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Elaboration of a reusable immunosensor for the detection of staphylococcal enterotoxin A (SEA) in milk with a quartz crystal microbalance

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ABSTRACT

An immunosensor with quartz crystal microbalance (QCM) detection operating in flow-through mode was set up for assaying staphylococcal enterotoxin A (SEA) in model buffer medium and in milk. This biosensor is label-free and operates in the direct format. Each step of construction of the sensing layer comprising the capture anti-SEA antibody was investigated at the molecular level and optimized. The molecular layer was built using either amine- or acid-terminated thiols, with and without adding protein A to immobilize anti-SEA. The most efficient strategy to immobilize the antibody was selected on the basis of the biosensor's response to a standard solution of SEA. The optimized sensing layer was successfully used for the direct and fast (15 min) assay of SEA in phosphate buffer by QCM within a working range of 0.05–1 mg L^{-1} and a limit of detection of 0.02 mg L^{-1}. Using a sandwich type assay, the response was amplified by a factor of 2 and consequently the lowest measurable concentration dropped down to 0.007 mg L^{-1} for a total assay time of 25 min. Furthermore, sensor regeneration was achieved in good conditions with low pH buffer releasing solution. Two levels of regeneration were reached, the first one, up to the binding protein level, necessitating the rebinding of anti-SEA. In this case the response of the regenerated sensor was 73% in the first cycle then stabilized at 60% of the primary response. For the second level, the capture antibody was cross-linked to the underlayer of protein A and no further grafting was needed. In this second case, the response of the regenerated sensor was 65% of the primary response. Eventually, the piezoelectric immunosensor was successfully applied to the direct assay of SEA spiked in real milk samples.

1. Introduction

Food poisoning is caused by the consumption of foodstuff that has been contaminated at some stage of the food chain by bacterial pathogens. Among the predominant bacteria involved in these diseases, *Staphylococcus aureus* is one of the leading causes of gastroenteritis [1]. Under particular conditions, some *S. aureus* strains produce staphylococcal enterotoxins (SEs) and it is generally admitted that ingestion of 100 ng of toxin may be sufficient to cause intoxication symptoms [2]. Twenty-one different SEs have been identified to date [3] with staphylococcal enterotoxin A (SEA) being the most commonly encountered toxin in food poisoning outbreaks by *S. aureus* [4]. SEA is a small monomeric protein (28 kDa) with high thermal and proteolytic stability [5]. The detection of SEs in food matrices (milk and cheese) is a rather difficult task since it is usually present at very low concentration and the matrix contains many other potentially interfering protein species that may lead to false results. Immunoanalytical kits are available from various companies to assay SEs in foodstuffs [6]. These immunological assays, of the ELISA type, though sensitive, take at least 3 h and 1/2 for completion (excluding sample extraction) because of their multi-step procedure [7].

Within the last ten years, a significant number of papers have been published on the set up of label-free immunosensors for the detection of food pathogens and toxins [8]. Compared to ELISA-type assays, these analytical devices provide a quantitative response in a much shorter time. As regards SEs, label-free immunosensors operating with optical [4,9–13], acoustic [14–16] or electrical [17] transduction modes have been reported with variable working range and sensitivity. Let us note however that most papers dealt with the assay of staphylococcal enterotoxin B (SEB) since this toxin is considered as a potential biological weapon. The

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immunosensing devices designed for the direct detection of SEA allowed its quantification down to 0.1 mg L^{-1} [9,12].

The quartz crystal microbalance (QCM), which mass measurement principle is based on the piezoelectric effect, has enjoyed noticeable successes in the field of biosensors [18]. Indeed this technique enables quantitative studies of biochemical associations at the solid–liquid interface and in real-time without the need of an external label as the frequency of oscillation of the quartz crystal is at first approximation proportional to mass uptake at the ng level [19]. QCM sensitivity can be further increased by using high-frequency electrodeless quartz crystal sensors [20]. In addition the QCM can provide useful information on the energy dissipating properties of thin films deposited at the surface of the quartz crystal [21].

In previous papers, we highlighted the importance of controlling each step of assembling of the sensing layer at the molecular level so as to build up an efficient biosensor [22,23]. The feasibility of detection of SEA with a direct, label-free immunosensor using a quartz crystal microbalance with dissipation measurement (QCM-D) was recently demonstrated [24]. In this work, the elaboration and optimization of the sensing layer were investigated in more detail, stressing on the antibody immobilization strategy on the gold transducer. The optimized immunosensing platform was applied to the direct assay of SEA within a working range of 0.05–1 mg L^{-1} in buffered medium. Furthermore, sensor regeneration was achieved by applying a low pH releasing solution. Eventually, the optimized biosensor was successfully applied to the detection of SEA in spiked milk samples.

2. Materials and methods

2.1. Reagents

11-Mercaptoundecanoic acid (MUA), cysteamine (CEA), 1,4-phenylene diisothiocyanate (PDITC), dimethyl pimelimidate·2HCl, *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate (TSTU), diisopropylethylamine (DIPEA) and staphylococcal enterotoxin A were purchased from Sigma–Aldrich. A stock solution (97 mg L^{-1}) of SEA was prepared in water, aliquoted in 50 μL fractions and stored at −30 °C. The actual concentration of SEA was determined by UV measurement taking ε_{280nm} = 38,000 M^{-1} cm^{-1}. Recombinant protein A was purchased from Pierce Chemicals. Affinity-purified rabbit anti-SEA antibody (anti-SEA_{TT}, ref. LAI101) was purchased from Toxin Technology (Sarasota, FL, www.toxintechnology.com). Monoclonal mouse anti-SEA antibody (anti-SEA_{MLS}, ref. C86205M) was purchased from Meridian Life Science (meridianlifescience.com/). Phosphate buffered-saline PBS pH 7.4 (0.71 g $Na_2HPO_4·2H_2O$, 0.17 g KH_2PO_4, 4 g NaCl and 0.1 KCl in 0.5 L water) was used as running buffer.

2.2. Apparatus

Piezoelectric measurements were performed on AT-cut gold-coated quartz crystals with nominal frequency *F* of 5 MHz (Lot-Oriel, France) in a flow-through mode with a quartz crystal microbalance with dissipation monitoring QCM-D (E1 model, Q-sense AB, Sweden) at 22 ± 0.1 °C. The experimental setup is described in Ref. [25]. Solutions were flowed at 50 μL min^{-1} over the sensing area with a peristaltic pump. Mass uptakes Δm were calculated with the Sauerbrey equation (1) using the frequency at the fifth harmonics assuming the deposited films behave as an elastic mass [26].

$$\Delta F = -N \times \frac{\Delta m}{C_f} \quad (1)$$

where C_f (=17.7 ng cm^{-2} Hz^{-1}) is the mass sensitivity factor at *F* = 5 MHz and *N* (=1, 3, 5, 7, . . .) the overtone number.

2.3. Functionalization of the QCM sensor chips

The QCM electrodes were dipped in a freshly prepared solution of cysteamine (10 mM in water), or MUA (1 mM in EtOH) for 18–24 h. After washing with the same solvent, the cysteamine-treated electrodes were dipped in a solution of PDITC (200 mg L^{-1} in pyridine/DMF 1:9) for 30 min and the MUA-treated electrodes were dipped into a 50 mM solution of TSTU and DIPEA in DMF for 30 min so as to convert the surface carboxylic acid or amine functions into amine-reactive *N*-succinimidyl ester or isothiocyanate functions, respectively. The sensors were extensively washed in absolute ethanol and dried under a flow of argon [27]. Activated electrodes were mounted in the QCM and protein A (20 mg L^{-1}) was flowed for 5 min. Anti-SEA_{TT} (10 mg L^{-1}) was then applied for 10 min. Alternatively, the protein A step was omitted and anti-SEA_{TT} (10 mg L^{-1}) was flowed over the activated electrodes for 10 min. For the sensor regeneration experiment, a 20 mM solution of dimethyl pimelimidate in TEA buffer pH 8.3 was flowed for 13 min. The sensor was washed for 5 min with TEA buffer after which reactive imidoester functions were deactivated with 1 M ethanolamine. HCl pH 8.5 for 27 min. Non-specific binding was finally blocked with flowing PBS containing 1000 mg L^{-1} BSA (PBS–BSA) for 10 min.

2.4. Detection of SEA in the direct format

Solutions of SEA at 0, 0.0097, 0.0485, 0.194, 0.485, 0.97 and 1.94 mg L^{-1} were prepared in PBS–BSA from the aqueous stock solution. These solutions were flowed for 10 min and the sensors were flushed with PBS–BSA for 5 min. The variation of frequency between the beginning of injection and the end of the washing step was measured on each sensorgram. Alternatively, the slope of the frequency shift was determined mathematically on the linear portion of the trace.

2.5. Detection of SEA in the amplified sandwich assay format

A solution of anti-SEA_{MLS} (15 mg L^{-1} in PBS–BSA) was flowed for 5–8 min, followed by a washing step with PBS–BSA for 5 min. The variation of frequency between the beginning of injection of SEA and the end of the washing step was measured on each sensorgram.

2.6. Sensor regeneration

After the antigen capture step, 100 mM glycine·HCl buffer pH 2.5 was flowed for 10 min (until a steady baseline was reached).

2.7. Detection of SEA in milk

Milk was freshly prepared as 5% w/v skimmed milk powder in water. SEA was spiked in milk at 0.097, 0.194, 0.485, and 0.97 mg L^{-1}. After the anti-SEA antibody immobilization step, unspiked skimmed milk was flown over the sensors for 5–8 min, followed by injection of the spiked milk samples for 10 min. The sensors were flushed with running buffer until a stable baseline was reached.

3. Results and discussion

3.1. Design of the sensing layer

The construction of an immunosensor for the detection of SEA operating in the direct format requires the immobilization of the