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Acoustic Microsensors III. Direct detection of Staphylococcal Enterotoxin B employing a piezoelectric crystal immunosensor with a flexible carboxylated dextran matrix as the biochemical interface

TNO Prins Maurits Laboratory

Lange Kleiweg 137
P.O. Box 45
2280 AA Rijswijk
The Netherlands

Phone +31 15 284 28 42
Fax +31 15 284 39 63

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Author(s)
J.L.N. Harteveld

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Titel	:	Acoustic Microsensors III. Direct detection of Staphylococcal Enterotoxin B employing a piezoelectric crystal immunosensor with a flexible carboxylated dextran matrix as the biochemical interface
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In het kader van het project 'Akoestische Microsensoren' (A93KL448) wordt onderzoek gedaan naar een biosensorsysteem gebaseerd op immunochemische reacties die worden gedetecteerd met behulp van piëzo-elektrische kwartskristallen. Dit werk wordt uitgevoerd in samenwerking met andere NATO-landen in de zogenaamde Project Group 33 (PG33) waaraan het TNO Prins Maurits Laboratorium (TNO-PML) namens Nederland deelneemt. Andere deelnemende landen zijn Canada, Frankrijk, Duitsland, de Verenigde Staten en het Verenigd Koninkrijk. PG33 streeft ernaar een biosensorsysteem te ontwikkelen voor de 'real time' monitoring van biologische strijdmiddelen en aanverwante verbindingen. TNO-PML onderzoekt de mogelijkheden van een immunoreactie op een piëzo-elektrisch kwartskristal gebruikmakend van 'bulk acoustic waves' (BAW). Dit sensorsysteem vereist in principe geen gelabelde antilichamen, lange incubatietijden of gecompliceerde immobilisatieprocedures.
Staphylococcus Enterotoxin B (SEB) is één van de toxinen die geproduceerd wordt door de bacterie *Staphylococcus aureus*. Het is een polypeptide bestaande uit 239 aminozuren en heeft een moleculairgewicht van circa 28000 g/mol. Het toxine, dat voedselvergiftiging veroorzaakt, behoort tot de biologische strijdmiddelen. SEB werd door PG33 gekozen als teststof om de diverse sensorsystemen van de aan PG33 deelnemende landen te vergelijken.
In een vorig TNO-rapport PML 1996-A86 werd de detectie van SEB beschreven, waarbij de kwartskristal immunosensor gebruikt werd in een flow-injectiesysteem. De detectie was gebaseerd op een competitieschema: polyclonale anti-SEB antilichamen werden gemengd met SEB in oplossing, waarna de resterende niet aan SEB gebonden antilichamen konden reageren met SEB aan het sensoroppervlak. De detectiegrens bedroeg 100 ng/ml, waarbij de inhibitie van de sensorrespons ongeveer 10% van de maximale respons bedroeg. Veel verbetering werd bereikt door de toepassing van een flow-injectiesysteem, vooral door de eliminatie van aspecifieke effecten. De detectie kan echter niet als een 'real time assay' beschouwd worden, aangezien een pre-incubatieperiode van 20 minuten noodzakelijk is. Zoals genoemd in het vorige rapport in deze serie, blijft het doel de directe detectie van SEB en andere antigenen door aan het sensoroppervlak gebonden antilichamen.

In de literatuur worden veel procedures beschreven voor de immobilisatie van antilichamen aan glas- en metaaloppervlakken. De zogenaamde hydrogelmatrix, zoals beschreven door Löfas en Johnson, leek een veelbelovende methode. Deze immobilisatietechniek wordt toegepast in de Biacore, een handelsnaam voor het biosensorsysteem van Pharmacia, Zweden.
De belangrijkste kenmerken van de hydrogelmatrix, opgebouwd uit een carboxygemethyleerde dextraanlaag, zijn:

- een veel grotere immobilisatiecapaciteit dan bij coatings die uit een monolaag bestaan: de dextraanlaag is in bufferoplossing ongeveer 100 nm dik (afhankelijk van pH en ionsterkte);
- de dextraanlaag levert een flexibele, niet gecrosslinkte open structuur waarin liganden door de hele laag kunnen binden;
- weinig aspecifieke binding.

Op basis van de publicatie van Pharmacia werd besloten om de dextraanlaag op de piëzo-elektrische kristallen aan te brengen, waarna deze kristallen gebruikt werden voor de directe detectie van SEB in een flow-injectiesysteem.
Het systeem werkte erg goed voor de directe detectie van SEB. Er was geen preincubatiestap meer nodig en er werd een detectiegrens bereikt van 7 ng/ml, hetgeen een grote verbetering is vergeleken met de detectiegrens van 100 ng/ml voor de competitieassay. Een ander voordeel van de directe detectiemethode ten opzichte van de competitie assay is het uitsparen van dure antilichamen. Het kristal kan eenvoudig geregenereerd worden door middel van een één minuut durende blootstelling aan 0,1 M HCl, waarna de respons goed kan worden gereproduceerd.
In de toekomst zal het onderzoek zich richten op de detectie van grote antigenen die tot de biologische strijdmiddelen behoren, zoals sommige virussen en bacteriën. De hydrogelmatrix zal hiervoor waarschijnlijk niet geschikt zijn, omdat de poriën van deze matrix te klein zijn voor de penetratie van dergelijke grote deeltjes. Andere immobilisatieprocedures voor de piëzo-elektrische kristallen zullen worden onderzocht.

Contents

1 Introduction

1.1 General

Within the framework of the project 'Acoustic microsensors' (A93KL448) the possibilities of a biosensor system based on an immunological reaction on a piezoelectric crystal sensor using bulk acoustic waves (BAW) are investigated at the TNO Prins Maurits Laboratory (TNO-PML) [1]. These studies are carried out in close collaboration with other NATO nations in the so-called Project Group 33 (PG33) sponsored by the NATO Army Armament Group (NAAG). The aim of PG33 is the development of a biosensor system for the real-time monitoring of BW agents and related substances. Staphylococcal Enterotoxin B (SEB) is one of the toxins produced by the bacterium *Staphylococcus aureus*. SEB is a polypeptide consisting of 239 amino acids and has a molecular weight of around 28,000 Dalton. The substance is known to cause food poisoning and is within the definition of biological warfare [2]. SEB was selected by PG33 as a test substance for comparing the various biosensor systems under development by the participating nations (Canada, France, Germany, the Netherlands, United Kingdom and United States). In a previous report the detection of SEB was described employing a piezoelectric crystal immunosensor in a flow injection system [3]. The detection was based on a competition scheme: polyclonal anti-SEB antibodies were mixed with SEB in solution after which the remaining antibodies, which did not react with SEB, were allowed to react with SEB on the sensor surface. The detection limit was 0.1 µg/ml where the inhibition of the sensor response was approximately 10% of the maximum response. Much improvement had been made by the application of a flow injection system, mainly due to the elimination of unspecific effects. However, since the detection requires a pre-incubation period of 20 minutes it could not be considered to be a real time assay. As it was stated in the previous report [3], the direct detection of SEB and other antigens by surface bound antibodies remains our goal.

Many procedures for the immobilisation of antibodies on glass and metal surfaces are described in the literature. A very promising method seemed to be the hydrogel matrix as was described by Löfas and Johnson [4]. This technique is incorporated in the Biacore, a tradename for the biosensor from Pharmacia, Sweden. The hydrogel method is introduced in more detail in Section 1.2.

On the basis of Pharmacia's publication it was decided to incorporate the hydrogel matrix on our piezoelectric crystals. Subsequently, these crystals were used for the direct detection of SEB in a flow injection system.

1.2 The hydrogel matrix as biochemical interface

The hydrogel matrix was developed for the surface plasmon resonance (SPR) sensor. This optical sensor is comparable to the piezoelectric crystal sensor with respect to the monitoring of immunoreactions. By immobilizing one component of a biospecific pair (antibody-antigen), the technique can be used for detection and measurement of the concentration of the counterpart, by monitoring the change in optical properties during binding and dissociation on a surface. In the SPR technique, one component of the biospecific pair is immobilised on a glass plate which is coated with a thin layer of evaporated gold. The immobilization must therefore be carried out on a gold surface, the same as for the piezoelectric crystal.
The first step in the construction of the matrix is the formation of a so-called self assembling monolayer (SAM) of 1-mercapto-16-hexadecanol [5]. This compound forms an extremely hydrophilic layer because adsorption takes place with the thio-terminus on the gold surface and the hydroxy-terminus standing away from the surface (Figure 1).

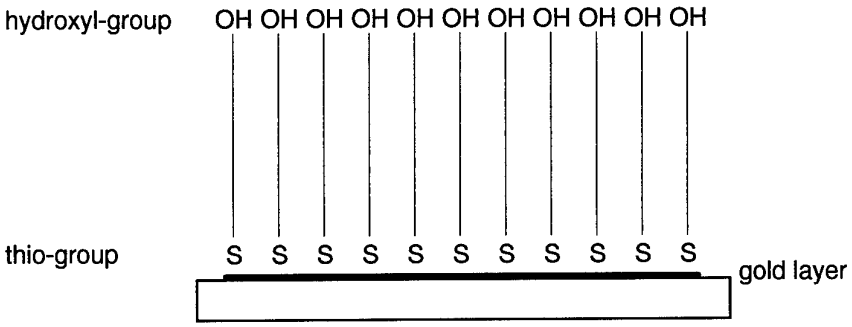

Figure 1: Schematic presentation of an ordered monolayer of 1-mercapto-16-hexadecanol on a gold surface.

This very dense layer serves mainly as a functionalized structure for further modification of the surface. In addition this layer also prevents proteins and other ligands to come into contact with the metal surface.
In the next step the hydroxyl-groups are activated with epichlorohydrin under basic conditions to form epoxides which can then be used for the covalent coupling of a dextran polymer. Further functionalization to a carboxymethyl-modified matrix can be done by reaction with bromoacetic acid. This negatively charged hydrogel-covered surface can be used for covalent coupling of a large variety of compounds, including proteins. In our case polyclonal anti-SEB antibodies were covalently bound to the matrix by carbodiimide chemistry. A schematic presentation of the total structure of the modified gold surface is given in Figure 2.

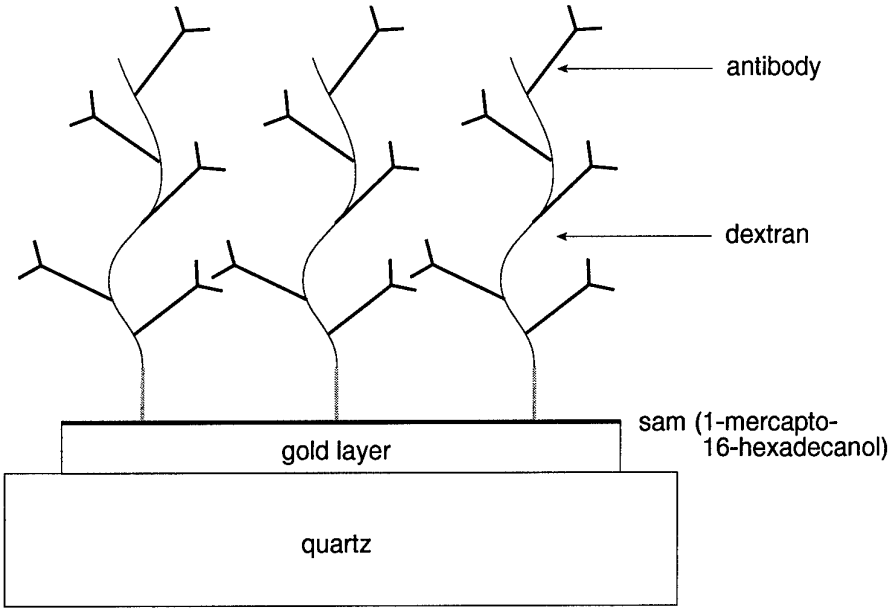

Figure 2: Schematic presentation of the modified gold surface.

The fast and efficient immobilization of the antibodies is possible by utilizing electrostatic attraction forces for the ionic preconcentration of the positively charged antibodies into a negatively charged hydrophylic matrix. The pH of the protein solution must therefore be kept below the isoelectric point of the protein. On the other hand the optimum pH for coupling the antibodies to carboxylic groups by the carbodiimide reaction is 6. The efficiency of the carbodiimide reaction decreases at lower pH.
The main features of the hydrogel matrix are:

- an increased immobilization capacity as compared to monolayer based coatings: the dextran layer is about 100 nm thick in buffer solution (depending on pH and ionic strength);
- the dextran layer gives a flexible, uncrosslinked, open structure where ligands can bind all through the matrix;
- low unspecific binding.

1.3 Working program

The working program was carried out in close collaboration with TNO Nutrition and Food Research. They perform experiments with the Biacore biosensor system for the detection of mycotoxins.
It was decided to use the Biacore biosensor system as a model to determine whether the SEB/anti-SEB system works on the hydrogel matrix. When successful on the Biacore, experiments with the piezoelectric crystal sensor could be started. The working program involved the following steps.

Biacore

1 The ionic preconcentration of the anti-SEB antibodies. These experiments were performed to determine the optimal buffer solution for the coupling of the anti-SEB antibodies.
2 Coupling of anti-SEB antibodies to the dextran matrix.
3 Reaction of SEB with anti-SEB antibodies.

Piezoelectric crystal sensor

1 The synthesis of the thiol-compound, 1-mercapto-16-hexadecanol, that serves as linker layer between the gold surface and the dextran matrix. The starting compound was commercially available 1,16-hexadecanediol.
2 Synthesis of the dextran layer on the gold surface.
3 Coupling of the anti-SEB polyclonal antibodies to the dextran matrix.
4 Reaction of SEB with anti-SEB.

2 Experimental

2.1 Ionic preconcentration of anti-SEB antibodies

Anti-SEB antibodies were dissolved to a concentration of 50 µg/ml in 10 mM acetate buffer solutions with the following values of the pH: 3.55, 3.61, 3.77, 3.98, 4.22, 4.43, 4.61, 4.82, 5.03, 5.24 and 5.46.
Of each solution 30 µl was injected subsequently with a flow rate of 10 µl/min using a flow injection system. The response on the Biacore biosensor system (Pharmacia, Sweden) in arbitrary radiation units was followed and stored on disk.

2.2 Coupling of anti-SEB antibodies to the dextran matrix on the Biacore sensor

2.2.1 Chemicals

- Acetic acid (Merck, 00063, 1000)
- EDTA (ethylene diamine tetra acetic acid) (Sigma, ED)
- Ethanolamine hydrochloride (Sigma, E-6133)
- HEPES (N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]) (Sigma, H-3375)
- N-(3-Dimethylaminopropyl) -N'-ethylcarbodiimide hydrochloride (EDC) (Fluka, 03451)
- N-Hydroxysulfosuccinimide sodium salt (Sulfo-NHS) (Fluka, 56485)
- Sodium acetate trihydrate (Baker, 0256)
- Sodium chloride (Merck, 6404.5000)
- Sodium hydroxide (Merck, 6498.5000)
- Polyclonal anti Staphylococcal enterotoxin B (TTI, LBI202)
- Staphylococcal enterotoxin B (SEB) (TTI, BT202)
- Surfactant P20 (Biacore, BR-1000-54)

2.2.2 Solutions

- EDC/NHS: 750 mg EDC and 115 mg NHS in 10 ml demineralized (demi) water.
- 0.1 M Sodium acetate buffer: 13.61 g sodium acetate trihydrate in 1 l demi water. The pH was adjusted to 4.8 with acetic acid.
- Antibody solution: 50 µg anti-SEB antibodies were dissolved in 1 ml sodium acetate buffer.
- Ethanolamine: ethanolamine was diluted 10 times with demi water and the pH was adjusted to 8.5 with hydrochloric acid.

- HBS (Hepes Buffered Saline) buffer: 0.01 M HEPES pH 7.4; 0.15 M NaCl; 3 mM EDTA; 0.005% v/v Surfactant P20.

2.2.3 Procedure

During the incubations the sensor chip was contained in a small plastic box with a wetted tissue inside. For each incubation 50 µl of liquid was pipetted on the surface of the sensor chip in the following sequence:

- incubation of the sensor chip (gold surface with dextran layer) in EDC/NHS solution for 30 min at room temperature (RT);
- washing with 0.1 M sodium acetate buffer;
- incubation in antibody solution for 30 min at RT;
- incubation in ethanolamine solution for 10 min at RT;
- washing with HBS buffer;
- storage in HBS buffer.

2.3 Reaction of SEB with anti-SEB antibodies on the Biacore

SEB was dissolved in HBS buffer in the following concentrations: 0.5, 0.2, 0.1, 0.05, 0.02 and 0.01 µg/ml. 50 µl of each solution was injected with a flow rate of 10 µl/min using the Biacore's flow injection system. After each injection the surface was regenerated with 10 µl GuCl (Guanidine adjusted to pH 4 with HCl).

2.4 Synthesis of 1-mercapto-16-hexadecanol

2.4.1 Chemicals

1, 16-Hexadecanediol was purchased from Aldrich, Belgium. Silica gel (type: 60), methanesulfonyl chloride, potassium thioacetate, THF (tetrahydrofuran), triethylamine, dichloromethane, methanol, ethanol and 37% hydrochloric acid were purchased from Fluka, Switzerland. Acetic acid and acetyl chloride were purchased from Merck, Germany.

2.4.2 Analysis

Synthesis products were checked by nuclear magnetic resonance (NMR) spectrometry. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian (Palo Alto, CA, USA) VXR 400S spectrometer operating at 400 MHz and 100.6 MHz, respectively. Chemical shifts are given in ppm relative to TMS.

2.4.3 Synthesis of 1-O-methanesulfonyl-16-hexadecanol

1-O-methanesulfonyl-16-hexadecanol was prepared as described by Frisch et al. [6] with a few modifications. Briefly, a solution of 1,16-hexadecanediol (21 mmol, 5.5 g) in a mixture of THF (200 mL) and triethylamine (21 mmol, 2.95 mL) was added dropwise to a solution of methanesulfonyl chloride (21 mmol, 2.4 g) in THF

(10 mL). After stirring for 60 h, the precipitate was removed by filtration and the filtrate was concentrated under reduced pressure and the residue redissolved in dichloromethane (10 mL). Thin Layer Chromatographic (TLC) analysis (1% methanol in dichloromethane) showed three spots: the doubly mesylated compound with Rf 0.68, the desired monomesylated compound with Rf 0.20 and the remaining starting material with Rf 0.00. The monomesylated compound was isolated by silica gel column chromatography applying a gradient from dichloromethane to 3% methanol in dichloromethane. 1-O-methanesulfonyl-16-hexadecanol was obtained as a white solid (1.6 g, 4.7 mmol; 22%).
^{1}H-NMR ($CDCl_3$): 4.22 (t, 2H, CH_2OS), 3.65 (m, 3H, CH_2OH + OH), 3.00 (s, 3H, CH_3), 2.0 - 1.0 (m, 28H, 14x CH_2).
^{13}C-NMR ($CDCl_3$): 70.2 (CH_2OS), 63.2 (CH_2OH), 40 - 20 (CH_3 + 14x CH_2).

2.4.4 Synthesis of S-acetyl-1-mercapto-16-hexadecanol

A solution of potassium thioacetate (7 mmol, 800 mg) in ethanol (25 mL) at 75 °C was added dropwise to a stirred solution of 1-O-methanesulfonyl-16-hexadecanol (1.6 g) in ethanol (25 ml), according to a method described by Sjöberg [7]. After 1 h, TLC analysis (1% methanol/dichloromethane) showed the conversion of the starting compound into a higher running compound. The reaction mixture was quenched by the addition of acetic acid (0.2 mL). After filtration of the reaction mixture and concentration of the filtrate, the residue was dissolved in dichloromethane (5 mL) and purified by silica gel column chromatography, applying a gradient from dichloromethane to 3% methanol in dichloromethane. S-acetyl-1-mercapto-16-hexadecanol was obtained as a white solid (4.2 mmol, 1.3 g; 89%).

2.4.5 Synthesis of 1-mercapto-16-hexadecanol

S-acetyl-1-mercapto-16-hexadecanol (4.2 mmol, 1.3 g) was added to a solution of 1% HCl in methanol, which was prepared by addition of acetyl chloride (2.1 mL) to methanol (100 mL) at 0 °C. The mixture was heated for 1 h at 60 °C. TLC analysis (1% methanol/dichloromethane) showed the conversion of the starting compound into a higher running compound. The reaction mixture was quenched with triethylamine (2 mL) and concentrated. After purification by silica gel column chromatography (eluent: dichloromethane) a white solid (3.7 mmol, 1 g; 88%) was obtained, purity: > 95%.
^{1}H NMR ($CDCl_3$): 3.63 (t, 2H, CH_2O), 2.53 (m, 2H, CH_2S), 1.7 - 1.0 (m, 28H, 14x CH_2). ^{13}C NMR ($CDCl_3$): 63.1 (CH_2O), 34.1 - 26.0 (m, 14x CH_2), 24.7 (CH_2S).

2.5 Synthesis of the dextran layer on the gold surface of the piezoelectric crystal

2.5.1 Chemicals

- Bromoacetic acid (Sigma, B-7130)
- Dextran T500 (Pharmacia Biotech, 17-0320-01)
- Diglyme (Sigma, M-8132)
- EDTA (Sigma, ED)
- Epichlorohydrin (Sigma, E-4255)
- HEPES (Sigma, H-3375)
- Sodium chloride (Merck, 6404.5000)
- Sodium hydroxide (Merck, 6498.5000)
- Perhydrol 30% (Merck, 07209.1000)
- Surfactant P20 (Biacore, BR-1000-54)
- Sulfuric acid (96%) (Merck, 00731.1000)

2.5.2 Solutions

- Piranha: Sulfuric acid/Perhydrol = 1:1;
- 0.005 M 1-mercapto-16-hexadecanol: 0.14 g was dissolved in 80 ml ethanol followed by addition of 20 ml demi water;
- Epichlorohydrin: 200 µl of the compound in 4 ml 1:1 0.4 M NaOH and diglyme;
- Dextran: 3 g dextran T500 in 10 ml demi water;
- Bromoacetic acid: 3.5 g in 25 ml 2 M NaOH;
- HBS buffer: 0.01 M HEPES pH 7.4; 0.15 M NaCl; 3 mM EDTA; 0.005% v/v Surfactant P20.

2.5.3 Procedure

The following sequence was employed:

- incubation of the crystal in Piranha for at least 1 hour at 80 °C;
- washing with demi water and dried in a flow of nitrogen gas;
- incubation in the solution of 1-mercapto-16-hexadecanol for 16 hours at RT;
- washing twice with ethanol and twice with demi water;
- incubation in epichlorohydrin solution for 4 hours at RT;
- washing thoroughly with demi water, ethanol and demi water;
- incubation in the dextran solution for 20 hours at RT;
- washing thoroughly with demi water, ethanol and demi water;
- incubation in bromoacetic acid solution for 16 hours at RT;
- washing thoroughly with demi water, ethanol and demi water;
- storing the crystal in HBS buffer.

2.6 Coupling of anti-SEB polyclonal antibodies to the dextran matrix on the piezoelectric crystal sensor

The solutions and procedure were the same as described in Paragraph 2.2 for the Biacore system with the following alterations: the piezoelectric crystals were placed in a conical reaction vial of 10 ml (Pierce cat. no. 13225). For each incubation 750 µl of the reagent solution was added to the vial. Four crystals were treated simultaneously with the same solution in this manner.

2.7 Reaction of SEB with anti-SEB on the surface of the piezoelectric crystal sensor

The sensor test system is described in detail in a previous report [3]. For the experiments described in this report some of the equipment was changed. The solvent delivery system was a Vickers Medicals digital infusion pump model IP5. The syringe holder was adapted to fit a 10 ml syringe. The flow cell was connected to a ISMATIC ASIA FIA (flow injection analysis) system equipped with a sample loop of 500 µl. The flow rate was 50 µl/min.
SEB was dissolved in HBS buffer resulting in the following concentrations: 3.1, 6.25, 7.81, 10.0, 12.5, 15.63, 20.83, 31.25, 62.5, 125, 250 and 1000 ng/ml. Regeneration of the sensor surface (desorption of SEB) was accomplished by the injection of 50 µl 0.1 M HCl, a so-called 1 minute pulse of HCl. The specifity of the sensor system was checked by the injection of 1 mg/ml BSA.

3 Results and discussion

3.1 Coupling of anti-SEB antibodies on the Biacore biosensor

The effect of the pH on the amount of antibody that was sorbed into the dextran matrix by electrostatic forces was determined indirectly by measuring the response from the Biacore (Figure 3). This response, measured in arbitrary radiation units (RU), is proportional to the mass surface density of the SPR sensor surface.

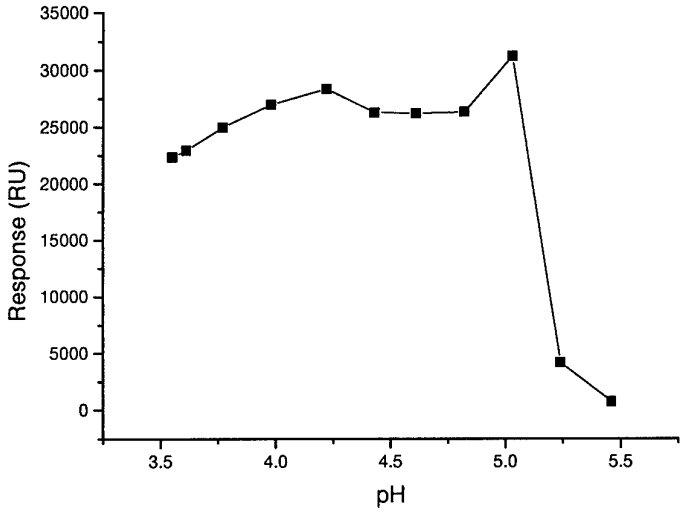

Figure 3: Effect of the pH on the amount of anti-SEB antibodies sorbed into the dextran matrix.

As may be concluded from Figure 3, the amount of sorbed antibodies decreases dramatically at pH values above 5 and is nearly constant between 3.5 and 5. To favour the carbodiimide reaction the highest possible pH has to be selected. In practice, considering the steep fall at this pH (Figure 3), a somewhat lower pH was chosen for the preparation of the buffer solution because the real value of the pH of this solution might appear to be higher. Therefore, for the coupling of anti-SEB antibodies a pH of 4.8 was selected.

3.2 Reaction of SEB with anti-SEB antibodies on the Biacore

Response curves to SEB as measured by the Biacore system are shown in Figure 4. At point 1 the sample was introduced. The duration of the sample pulse is five minutes as can be calculated from sample volume and flow rate (50 µl/10 µl.min^{-1}). This calculated value is in agreement with the experimental value. At point 2 in Figure 4 a small decrement in the response indicates the

desorption of a small amount of reversibly bound SEB by clean buffer solution and hence the end of the sample pulse, exactly five minutes after point 1. The agreement between experimental and calculated response time indicates that no mixing in the flow system occurs.
The regeneration of the sensor surface with a one minute pulse (10 µl, 10 µl.min^{-1}) of Guanidine chloride is clearly shown in Figure 4 starting at point 3. After one minute the sensor signal returns to its baseline which indicates an effective regeneration.

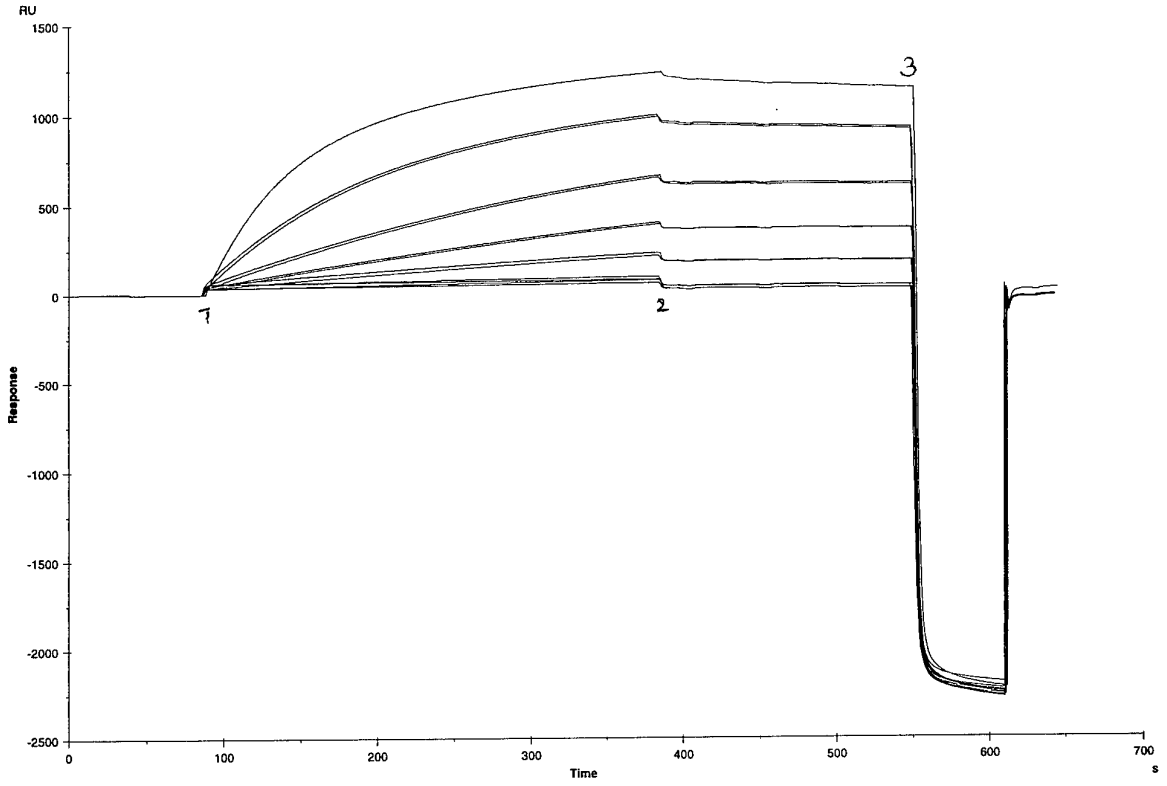

Figure 4: Response of the Biacore to a = 0.5; b = 0.2; c = 0.1; d = 0.05; e = 0.02 and f = 0.01 µg/ml SEB.

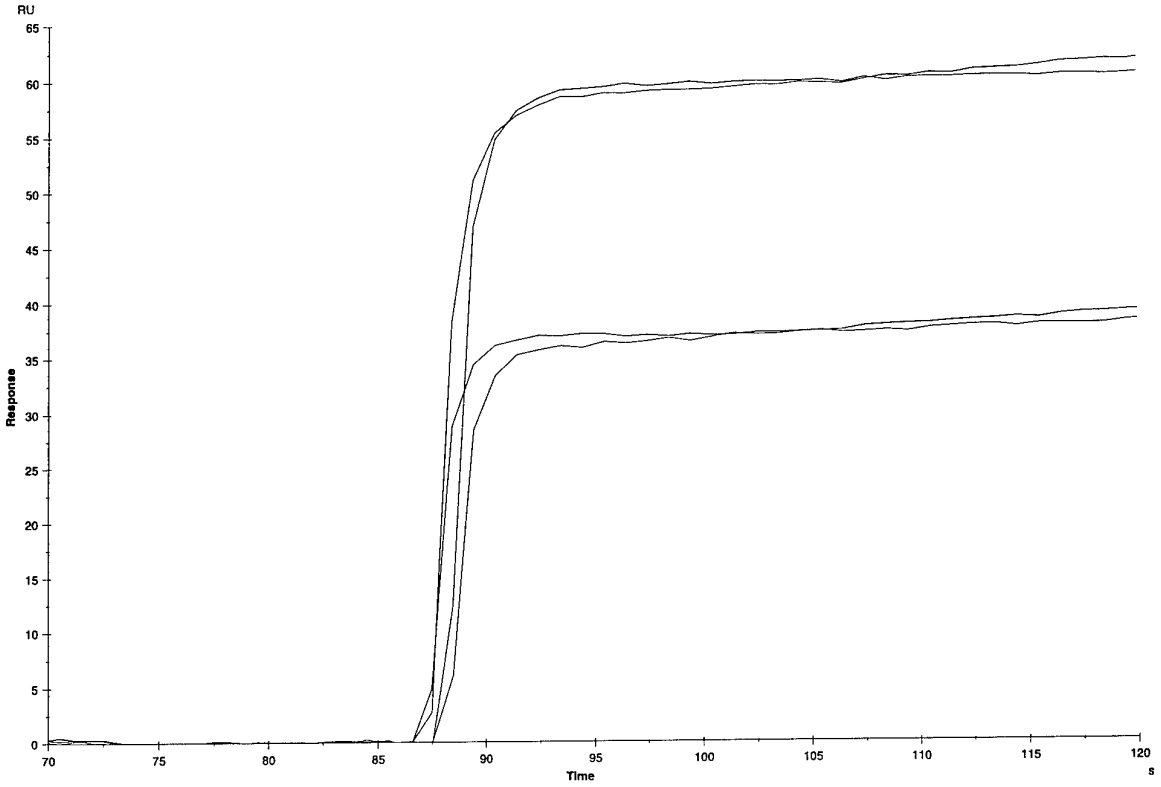

Figure 5: Response of the Biacore to 0.02 and 0.01 µg/ml SEB (upper and lower curve respectively).

To indicate the detection limit of the Biacore for this application the response curves at 0.02 and 0.01 µg/ml are amplified in Figure 5.
The signal to noise ratio is still quite large for 0.01 µg/ml, so the detection limit of the Biacore for SEB can be much lower.

3.3 Coupling of anti-SEB antibodies on the surface of the piezoelectric crystal sensor

The above described experiments with the Biacore biosensor system demonstrate that anti-SEB antibodies could successfully be incorporated in a dextran layer on a gold surface. Based on these results the same biochemical interface was built on the gold surface of the piezoelectric crystal sensor starting with the synthesis of 1-mercapto-16-hexadecanol used as a linker layer followed by the formation of the carboxylated dextran layer and the coupling of anti-SEB polyclonal antibodies.
No methods were available to examine the properties of the dextran layer on the crystal. However, after coupling of antibodies, the quality of the layer was demonstrated in the performance of the crystal as immunosensor.

3.4 Reaction of SEB with anti-SEB on the surface of the piezoelectric crystal sensor

The frequency-time curve of the piezoelectric crystal sensor for some of the measured SEB concentrations is presented in Figure 6. After regeneration with 0.1 M HCl a 1.6 ng/ml SEB solution was injected at point 2. Since no response was detected the concentration was increased to 3.1 ng/ml at point 3. However, the response at this concentration was not very significant considering the small slope of the curve. Regeneration with 0.1 M HCl at point 4 returned the baseline to its original value. Injection of a solution with a concentration of 15.6 ng/ml SEB resulted in a clear response with a total response time of 16 minutes. The experimental response time was six minutes longer than the theoretical response time, calculated from sample volume and flow rate, which indicated mixing of sample and buffer in the flow cell. This mixing can be eliminated by using a higher flow rate but then, as a consequence, the contact time between antigen and antibody decreases, the sensitivity decreases simultaneously. The injection of 1 mg/ml BSA (point 8) returned no response at all which indicates the absence of aspecific sorption of proteins. SEB solutions with other concentrations were measured in the same manner. After the binding of SEB was observed as a response, the sensor surface was always regenerated with 0.1 M HCl. The resulting calibration curve is presented in Figure 7. The sensor offers a very large dynamic range of three orders of magnitude, whereas saturation occurs at approximately 1 µg/ml.
The detection limit for SEB lies between 5 and 10 ng/ml as can be derived from the signal to drift and noise ratios from the individual response curves.

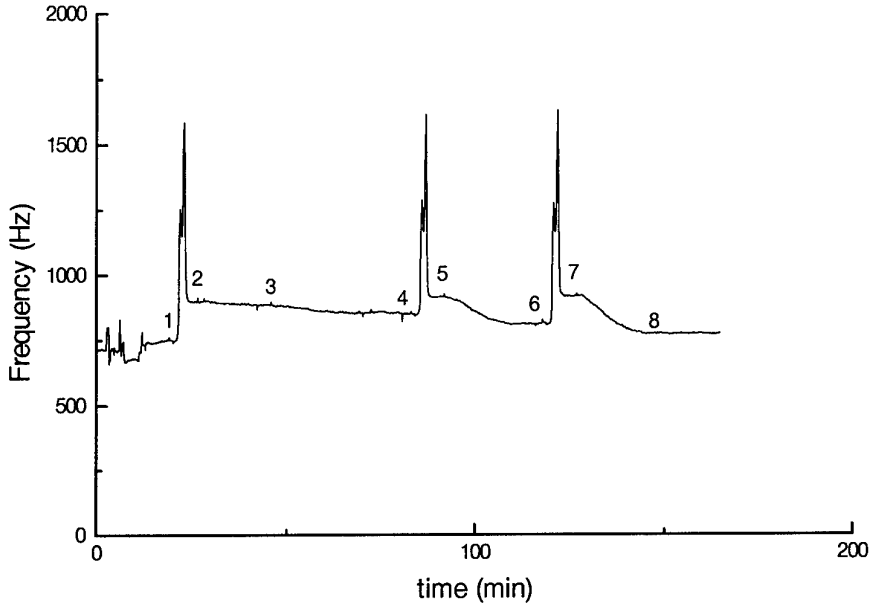

Figure 6: *Response of the piezoelectric crystal sensor to 1 = 0.1 M HCl, 2 = 1.6 ng/ml SEB, 3 = 3.1 ng/ml SEB, 4 = 0.1 M HCl, 5 = 15.6 ng/ml SEB, 6 = 0.1 M HCl, 7 = 20.8 ng/ml SEB and 8 = 1 mg/ml BSA.*

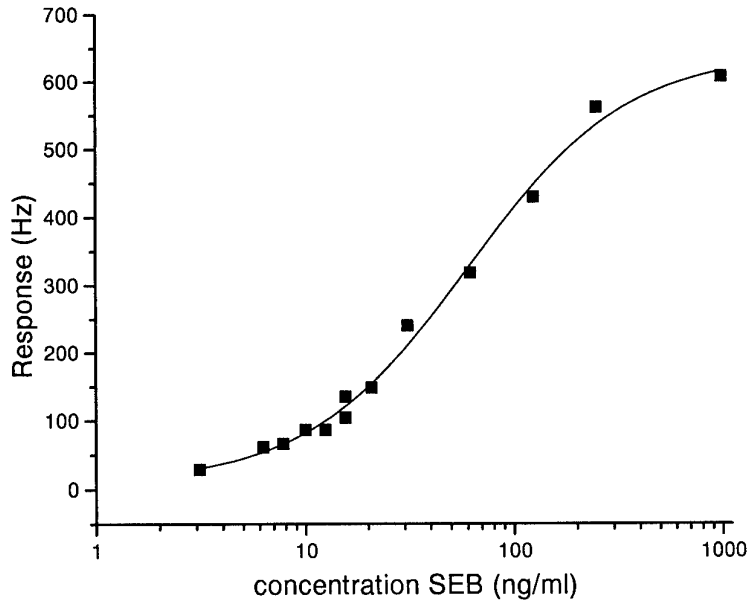

Figure 7: *Calibration curve of the piezoelectric crystal sensor for SEB.*

To investigate the reproducibility of the sensor, SEB was injected several times at a concentration of 50 ng/ml, which is the concentration around the inflection point in the calibration curve. It is clear from Figure 8 that the sensor shows a large degree of reproducibility and performance: the baseline returns to its original value after regeneration with 0.1 M HCl without any significant drift which allows for the immediate injection of the next sample. The mean response resulting from eight injections of SEB was 221 Hz with a standard deviation of 4 Hz (1.8%). This response was about 70 Hz lower than according to the calibration curve. This might be due to degeneration of the coating after three days of storage at room temperature.

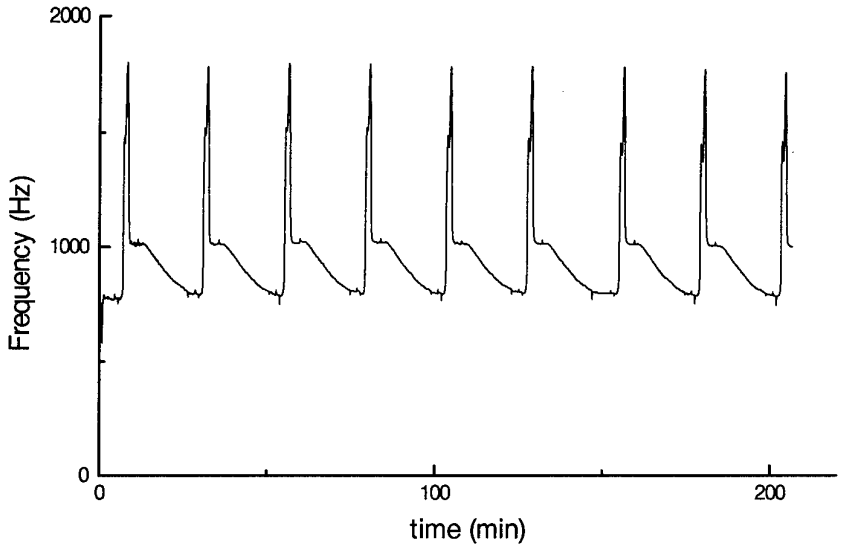

Figure 8: Eight injections of 50 ng/ml SEB on a dextran coated piezoelectric crystal sensor.

To investigate the reproducibility of the application of the biochemical interface on the piezoelectric crystal sensor, experiments with SEB in the same concentration range were performed on four crystals, all prepared as described in Paragraphs 2.5 and 2.6. The resulting calibration curves are plotted in one plot in Figure 9.

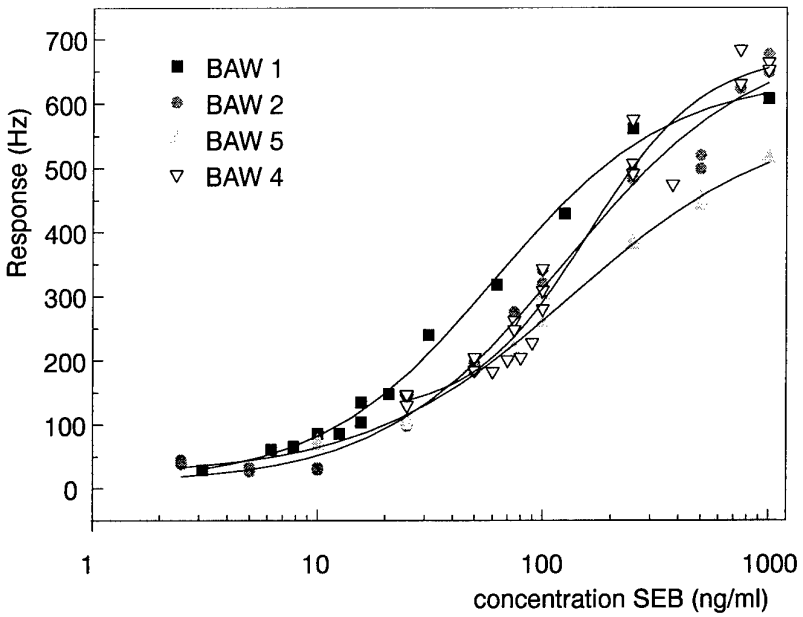

Figure 9: Calibration curves for SEB on four piezoelectric crystal sensors.

The relative deviation in the responses shown in Figure 9 ranged from 30 to 40% and is independant of the concentration of SEB. Considering the many steps involved in the preparation of the biochemical interface on the quartz crystal this result is quite acceptable.

4 Conclusions and recommendations

A piezoelectric crystal immunosensor for the direct detection of SEB has been developed. The sensor was provided with a biochemical interface based on the hydrogel matrix as described by Löfas and Johnson [4]. As compared to the competition assay described in a previous report [3] much improvement has been made. The detection of SEB does not need a preincubation step and the detection limit has been lowered from 100 to about 7 ng/ml. Another advantage of the direct over the competition assay is the saving of expensive antibodies. The crystal can be regenerated by a simple one minute injection of 0.1 M HCl, after which the response can be very well reproduced. The number of regenerations possible before the biochemical interface starts to detoriate remains to be investigated. Another item that has to be investigated is the way the coated crystals have to be stored with respect to the preservation of their immunochemical activity.
Future research will focus on the detection of larger antigens which fall within the definition of biological warfare, like viruses and bacteria. The hydrogel matrix will most probably not be suited for such large antigens because its network pores will be too small for penetration. Therefore, other antibody immobilisation procedures have to be investigated.

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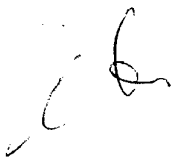
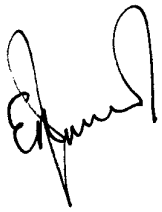
7 Authentication





J.L.N. Harteveld
Project leader/Author

E.R.J. Wils
Research Co-ordinator

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Acoustic Microsensors III. Direct detection of Staphylococcal Enterotoxin B employing a piezoelectric crystal immunosensor with a flexible carboxylated dextran matrix as the biochemical interface

11. AUTHOR(S)

J.L.N. Harteveld

12. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

TNO Prins Maurits Laboratory, P.O. Box 45, 2280 AA Rijswijk, The Netherlands
Lange Kleiweg 137, Rijswijk, The Netherlands

13. SPONSORING AGENCY NAME(S) AND ADDRESS(ES)

DMKL/OMAT/K10
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15. ABSTRACT (MAXIMUM 200 WORDS (1044 BYTE))

Within the framework of the project 'Acoustic Microsensors' (A93KL448) the possibilities of a biosensor system based on an immunological reaction on a piezoelectric crystal sensor are investigated at the TNO Prins Maurits Laboratory (TNO-PML). The results described are a continuation of previous reported work in which the toxin Staphylococcal Enterotoxin B (SEB) was detected in a competition assay with a preincubation period of at least 20 minutes and a detection limit of 100 ng/ml. A new biochemical interface based on a flexible carboxylated dextran matrix, to which antibodies are bound, was developed. With this biosensor system SEB could be detected directly without a pre-incubation step at a lowest concentration of approximately 7 ng/ml. The biosensor can be easily regenerated by a one-minute injection of 0.1 M HCl.

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