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Antibody immobilization strategy for the development of a capacitive immunosensor detecting zearalenone

Astrid Foubert[a,*], Natalia V. Beloglazova[a], Martin Hedström[b,c], Sarah De Saeger[a]

[a] Ghent University, Faculty of Pharmaceutical Sciences, Laboratory of Food Analysis, Ottergemsesteenweg 460, Ghent, Belgium
[b] CapSenze HB, Medicon Village, Scheelevägen 22, Lund, Sweden
[c] Lund University, Department of Biotechnology, Naturvetarvägen 14 / Sölvegatan 39 A, Lund, Sweden

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ABSTRACT

A highly sensitive flow-injection capacitive immunosensor was developed for detection of the mycotoxin zearalenone (ZEN). Different strategies for immobilization of an anti-ZEN antibody on the surface of a gold electrode, i.e. polytyramine or self-assembled monolayers (SAMs) of 3-mercaptopropionic acid (3-MPA) and lipoic acid (LA), were used and their performances were compared. The LA- and 3-MPA-based systems showed broad linear ranges for ZEN determination, i.e. from 0.010 nM to 10 nM and from 0.020 nM to 10 nM, respectively. Under optimal conditions, the LA-based immunosensor was capable of performing up till 13 regeneration-interaction cycles (with use of glycine HCl, pH 2.4) with a limit of detection (LOD) of 0.0060 nM, equivalent to 1.9 pg mL^{-1}. It also demonstrated a good inter-assay precision (RSD < 10%). However, the tyramine-based capacitive immunosensor showed a bad repeatability (only 4 regeneration-interaction cycles were possible) and inter-assay precision (RSD > 15%) which did not allow sensitive and precise measurements. The LA-based method was compared with a direct ELISA. These results demonstrated that the label-free developed capacitive immunosensor had a better sensitivity and shorter analysis time in comparison with the direct microwell-plate format.

1. Introduction

Zearalenone (ZEN), a phenolic resorcylic acid lactone, is a widespread mycotoxin mainly found in maize, wheat, barley, sorghum, rye and other grains [1,2]. ZEN is of major interest as it has proven to be hepatotoxic, immunotoxic, and carcinogenic for a number of mammalian species [3]. Moreover, ZEN and some of its metabolites i.e. α- and β-zearalenol (α- and β-ZEL), zearalanone (ZAN), and α- and β-zearalanol (α-ZAL and β-ZAL) can competitively bind to estrogen receptors, and therefore cause hyper-estrogenism and infertility in livestock on long-term [1]. To protect public health and livestock production, the European Commission has established maximum limits and recommendations for ZEN in feed and foods [4,5]. The toxic nature of ZEN makes its detection an absolute necessity. Several methods have been developed and among them (multi-analyte) LC-MS (/MS) [6,7] methods are still the most popular due to high accuracy and reliability. Despite all undeniable advantages, these methods require use of expensive equipment and an intense and time-consuming sample pretreatment. The existence of specific recognition elements like monoclonal antibodies, aptamers, etc. has led to the development of rapid, selective and sensitive screening tests. Several immunoassays for the determination of ZEN have been published, which allowed apparatus-based detection [8,9] and visual detection [10]. Electrochemical methods for the determination of ZEN (and other mycotoxins [11]) have been published as well [12–14] and they can be considered as a good alternative to chromatographic and plate- and membrane-based immunoassays [15]. In general, electrochemical methods possess the advantage of being simple, sensitive, selective, low cost, and can be in some cases miniaturized and integrated in automated devices [16–19]. Most of the electrochemical methods reported for ZEN detection are based on measurement of a current (amperometric) which was generated after interaction of ZEN (or its derivatives) and a specific recognition element (antibody [12,20,21] or dsDNA [13]). In this manuscript a capacitive biosensor, which belongs to a sub-category of impedance biosensors, was used [22]. It is known that impedance is an effective tool to exhibit the electron-transfer resistance feature of a surface-modified electrode. However, recording a full impedance spectrum within a wide range of frequencies is time consuming and data processing is complex [23], which is not the case for the capacitive biosensor. This capacitive system has already been successfully applied

* Corresponding author.
E-mail address: astrid.foubert@ugent.be (A. Foubert).

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for the detection of various analytes [24–29] with high sensitivity, selectivity, and with use of low sample volumes.

One of the most important challenges in the development of an electrochemical immunosensor is the immobilization of recognition elements on the surface of an electrode. For capacitive measurements gold electrodes are most often used. In this case a recognition element can be directly immobilized on the gold electrode by introducing a thiol group in the structure (e.g. SH-modified aptamers [30–33], DNA-probe [34–36], thiolated oligonucleotide [37]). Other strategies like electrodeposition (chitosan-gold nanoparticles [38], electrochemical reduction (4-carboxyphenyl [39], 4-nitrophenyl [40], graphene oxide [41]) and passive adsorption (OTA-BSA [42]) have also been used to functionalize a gold electrode. Until now, the most widely used technique for immobilization of antibodies onto a gold surface is based on the use of self-assembled monolayers (SAMs) (e.g. cysteamine [43–47], 4-mercaptobenzoic acid [48,49], 11-amino-1-undecanethiol [50,51], and a combination of mercaptoundecanoic acid/mercaptoethanol [52]). More specifically for gold electrodes that are applied in capacitive sensors lipoic acid (LA) [53,54], tyramine and thiourea [53] were used for immobilization of recognition elements.

To the best of our knowledge no reports have focused on the practical comparison of different immobilization strategies for the development of an antibody-based capacitive biosensor. In addition, research focused on the detection of ZEN by a capacitive immunosensor has not been published. In the present manuscript an anti-ZEN antibody was immobilized on a gold electrode by use of tyramine or SAMs (3-mercaptopropionic acid (3-MPA) or LA), and the performance characteristics were determined for each system followed by their comparison.

2. Materials and methods

2.1. Reagents

The mycotoxins ZEN and deoxynivalenol (DON) were purchased from Fermentek (Jerusalem, Israel). T2-toxin, phosphate buffered saline (PBS, pH 7.4), potassium dihydrogen phosphate, potassium phosphate monobasic, aceton, ethanol, sulfuric acid, tyramine, 3-mercaptopropionic acid (3-MPA), lipoic acid (LA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), 1-dodecanethiol, potassium hexacyanoferrate(III), potassium chloride, glycine, hydrochloric acid, Tween 20, bovine serum albumin (BSA), casein, carbonate buffered saline (CBS, 0.05 M, pH 9.6) capsules were purchased from Sigma Aldrich (Diegem, Belgium). Water was purified using a Milli-Q Gradient System (Millipore, Brussels, Belgium). Colorburst™ Blue 3, 3′, 5, 5′-tetramethylbenzidine (TMB) substrate solution containing hydrogen peroxide was supplied by Alercheck (Springvale, ME, USA). Rabbit anti-mouse immunoglobulin (IgG, protein concentration 2.1 g L^{-1}) was purchased from DakoCytomation (Heverlee, Belgium).

Monoclonal anti-ZEN antibody (ZEN mAb; clone number 2D8) was prepared in our laboratory. Cross-reactivity of the ZEN mAb was 69% with α-zearalenol, 42% with α-zearalanol, 22% with zearalanone and none at all (< 1%) with β-zearalenol and β-zearalanol [55]. Due to stability reasons the mycotoxins and ZEN mAb were stored at −20 °C. Stock solutions of ZEN mAb were prepared in PBS-1% glycerol. ZEN-horseradish peroxidase (HRP) was synthesized by our group, and the protocol is presented in the Supplementary material. Maxisorp polystyrene 96-well plates were purchased from Nunc (Roskilde, Denmark). Absorbance was measured by use of the Biorad 550 microplate reader (Temse, Belgium) and the Safire 2 spectrophotometer from Tecan (Mechelen, Belgium). Sputtered gold electrodes were provided by CapSenze HB (Lund, Sweden). The capacitance measurements were performed by use of an automated flow injection system developed by CapSenze HB (Lund, Sweden). Cyclic voltammetry (CV) was performed using an Autolab PGSTAT101 potentiostat/galvanostat (Eco Chemie, The Netherlands) coupled to an electrochemical cell which contained the modified working electrode, an Ag/AgCl reference electrode, and a platinum auxiliary electrode in a permeating redox system (10 mM $K_3[Fe(CN)_6]$ in 0.1 M KCl).

2.2. Functionalization of a gold electrode with antibody

Cleaning of the electrodes was performed prior to any functionalization procedure. The electrodes were cleaned by submerging them and sonicating for 10 min in acetone, ethanol, and Piranha solution (H_2O_2/H_2SO_4, 1/3, v/v), respectively. After each cleaning step the electrodes were rinsed with ample amounts of distilled water and ethanol, and dry-blown with O_2.

2.2.1. Surface modification by use of tyramine

Tyramine was dissolved in 2.5 mL of ethanol and then 7.5 mL of phosphate buffer was added (10 mM KH_2PO_4/K_2HPO_4 in ultrapure water, pH = 7.2), resulting in a 10 mM tyramine solution. A clean electrode was placed in a reaction cell and covered with 300 µL of 10 mM tyramine solution. Electropolymerization was conducted by performing 15 cycles of voltammetric sweeping between 0 V and 1.5 V, at 0.05 V s^{-1}. Afterwards, this electrode was rinsed with ethanol and blown dry with O_2. The tyramine layer introduced amine functional groups on the gold surface. These groups were activated with a 5% glutaraldehyde solution in PBS for 20 min, followed by an overnight incubation of the electrode with 25 µL ZEN mAb (1 mg mL^{-1}) at 4 °C. The electrode was cleaned with ultrapure water and PBS, and blown dry with O_2. The electrode was reacted with 10 mM 1-dodecanethiol ethanol solution for 20 min to block any pinholes on the electrode surface. Next, the electrode was treated with ethanolamine (0.1 M, pH 8) for 20 min; this step was to block all aldehyde groups that did not bind with the antibody. Next, the electrode was washed with ultrapure water and PBS and stored under N_2.

2.2.2. Formation of the self-assembled monolayers on the surface of a gold electrode

A clean gold electrode was immersed in a 250 mM 3-MPA solution for 12 h (the optimal immersion time) at room temperature. The electrode was thoroughly rinsed with distilled water and dry-blown with O_2. Next, the carboxylic groups were activated for an amine reaction by using EDC (0.05 M) and NHS (0.03 M) in dry acetonitrile for 5 h, followed by rinsing the electrode with PBS and drying with O_2. The electrode was further functionalized by placing 25 µL of ZEN mAb (1 mg mL^{-1}) on the surface, and reaction took place overnight at 4 °C.

Finally, the electrode was reacted with a 10 mM 1-dodecanethiol ethanolic solution for 20 min to block all bare spots on the electrode surface.

For the immobilization with LA, the clean electrode was immersed overnight in an LA solution (2% w/w in absolute ethanol). The next functionalization steps were the same as for the functionalization using 3-MPA. The electrochemical behavior and degree of insulation of the electrodes was examined after each modification step by use of CV.

2.3. Capacitance measurements

All capacitance measurements were performed in a flow-cell with a dead volume of 10 µL. The measurements were performed using the current pulse technique with an automated flow-injection system. The detailed scheme of the used sensor can be found in the article of Erlandsson et al. (2014) [23].

Prior to analysis, a regeneration solution (25 mM glycine-HCl, pH 2.4) was injected to clean the surface of the electrode. After a stable baseline was obtained, the standard solutions of different ZEN concentrations (250 µL) were injected at a flow rate of 50 µL min^{-1}, and in between the injections regeneration solution was injected. Binding between ZEN mAb and ZEN resulted in a decrease of capacitance. In