capacitive biosensor

6.4.2.1 Regeneration solution

To evaluate the performance of the regeneration solution, the residual activity of the lectin electrode was calculated from the capacitance change (ΔC) due to the binding between endotoxin and lectin (1×10^{-10} M of endotoxin standard) before and after regeneration (Figure 5.7, chapter 5). A screening for different types of regeneration methods was performed using three classes; (i) displacement with other carbohydrate moieties, (ii) ionic strength, and (iii) pH, as shown in Table 6.2. The use of carbohydrates and the effect of increased ionic strength as regeneration methods were shown to be ineffective as the capacitive signal did not elevated more than a few

percents, indicating that many lectin molecules still remained occupied by endotoxin analyte after the regeneration step. However, when applying a solution of low pH (*i.e.* glycine HCl, pH 2.20) significant regeneration effects were obtained.

The pH and concentration of glycine buffer solution suited for the regeneration of the lectin-endotoxin system was then optimized. Influence of pH of glycine buffer solution, ranging from 4.0 down to 2.0 was first studied (Table 6.2). Generally, the percent residual activity of the lectin electrode increased as the pH decreased. High values were obtained (53 to 72%) at pH 2.40 down to 2.00, and pH 2.40 was chosen to avoid the possible deterioration of the insulating SAM layer as a result of low-pH (Jiang *et al.*, 2003). To further optimize the dissociation of the lectin-endotoxin complex, the effect of concentration of glycine was examined. At higher glycine concentrations (*i.e.* $\geq$ 25 mM) the average of percentage of residual activity values above 90 % were achieved. Normally, if post-regeneration binding remains above 90 % compared to the binding efficiency before regeneration, the conditions should be seen as adequate. Therefore, any of these concentrations can be used. However, higher ionic strength solution may lead to the production of pinholes on the electrode surface due to the removal of SAM (Jiang *et al.*, 2003). Therefore, 25 mM glycine buffer solution, pH 2.40 was chosen as regeneration solution in the continued experiments.

Since low pH may destroy SAM (Jiang *et al.*, 2003) glycine buffer at higher pH (pH 4.00) was also tested at different concentrations. The increase concentration will increase the ionic strength of the solution and this may help to dissociate the reaction between endotoxin and lectin. However the results showed that the increase in concentration of glycine, pH 4.00 was not effective enough for the dissociation of endotoxin from lectin.

Table 6.2 Assayed and optimized values of the type, pH and concentration of regeneration solution. The efficiency of endotoxin removal from the lectin immobilized on the electrode was studied by injecting 0.1 nM of endotoxin standard.

Regeneration solution	Percentage of residual activity			
	First analysis	Second analysis	Third analysis	average ± SD
Type				
10 mM Lactose	39	30	19	29 ± 10
10 mM Glucose	41	34	34	36 ± 4
10 mM Isopropyl β-D-thiogalacto pyronoside	25	36	28	30 ± 6
10 mM *O*-Nitrophenyl β-*D*-galactopyranoside	36	48	55	46 ± 10
10 mM Sodium chloride	18	27	9	18 ± 9
10 mM Glycine buffer pH 2.20	54	61	57	57 ± 4
pH*				
10 mM Glycine buffer pH 4.00	14	17	-	16
10 mM Glycine buffer pH 3.50	21	18	-	20
10 mM Glycine buffer pH 3.00	32	29	-	31
10 mM Glycine buffer pH 2.80	38	35	-	37
10 mM Glycine buffer pH 2.60	45	51	-	48
10 mM Glycine buffer pH 2.40	53	60	-	57
10 mM Glycine buffer pH 2.20	65	51	-	58
10 mM Glycine buffer pH 2.00	51	72	-	62
Concentration*				
10 mM Glycine buffer pH 2.40	59	64	-	62
25 mM Glycine buffer pH 2.40	89	100	-	95
50 mM Glycine buffer pH 2.40	96	88	-	92
100 mM Glycine buffer pH 2.40	91	96	-	94
10 mM Glycine buffer pH 4.00	20	20	-	20
25 mM Glycine buffer pH 4.00	28	35	-	32
50 mM Glycine buffer pH 4.00	41	32	-	37
100 mM Glycine buffer pH 4.00	46	38	-	42

*Note; the effects of pH and concentration of regeneration solution were performed in duplicates due to the life time of the electrode.

6.4.2.2 Sample volume

Generally, an increase in response can be achieved with an increase in volume of injected sample. Therefore, the capacitance change obtained from different sample volumes of 1×10^{-10} M of endotoxin standard was studied. Figure 6.2 shows the change in capacitive signal (-nF cm^{-2}) that increased as the sample volume was increased from 5 up to 250 µl. At lower values the difference between each sample volume was significant ($P < 0.05$). At sample volume 100 and 250 µl the capacitance change reached a maximum plateau. So, 100 µl was chosen because it has lower analytic time (15 min) than at 250 µl (20 min).

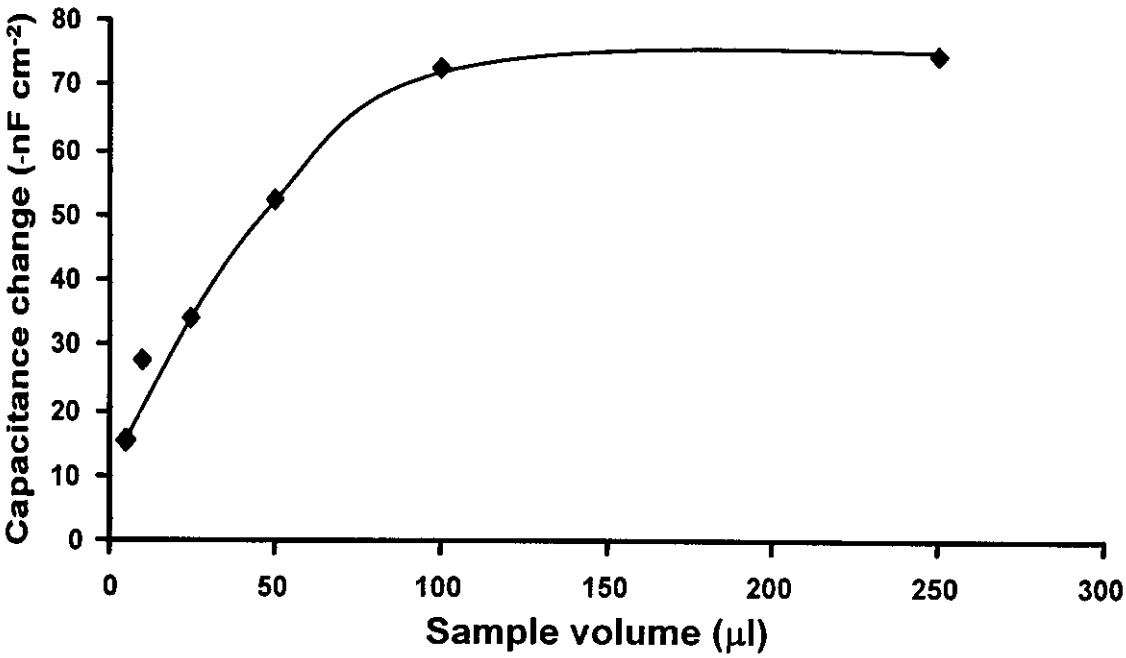

Figure 6.2 Responses of the capacitive biosensor system at different sample volume.

6.4.2.3 Flow rate

The flow rate of the buffer passing through the flow cell is the main factor affecting the yield of interaction between analyte and immobilized biorecognition element on the transducer. The capacitance changes obtained for different flow rates, ranging from 50 to 750 μl min^{-1}, were studied. The capacitance change resulting from injections of 1×10^{-10} M of endotoxin standard gave the highest response at a flow rate of 50 μl min^{-1} (Figure 6.3), which was chosen. A flow rate lower than 50 μl min^{-1} could not be tested because it was not obtainable due to the limitation of the peristaltic pump.

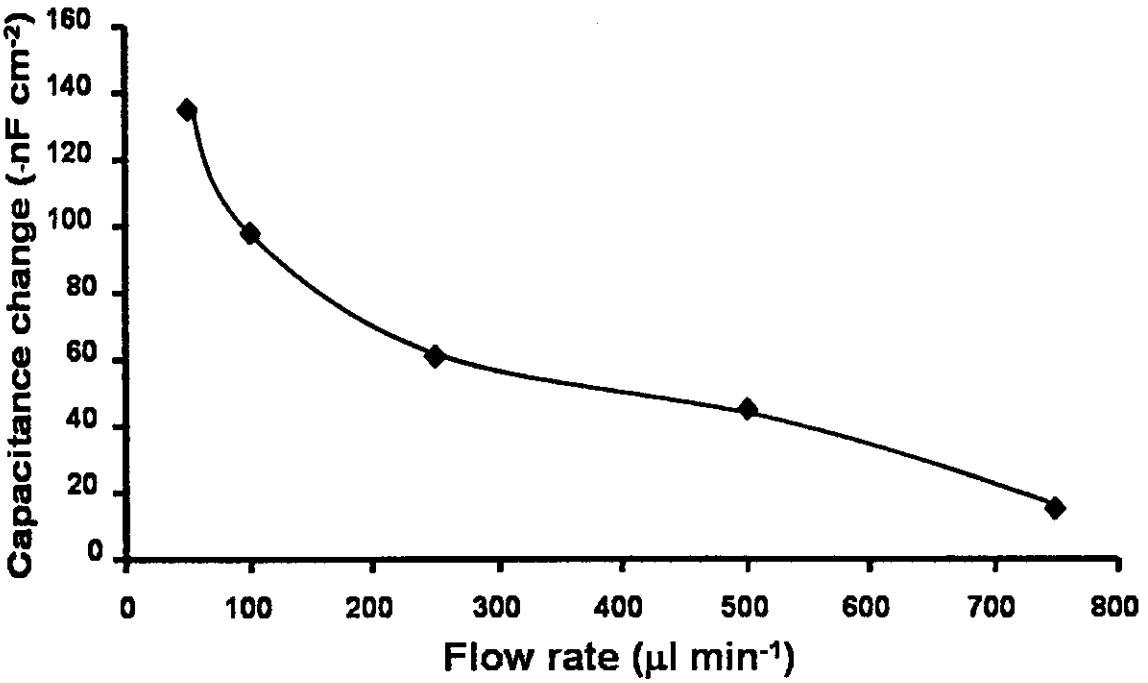

Figure 6.3 Responses of the capacitive biosensor system at different flow rates.

6.4.2.4 Buffer solution

The influence of the type of buffer used in the flow injection system; 10 mM phosphate buffer, pH 7.20 and 10 mM 2-[*N*-Merpholino] ethanesulfonic acid (MES) buffer, pH 7.20 (Chang *et al.*, 1997) were studied. At 10 mM concentration of the different buffer salts (pH 7.20), the sensitivity was not significantly affected (Figure 6.4). However, phosphate buffer gave a more stable base line and was chosen for further study.

Since the optimum range of pH for interaction between endotoxin and lectin is 6.80-7.50 (Levin, et. al., 1970). The effect of pH of the buffer solution was studied. The maximum change in capacitive signal for injections of 1×10^{-12} M of endotoxin standard was found to be at pH 7.20 (Figure 6.5).

Table 6.3 summarizes the optimized parameters, the values tested and the optimum values.

Table 6.3 Assayed and optimized values used in the study of the capacitive biosensor system as a tool for endotoxin analysis.

Parameters	Investigated values	Optimum
Sample volume (μl)	5, 10, 25, 50, 100, 250	100
Flow rate (μl ml^{-1})	50, 100, 250, 500, 750	50
Buffer solutions		
Type	10 mM Phosphate buffer, pH 7.20	10 mM Phosphate buffer,
	10 mM MES, pH 7.20	pH 7.20
pH	6.00, 6.60, 6.80, 7.00, 7.20, 7.40,	7.20
	7.60, 8.00	

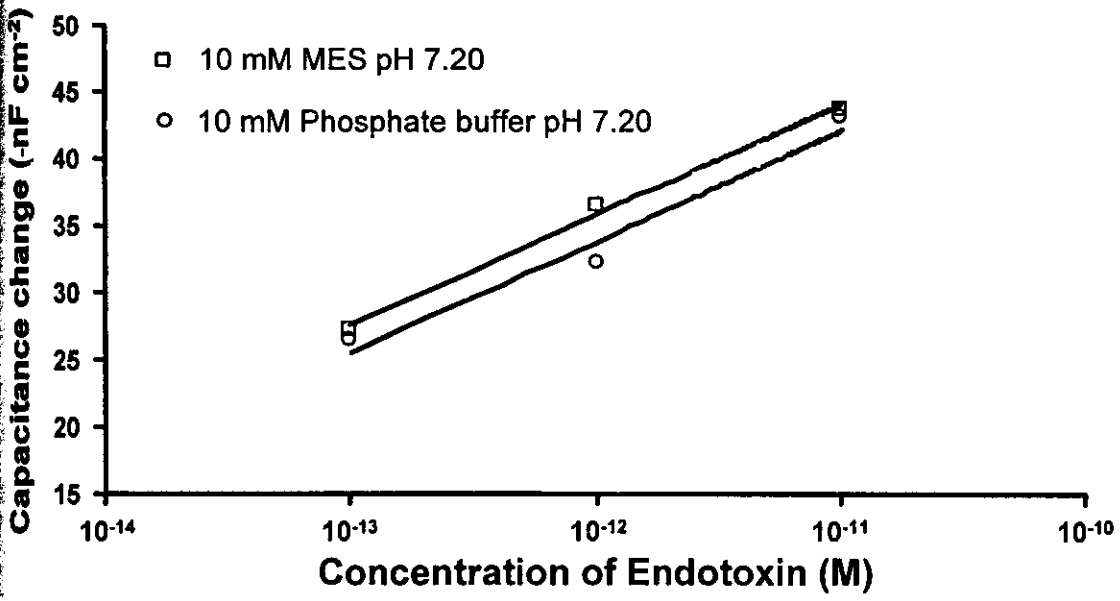

Figure 6.4 Responses of the flow injection capacitive biosensor system from different buffer solution.

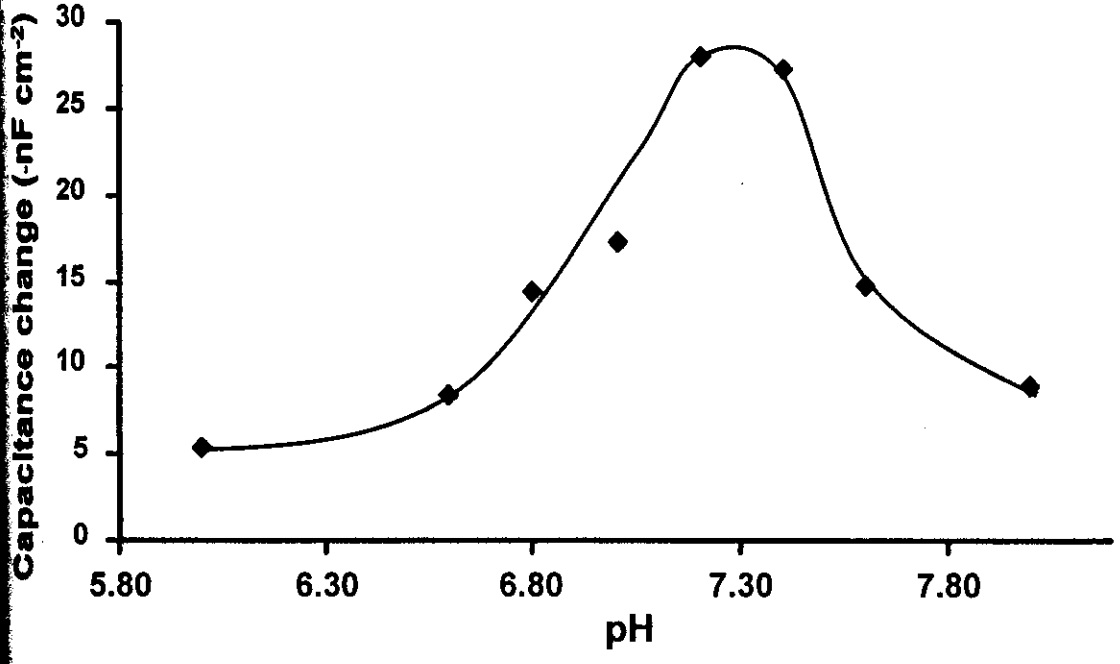

Figure 6.5 Effect of pH of phosphate buffer solution.

6.4.3 Linear dynamic range and detection limit

Endotoxin standard solutions ranging from 1×10^{-17} to 1×10^{-9} M were prepared from stock standard endotoxin solution (Sigma). Discrete pulse injections of these solutions (100 µl) with intermediate regeneration steps using 25 mM glycine-HCl buffer, pH 2.40 were performed. A linear relationship between the capacitance change and the logarithm of endotoxin concentration was obtained within a linear dynamic detection range of 1.0×10^{-13} to 1.0×10^{-10} M (Figure 6.6a). The linear regression equation was ΔC (-nF cm^{-2}) = 18 · log Endotoxin (M) + 253, with a correlation coefficient of 0.996. The detection limit was 1.0×10^{-13} M based on IUPAC Recommendation 1994 (see section 4.2). An attempt to improve the sensitivity (slope) of the lower concentration range was done by increasing the sample volume from 100 to 250 µl using the same flow rate of 50 µl min^{-1}. The sensitivity was increased with a LOD verified to be as low as 1.0×10^{-17} M based on IUPAC Recommendation 1994 (see section 4.2). The linear range was in this case determined to be between 1.0×10^{-17} and 1.0×10^{-13} M (Figure 6.6b). The linear regression equation was ΔC (-nF cm^{-2}) = 4 · log Endotoxin (M) + 77, with a correlation coefficient of 0.970. However, for concentration levels as extremely low as investigated in this part of the study, where only a few thousand molecules are registered, the reproducibility and analytical stability becomes rather poor (Figure 6.6b). Thus, this capacitive biosensor system under the second optimum conditions may only be suitable for an "order of magnitude" test of endotoxin.

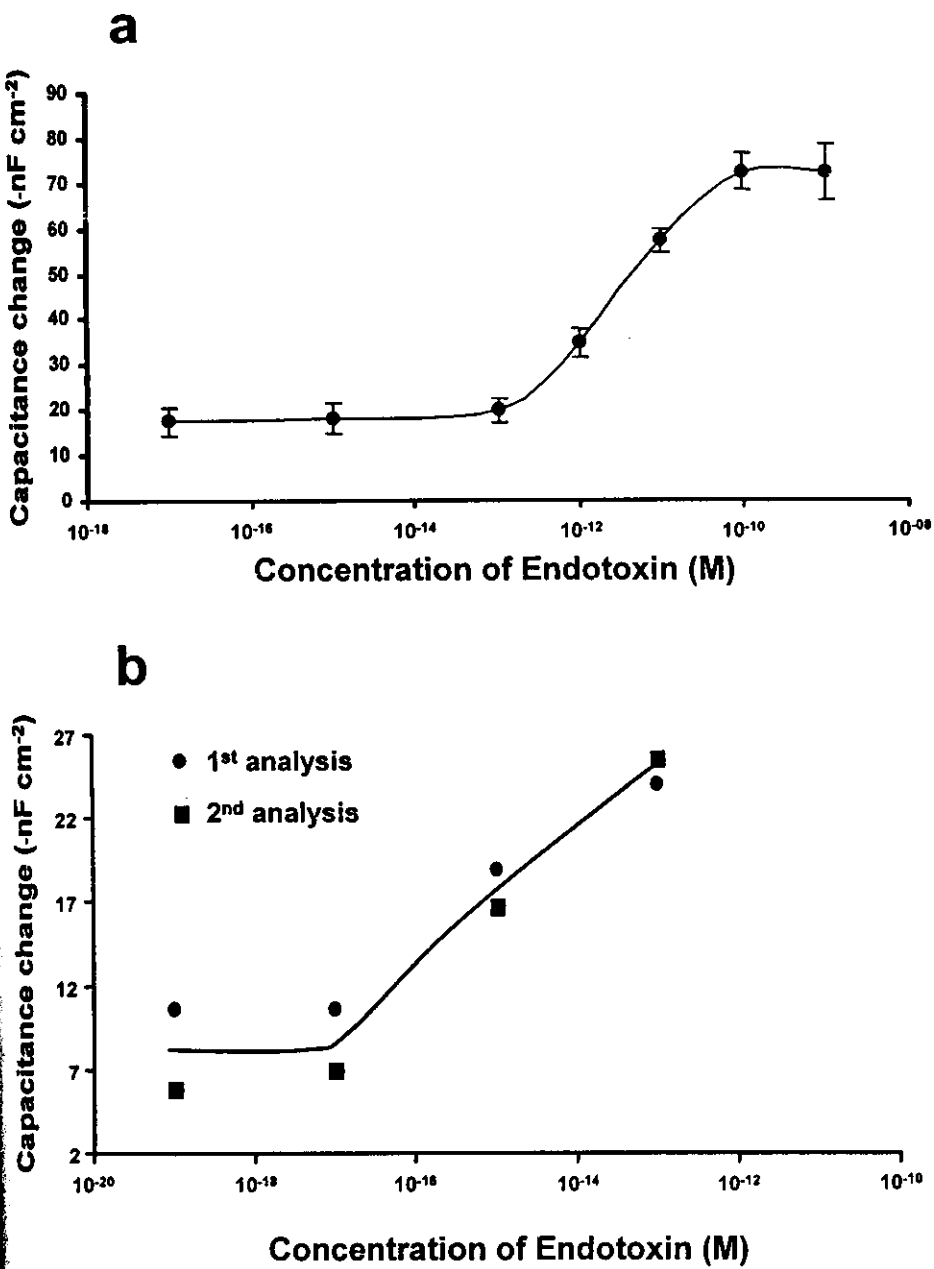

Figure 6.6 Capacitance change *vs.* the logarithm of endotoxin concentration for a surface with immobilized lectin. (a) the first optimum conditions; 50 µl min^{-1} flow rate of current buffer (10 mM phosphate buffer pH 7.20) and sample volume 100 µl, (b) the second optimum conditions; 50 µl min^{-1} flow rate of current buffer (10 mM phosphate buffer pH 7.20) and sample volume 250 µl. *Note; the second optimum conditions was performed in duplicates due to the life time of the electrode.

6.4.4 Comparison between the capacitive biosensor system and LAL-test

The analysis of endotoxin using the capacitive biosensor system and Chromogenic-LAL test were done on the same samples. The capacitance change from the biosensor system was used to calculated the concentration of endotoxin from the calibration equation done prior to the analysis. The results of Chromogenic-LAL test were obtained from Novozymes Biopharma AB, Lund, Sweden. The seven samples were, standard endotoxin at a concentration of 4.0×10^{-8} M (Std), endotoxin in supernatant (A), endotoxin in supernatant treated with lysozyme (B), and four samples of endotoxin in the extract from four different preparations (C, D, E, and F) (A and B were obtained as described in section 6.3.1, and C, D, E, F were the extractants obtained as described in 6.3.2). For the capacitive biosensor system, since the concentration of endotoxin in real samples was not known several dilution series (of 10 times) were prepared and analysed until the responses were within the linear range, at a dilution of 10^7 times.

The percent difference between the vial label of endotoxin standard concentration and the one obtained from the capacitive biosensor under optimum conditions (Figure 6.6a) was 11 % (Figure 6.7(std)). However, the results obtained from the Chromogenic-LAL test was 44% higher than the vial label indicating that the accuracy of the capacitive biosensor system is higher than the Chromoganic-LAL test.

Samples A and B (Figure 6.7) were endotoxin in the supernatant. In sample A the cells were disrupted using ultrasonication, where as in B, enzymatic disruption with lysozyme was concomitantly used with the ultrasonication step. Notable for sample B is the significantly diverging results generated with the capacitive biosensor compared to the reference method. This effect is supported by former studies (Brandenburg *et al.*, 1998), which shows that endotoxin molecules can bind to lysozyme with relatively high affinity. Hence, in the case of sample B, lysozyme-bound endotoxins could be transported through the biosensor flow cell without the ability to form affinity complex with the lectin molecules. Samples C, D, E and F (Figure 6.7) were endotoxin in extractants. The results of the endotoxin samples from the extractants obtained by the capacitive biosensor system were compared with those obtained using the reference Chromogenic-LAL method, the relative differences being in the range 2-15 %.

The capacitive biosensor system was validated by comparing the results to the reference Chromogenic-LAL method. These were done by using Wilcoson signed rank test. In Wilcoxon signed rank test, the null hypothesis (there is no difference between the two methods) is rejected at a significance level P value < 0.05 if the experimental value is less than or equal to the critical values (Triola, 1998). In this test, the null hypothesis is retained, that is, there is no evidence for a systematic difference between the results of the capacitive biosensor system and the reference Chromogenic-LAL method. Therefore, the concentrations determined by the capacitive biosensor system are in good agreement to the reference Chromeganic-LAL method.

The capacitive biosensor system under second optimum conditions was also tested by injecting the same seven samples, by diluting them about 10^{10} times before analysis. The capacitance change from the capacitive system was used to calculated the concentration of endotoxin from the calibration equation done prior to the analysis (The linearity between 1.0×10^{-17} and 1.0×10^{-13} M (Figure 6.6b)). The results for these samples obtained by the capacitive biosensor system were compared with those obtain using the reference Chromogenic-LAL method, the relative difference being in the range 8-112 %. Thus, the capacitive biosensor system is not suitable for quantitative analysis of endotoxin at this concentration range.

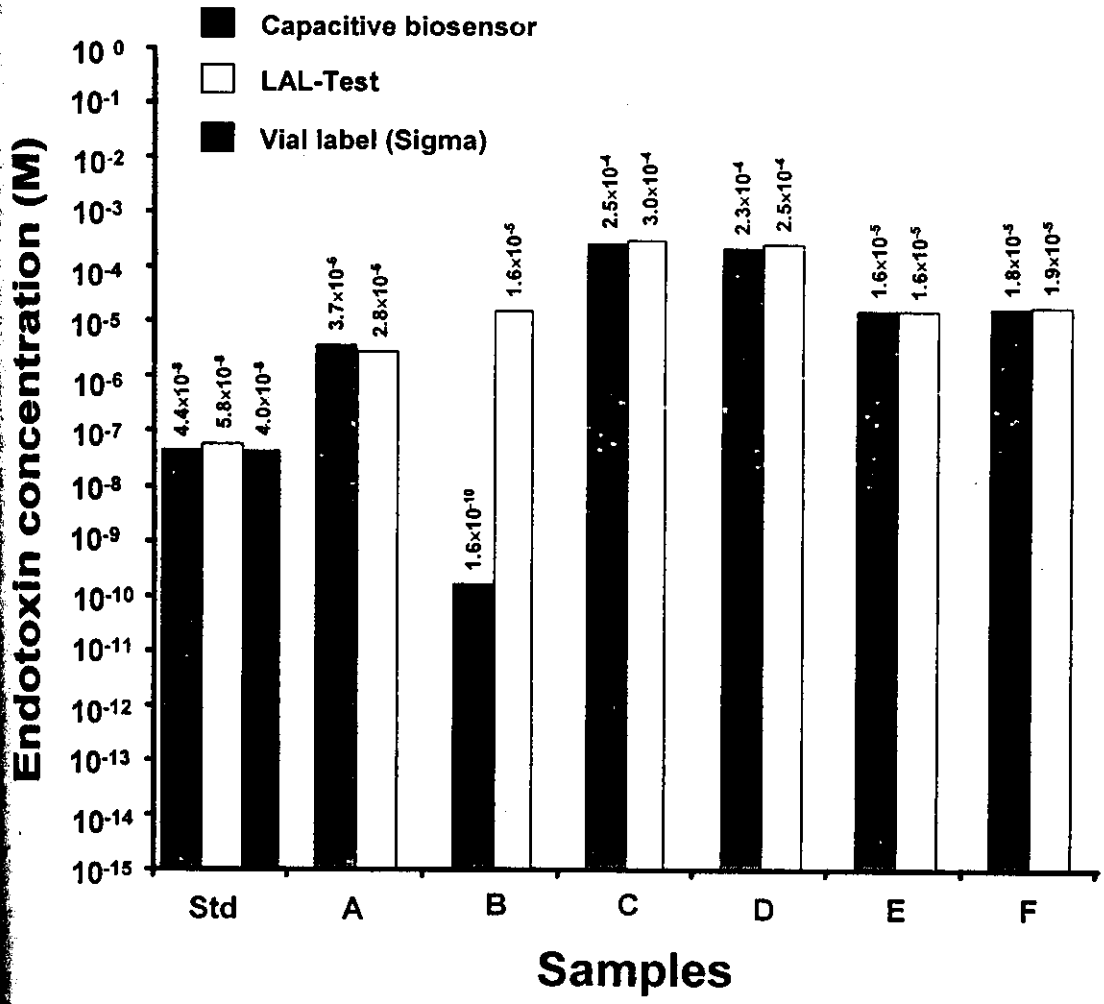

Figure 6.7 The logarithm of the endotoxin concentration for difference samples analyte . Samples; (Std) endotoxin standard with a concentration of 4.0×10^{-8} M; (A) endotoxin in supernatant; (B) endotoxin in supernatant treated with lysozyme; (C, D, E, and F) endotoxin in extractants from different preparations.

6.5 Conclusions

The flow injection capacitive biosensor system is potentially useful for direct assay of the interaction between bacterial endotoxin and lectin modified thioctic acid coupled to gold. This technique was sensitive to bacterial endotoxin and it was suitable for the quantitative analysis of ultra-trace amounts of bacterial endotoxin in fermentation liquids to provide quality assurance of the product. The regeneration of the activity of working electrode is also possible.