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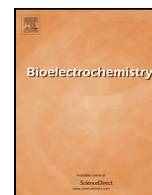

Label-free ITO-based immunosensor for the detection of very low concentrations of pathogenic bacteria

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ABSTRACT

Here we describe the fabrication of a highly sensitive and label-free ITO-based impedimetric immunosensor for the detection of pathogenic bacteria *Escherichia coli* O157:H7. Anti-*E. coli* antibodies were immobilized onto ITO electrodes using a simple, robust and direct methodology. First, the covalent attachment of epoxysilane on the ITO surface was demonstrated by Atomic Force Microscopy and cyclic voltammetry. The immobilization of antibody on the epoxysilane layer was quantified by Optical Waveguide Lightmode Spectroscopy, obtaining a mass variation of 12 ng cm^{-2} (0.08 pmol cm^{-2}). Microcontact printing and fluorescence microscopy were used to demonstrate the specific binding of *E. coli* O157:H7 to the antibody-patterned surface. We achieved a ratio of 1:500 *Salmonella typhimurium*/*E. coli* O157:H7, thus confirming the selectivity of the antibodies and efficiency of the functionalization procedure. Finally, the detection capacity of the ITO-based immunosensor was evaluated by Electrochemical Impedance Spectroscopy. A very low limit of detection was obtained (1 CFU mL^{-1}) over a large linear working range (10–10^6 CFU mL^{-1}). The specificity of the impedimetric immunosensor was also examined. Less than 20% of non-specific bacteria (*S. typhimurium* and *E. coli* K12) was observed. Our results reveal the applicability of ITO for the development of highly sensitive and selective impedimetric immunosensors.

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1. Introduction

The rapid and specific detection of pathogenic bacteria is crucial to ensure food safety and to safeguard public health. The pathogenic bacteria *Escherichia coli* O157:H7 is considered as one of the most dangerous foodborne pathogens. This bacterium can lead to hemorrhagic colitis, hemolytic-uremic syndrome, and other similar life-threatening complications [1,2]. The Economic Research Service (ERS) estimates the occurrence of about 73,480 cases of *E. coli* O157:H7 infection and 61 deaths each year in United States, with an associated annual cost of $488,771,183 [3]. Given the high specificity and sensitivity of biosensors and their capacity to deliver fast results [4,5], in the last decade these devices have emerged as an interesting alternative to traditional methods for the detection of pathogenic bacteria.

Impedance spectroscopy has been used to characterize the structure and functionality of various biosensors, including immunosensors, DNA sensors, and biocatalytic enzyme-based biosensors [6,7]. Impedimetric immunosensors have received particular attention in recent years, since they can perform direct and label-free measurements and have the potential to be used as multi-analyte sensors [8]. In addition, they are rapid detection devices that can be easily manipulated by unskilled personnel. Moreover, immunosensors have been widely used for pathogen detection due to the high sensitivity and specificity of antibodies [9–11].

Here we developed an impedimetric immunosensor based on indium tin oxide (ITO) surfaces for the detection of pathogenic bacteria *E. coli* O157:H7. ITO is considered to be a highly promising material because of its excellent optical transparency, high electrical conductivity, wide electrochemical working window, outstanding substrate adhesion, and stable electrochemical and physical properties [12]. All these properties make the ITO a highly appropriate material to develop a device for the detection of pathogenic microorganisms such as *E. coli*. There are few reports of immunosensors based on ITO surfaces for the

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detection of this pathogen. Adányi et al. [13] developed a label-free immunosensor using Optical Waveguide Lightmode Spectroscopy (OWLS), obtaining a limit of detection (LOD) of 3×10^4 CFU mL^{-1} (colony forming unit mL^{-1}) over a linear response between 3×10^4 and 3×10^7 CFU mL^{-1}. Ruan et al. [14] described a label-dependent immunosensor based on Electrochemical Impedance Spectroscopy (EIS), in which alkaline phosphatase-labeled secondary antibodies were used to amplify the signal by catalyzing the formation of an insoluble precipitate. They reported a linear response between 6×10^4 and 6×10^7 CFU mL^{-1}, with a LOD of 6×10^3 CFU mL^{-1}. Yang et al. [15] described a label-free EIS immunosensor using an interdigitated array microelectrode, reporting a higher LOD (1×10^6 CFU mL^{-1}). Nonetheless, the high virulence and the extremely low infectious dose of *E. coli* O157:H7 required to cause disease [16,17] call for the development of a more sensitive and specific immunosensor.

Here we describe a label-free immunosensor for the detection of very low concentrations of *E. coli* O157:H7. ITO substrates were deposited on glass surfaces by means of DC Magnetron Sputtering, and a direct and robust functionalization method was used to efficiently immobilize the antibodies onto the surface. The surface morphology and functionalization were studied by means of Atomic Force Microscopy (AFM), while the formation of the antibody monolayer on the ITO surface was monitored by OWLS. The specificity of the antibodies was demonstrated using microcontact printing and fluorescent microscopy. EIS was used for the label-free detection of a range of concentrations of *E. coli* O157:H7. The sensors showed a very low LOD (1 CFU mL^{-1}) over a large linear detection range (10–10^6 CFU mL^{-1}). This low LOD could be attributable to the optimized surface functionalization protocol, the physical and electrical properties of the ITO surfaces, the binding efficiency of the immobilized anti-*E. coli* O157 antibody to the bacteria, and the sensitivity of the EIS technique.

2. Experimental

2.1. Reagents and solutions

Phosphate-buffered saline (PBS), 3,3′,5,5′-tetramethylbenzidine (TMB), 3-Glycidoxypropyldimethoxymethylsilane, 2-(2-aminoethoxy) ethanol (AEE), albumin from bovine serum (BSA), ammonium hydroxide (30%), and polyoxyethylenesorbitan monolaurate (Tween 20) were purchased from Sigma Aldrich (St. Louis, Mo, USA). Sodium hydrogen carbonate, hydrogen peroxide, sulfuric acid, acetone, and absolute ethanol were supplied by Panreac (Barcelona, Spain). Potassium hexacyanoferrate(III) ($K_3Fe(CN)_6$) and potassium hexacyanoferrate(II) ($K_4Fe(CN)_6$) were from Fluka (Buchs, Switzerland).

Mouse monoclonal anti-*E. coli* O157 antibodies (primary capture antibody) and rabbit polyclonal anti-*Salmonella* horseradish peroxidase (HRP) conjugate were supplied by Abcam (Cambridge, UK). Polyclonal anti-*E. coli* HRP conjugate and polyclonal anti-*E. coli* FITC (fluorescein isothiocyanate) conjugate were purchased from Thermo Fisher Scientific (Rockford, IL, USA). Goat anti-rabbit IgG (Alexa Fluor 546) and Alexa Fluor 594 FluoroNanogold Fab' fragment of goat anti-mouse IgG were obtained from Invitrogen (Eugene, OR, USA).

A 10 mM PBS solution was used for all the experiments, coating buffer was prepared with 50 mM carbonate/bicarbonate at pH 9.6, and PBS-T buffer solution was PBS with 0.05% Tween 20.

2.2. Bacteria and culture plating methods

E. coli O157:H7 Δ*stx*, *E. coli* K12 and *Salmonella* enterica serivar Typhimurium (*S. typhimurium*) wild-type SV5015 were routinely grown in Luria–Bertani (LB) medium at 37 °C. For cell quantification, overnight cultures were pelleted by centrifugation at 5000 ×*g* for 5 min, and cells were re-suspended in a 10 ml of PBS. The concentration of viable cells (CFU mL^{-1}) was obtained by plating a range of aliquots on LB agar plates.

2.3. ITO deposition and characterization

The ITO thin films were deposited on 10×10 mm^2 glass (NTB, Switzerland) substrates by means of the DC Magnetron Sputtering system [18] from a 75 mm oxide sputtering target, In_2O_3:SnO_2 = 90:10 wt.% (acquired from Goodfellow), without intentional heating. Before film deposition, the sputtering chamber was pumped down to 3.5×10^{-5} mbar using a combinatory system of a rotary pump (Alcatel ANNECY Ty-2033, PS — 33 m^3 h^{-1}) and a diffusion pump (Edwards Vapor Pump EO4, PS — 600 L s^{-1}), which work together to maintain a high vacuum. Prior to each deposition, the ITO ceramic target was pre-sputtered for 5 min. Table A.1. (see Appendix A) shows a summary of the deposition parameters used.

The surface morphology and roughness were analyzed by a commercial Dimensional 3100 AFM (Veeco Instruments, USA). The AFM measurements were performed in air using a silicon tip (NSC15/AIBS, MikroMasch, Estonia) in tapping mode and at room temperature. AFM images were analyzed with the WSxM software (Nanotec Electronica SL) [19].

2.4. Surface cleaning and functionalization procedures

The ITO substrates were rinsed with acetone, absolute ethanol, and Milli-Q water (Millipore, USA). Afterwards, they were immersed in a solution of 1:1:5 (v/v) H_2O_2(30%)/NH_4OH(30%)/H_2O for 1 h, washed extensively with Milli-Q water, and dried under a stream of nitrogen. ITO substrates were next immersed in a 3-Glycidoxypropyldimethoxymethylsilane ethanolic solution (1%) for 20 min. Subsequently, the samples were dried and placed in an oven at 110 °C for 1 h. They were then thoroughly washed with ethanol and dried. Afterwards, the substrates were incubated with anti-*E. coli* O157 antibodies (15 μg mL^{-1} solution in PBS) for 1 h at room temperature. Finally, the substrates were washed with PBS and immersed in a 100 mM AEE in sodium bicarbonate buffer (pH 8) for 30 min, washed, and stored in a PBS solution at 4 °C.

2.5. OWLS experiments

A commercial OWLS instrument from MicroVacuum Ltd. (Budapest, Hungary) was used for optical experiments on the ITO-coated sensor chips (OW 2400c Sensor Chip, MicroVacuum, Hungary). The solutions were injected with a peristaltic pump (Ismatec, Switzerland) at a flow of 56 μL min^{-1}. A solution of anti-*E. coli* O157 antibodies (15 μg mL^{-1} in PBS) was injected and passed over the surface of epoxysilane-functionalized ITO chips. After blocking the surface with a solution of AEE, various concentrations of *E. coli* O157:H7 were injected.

2.6. Patterning of anti-*E. coli* O157 antibodies for bacteria detection

An anti-*E. coli* O157 antibody array was patterned on ITO surfaces using microcontact printing. Firstly, a poly(dimethylsiloxane) (PDMS, Sylgard 184, Dow Corning) stamp with a regular array of square posts of 10 μm and a pitch of 10 μm was replicated from a silicon master (Centre Nacional de Microelectrònica — CNM, Spain), as described elsewhere [20]. The stamp was incubated for 15 min with an anti-*E. coli* O157 solution (15 μg mL^{-1} in PBS) and dried under a nitrogen flow. Afterwards, the PDMS stamp was placed in contact with the epoxysilane-modified ITO substrate for 2 min (see Section 2.4). Non-patterned regions were blocked with 100 mM of AEE solution for 30 min. A second antibody labeled with Alexa Fluor 594 was used to confirm the patterned antibody (FluoroNanogold Fab' fragment of goat anti-mouse IgG).

The anti-*E. coli*-patterned substrate was incubated for 45 min with 400 μL of *E. coli* O157:H7 in PBS (10^8 CFU mL^{-1}). A sandwich structure was obtained by incubation with a second anti-*E. coli* polyclonal antibody labeled with a FITC fluorescent dye (0.1 mg mL^{-1}) for 1 h. To confirm the selectivity of the system, the same protocol was followed using

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