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Label-Free Detection of Bacteria by Electrochemical Impedance Spectroscopy: Comparison to Surface Plasmon Resonance

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The low but known risk of bacterial contamination has emerged as the greatest residual threat of transfusion-transmitted diseases. Label-free detection of a bacterial model, *Escherichia coli*, is performed using nonfaradic electrochemical impedance spectroscopy (EIS). Biotinylated polyclonal anti-*E. coli* is linked to a mixed self-assembled monolayer (SAM) on a gold electrode through a strong biotin−neutravidin interaction. The binding of one antibody molecule for 3.6 neutravidin molecules is determined using the surface plasmon resonance (SPR). The detection limit of *E. coli* found by SPR is 10^7 cfu/mL. After modeling the impedance Nyquist plot of *E. coli*/anti-*E. coli*/mixed SAM/gold electrode for increasing concentrations of *E. coli* (whole bacteria or lysed bacteria), the main parameter that is modified is the polarization resistance R_P. A sigmoid variation of R_P is observed when the log concentration of bacteria (whole or lysed) increases. A concentration of 10 cfu/mL whole bacteria is detected by EIS measurements while 10^3 cfu/mL is detected for lysed *E. coli*.

The detection of pathogenic bacteria remains a challenging and important issue for ensuring food safety and security, for controlling water and soil pollution, and for preventing bioterrorism and nosocomial diseases. It has been reported that 1.5 billion people around the world are infected by bacteria each year. These agents pose threats not only to the general population but also to recipients of blood transfusions.[1]

The effective testing of bacteria requires methods of analysis that meet a number of challenging criteria.[2] Analysis time and sensitivity are the most important limitations related to the usefulness of microbiological testing. An extremely selective detection methodology is also required, because low numbers of pathogenic bacteria are often present in a complex biological environment along with many other nonpathogenic organisms. Traditional methods for the detection of bacteria involve the following basic steps: preenrichment, selective enrichment, biochemical screening, and serological confirmation. Hence, a complex series of tests is required, and the results are often difficult to interpret and not available in the time scale desired in a clinical laboratory. In response to this problem, a number of instruments have been developed using various principles of detection, e.g., flow cytometry,[3,4] polymerase chain reaction,[5−8] immunomagnetic separations,[3,9] bioluminescence,[10,11] mass spectrometry, etc. However, many of these methods are still time-consuming and very expensive.

Emerging technologies are promising diagnostic tools for a wide range of clinical applications, including blood donation screening. Bacterial contamination of blood components has emerged as the greatest transfusion-associated risk of infection.

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(1) Brecher, M. E.; Hay, S. N. *Clin. Microbiol. Rev.* **2005**, *18*, 195−204.

(2) Ivnitski, D.; Abdel-Hamid, I.; Atanasov, P.; Wilkins, E. *Biosens. Bioelectron.* **1999**, *14*, 599−624.

(3) Hibi, K.; Abe, A.; Ohashi, E.; Mitsubayashi, K.; Ushio, H.; Hayashi, T.; Ren, H.; Endo, H. *Anal. Chim. Acta* **2006**, *173−174*, 158−163.

(4) Kempf, V. A. J.; Mändle, T.; Schumacher, U.; Schäfer, A.; Autenrieth, I. B. *Int. J. Med. Microbiol.* **2005**, *295*, 47−55.

(5) Hoshino, T.; Kawaguchi, M.; Shimizu. N.; Hoshino, N.; Ooshima, T.; Fujiwara, T. *Diagn. Microbiol. Infect. Dis.* **2004**, *8*, 195−199.

(6) Lazaro, D. R.; Hernandez, M.; Esteve, R.; Hoofar, J.; Pla, M. *J. Microbiol. Methods* **2003**, *54*, 381−390.

(7) Mohammadi, T.; Pietersz, R. N. I.; Vandenbracke-Grauls, C. M. J. E.; Savelkoul, P. H. M.; Reesink, H. W. *Transfusion* **2005**, *45*, 731−736.

(8) Greisen, K.; Loeffelholz, M.; Purohit, A.; Leong, D. *J. Clin. Microbiol.* **1994**, *32*, 335−351.

(9) Yang, L.; Li, Y. J. *Microbiol. Methods* **2006**, *64*, 9−16.

(10) Prosser, J. I.; Killham, K.; Glover, L. A.; Rattray, E. A. S. *Crit. Rev. Biotechnol.* **1996**, *16*, 157−183.

(11) Ramanathan, S.; Shi, W. P.; Rosen, B. P.; Daunert, S. *Anal. Chim. Acta* **1998**, *369*, 189−195.

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Frequency of transmission of HIV and HCV has declined by more than 4 orders of magnitude over the past two decades while the risk of bacterial contamination remains unchanged. In line with the actively pursued zero risk policy for pathogen and immunological risk in the transfusion of blood components, scientific and technological innovation is constantly surveyed by blood transfusion centers. Until the late 1990s, mandatory blood screening for transmissible infectious agents depended entirely on ELISA assays. The recent emergence of nucleic acid amplification technologies has revolutionized the viral diagnosis. In more complex biological situations, when a broad spectrum of pathogens must be screened, the limitations of these first-generation technologies became apparent.[12]

Over the past decade, many attempts have been made to use biosensor technology as a sensitive and reliable detection protocol.[2,13] The importance of biosensors results from the elimination or the simplification of the sample preparation steps, and from their high specificity and sensitivity, which allow the detection of a broad spectrum of analytes in complex sample matrixes (blood, serum, water, or food). Immunological-based biosensor technologies, such as amperometric immunosensors,[14−16] potentiometric immunosensors,[17] piezoelectric biosensors,[18,19] electrical impedance biosensors,[20−22] and optical biosensors,[23−25] have been applied to the detection of bacteria.

The aim of this work is to develop an impedancemetric immunosensor for the direct detection of transmissible bacterial agents using a model bacterium, *Escherichia coli* (CIP 76.24). Electrochemical impedance spectroscopy (EIS) offers a method of transducing analyte interaction on derivatized surfaces. It is a rapidly developing and effective electrochemical technique for the characterization of biocatalytic transformations on electrode surfaces and especially for the transduction of biosensing events on electrodes. Compared to other electrochemical techniques, one of the advantages of EIS is the small-amplitude perturbation from steady state, which makes it possible to treat the response theoretically by linearized or otherwise simplified current−potential characteristics.

The chemistry of the biosensor surface on which the biological receptors are immobilized is an important factor affecting detection sensitivity and specificity. Various types of chemistries have been researched for their applications as platforms for antibody-based biosensors. Self-assembled monolayers (SAMs) have been investigated extensively since 1983.[26] They were exploited to provide model surfaces for investigating cell behavior in bioanalysis and tissue engineering. The design flexibility of the SAMs technique allows the immobilization of biological macromolecules and living organisms, such as cells, proteins, antibodies, and DNA, on different substrates. Of the various kinds of SAMs, alkanethiolate SAMs are widely used mainly on gold surfaces due to their many advantages: they are resistant to nonspecific adsorption and form a well-ordered and dense monolayer structure that can easily be prepared on a gold surface by mild incubation for a sufficient time period.

To design the biosensor, we used mixed self-assembled monolayers on gold electrodes, formed by a biotinylated thiol ($HSCH_2(CH_2)_9CH_2NHCOBiotin$) and a spacer alcohol thiol (6-mercapto-1-hexanol, $HSCH_2(CH_2)_4CH_2OH$). We investigated a multilayer system relevant to biosensor research to anchor *E. coli*, using neutravidin and a biotin-labeled specific antibody. The strong affinity of biotin−neutravidin interaction makes this immobilization strategy much more efficient than chemistry-based methods. The bond formation is rapid and extremely stable, allowing a good orientation of biotinylated antibodies.[27] The formation of the self-assembled multilayers and the antibody anchoring were monitored by the SPR technique, and *E. coli* detection was performed either by surface plasmon resonance or by electrochemical impedance spectroscopy. Atomic force microscopy (AFM) is the workhorse for imaging surfaces at nanometric scales with high resolution and is very useful in many different fields of science, especially in microbiology studies. It was used to characterize the multilayer film in this study since it could give access to the molecular architecture and allows the topography of a living microorganism to be imaged in vitro.

EXPERIMENTAL SECTION

Microorganisms and Antigenic Lysed Samples. The two bacterial strains used in this study originate from the bacterial collection of the Pasteur Institute, Paris. *E. coli* (CIP 76.24) and *Staphylococcus epidermidis* (CIP 68.21) were selected as a Gram-negative and a Gram-positive bacterial model, respectively. Following overnight culture on Mueller-Hinton agar plates, liquid culture was perfomed in trypticase soy broth. The culture turbidity was adjusted to match a 0.5 McFarland standard and dilutions (10^8−10 cfu/mL) were performed in phosphate-buffered saline (PBS), pH 7.4. Quantitative bacterial counts were performed in duplicate by plating 0.1-mL aliquot of the limited dilutions (10^3 and 10^2 cfu/mL) onto Mueller-Hinton agar plates to confirm the bacterial concentrations. The bacterial suspensions were aliquoted and stored at −80 °C prior to analysis.

The procedures used for antigen preparation have previously been described by Lin et al.[28] Briefly, 1 mL of liquid culture was centrifuged at 1600*g* for 15 min at 4 °C, and the supernatant was removed. The bacterial pellet was washed once in PBS (pH 7.6). The cell pellet was then resuspended in 50 μL of RIPA buffer (10 mM Tris HCl pH 8, 150 mM NaCl, 0.5% Triton X-100) containing

(12) Fournier-Wirth, C.; Coste, J. *Developments in Biologicals*; Advances in Transfusion Safety Vol. IV; Ed.; Karger: New York, 2007; pp 61−70.
(13) Deisingh, A. K.; Thompson, M. *Can. J. Microbiol.* **2004**, *50*, 69−77.
(14) Abdel-Hamid, I.; Ivnitski, D.; Atanasov, P.; Wilkins, E. *Biosen. Bioelectron.* **1999**, *14*, 309−316.
(15) Hasebe, Y.; Yokobori, K.; Fukasawa, K.; Kogure, T.; Uchiyama, S. *Anal. Chim. Acta* **1997**, *357*, 51−54.
(16) Brewster, J. D.; Mazenko, R. S. *J. Immunol. Methods* **1998**, *211*, 1−8.
(17) Gehring, A. G.; Patterson. D. L.; Tu, S. I. *Anal. Biochem.* **1998**, *258*, 293−298.
(18) Chang, B. W.; Hsu, Y. M.; Chang, H. C. *Sens. Actuators, B* **2000**, *65*, 105−107.
(19) Su, X. L.; Li, Y. *Biosens. Bioelectron.* **2004**, *19*, 563−574.
(20) Timms, S.; Colquhoun, K. O.; Fricker, C. R. *J. Microbiol. Methods* **1996**, *26*, 125−132.
(21) Yang, L.; Ruan, C.; Li, Y. *Biosens. Bioelectron.* **2003**, *19*, 495−502.
(22) Radke, S. M.; Alocilja, E. C. *Biosens. Bioelectron.* **2005**, *20*, 1662−1667.
(23) Subramanian, A.; Irudayaraj, J.; Ryan, T. *Sens. Actuators, B* **2006**, *114*, 192−198.
(24) Subramanian, A.; Irudayaraj, J.; Ryan, T. *Biosens. Bioelecton.* **2006**, *21*, 998−1006.
(25) Moreno-Bondi, M. C.; Taitt, C. R.; Shriver-Lake, L. C.; Ligler, F. S. *Biosens. Bioelectron.* **2006**, *21*, 1880−1886.
(26) Nuzzo, R. G.; Allara, D. L. J. *Am. Chem. Soc.* **1983**, *105*, 4481−4483.
(27) Huang, T.; Sturgis, J.; Gomez, R.; Geng, T.; Bashir, R.; Bhunia, A. K.; Robinson, J. P.; Ladisch, M. R. *Biotechnol. Bioeng.* **2003**, *81*, 618−624.
(28) Lin, F. Y. H.; Sabri, M.; Alirezaie, J.; Li, D.; Sherman, P. M. *Clin. Diagn. Lab. Immunol.* **2005**, *12*, 418−425.

a mixture of proteinase inhibitors for 40 min at 4 °C. The lysate was then recentrifuged at 10000*g* for 15 min at 4 °C, and the supernatant with whole bacterial proteins was collected and stored at −80 °C.

Biomaterials and Chemicals. Rabbit anti-*E.coli* polyclonal antibody−biotin conjugated was obtained from Abcam as a solution of 4 mg/mL concentration.

The biotin thiol ($HSCH_2(CH_2)_9CH_2NHCOBiotin$) and the spacer thiol (6-mercapto-1-hexanol, $HSCH_2(CH_2)_4CH_2OH$) were purchased from ProChimia Surfaces and Sigma-Aldrich, respectively. Bovine serum albumin (BSA), which was used as a blocking reagent, was bought from Sigma-Aldrich. Neutravidin was obtained commercially from Pierce. Solvents such as ethanol and chloroform were both bought from Fluka (purity >99.8%).

Potassium dihydrogen phosphate, disodium hydrogen phosphate, and sodium chloride all from Prolabo, potassium chloride from Fluka, and sodium hydroxide from Aldrich Chemical Co. were used to prepare the PBS, consisting of 1.8 mM KH_2PO_4, 0.1 mM Na_2HPO_4, 140 mM NaCl, and 2.7 mM KCl, pH 7.0. All of them were analytical grade reagents (>99%). Solutions were prepared with water that had been purified by Elga, an ultrapure water system. This buffer solution was used for all experiments.

Immunoblot analysis. Whole bacteria from the pure cultures *of E. coli* (10^7−10^3 cfu/mL) and antigen preparations from the bacterial lysates (10^7 and 10^6 cfu/mL) were assayed by immunoblot analysis. The samples were rendered soluble and separated by electrophoresis under reducing conditions on a 12% acrylamide gel (NuPAGE Bis-tris, Invitrogen). After transfer to a nitrocellulose membrane (Hybond ECL, GE Healthcare) and immunodetection with a rabbit anti *E. coli* polyclonal biotin-conjugated antibody (1:800 dilution), the rabbit IgG was then probed with horseradish peroxidase-labeled goat anti-rabbit antibodies (1:10 000 dilution), and the proteins were visualized by using an enhanced chemiluminescence detection method.

Surface Plasmon Resonance (SPR). A commercial version of the SPR device Biosuplar-3 from Biosuplar was used to monitor the whole procedure of multilayer assembly and its application to immunosensing in real time. This instrument utilizes attenuated total reflection prism coupling in the Kretschmann−Raether geometry for excitation of surface plasmons. The prism is mounted on a swivel carriage that rotates around the horizontal axis of the plane of the working facet of the prism. Rotation is performed automatically by the computer-controlled precision rotating mechanism. Maximum angle scan range is ±17 angular degrees. The reagents and samples are poured through the flow cell (0.6 cm^2) housing the sensor chip over the prism surface. The flow of reagents is controlled by a peristaltic pump. A solid-state laser (λ = 670 nm) serves as a modulated light source. The reflected light intensity is measured using a Si photodiode operating in a zero-voltage mode. The experimental setup was equipped with a computer-controlled data acquisition system. The SPR response was determined by the difference between the signal at the injection of the sample and the signal after washing.

Electrochemical Impedance Spectroscopy. Electrochemical impedance spectroscopy combines the analysis of both the resistive and capacitive properties of materials, based on the perturbation of a system at equilibrium with a small sinusoidal excitation signal. The obtained impedance spectra can be modeled with equivalent circuit models. which are useful for the interpretation of the electrical properties of the biolayer.

Electrochemical measurements were performed in a conventional electrochemical cell containing a three-electrode system using a Voltalab 80 analyzer (PGZ 402 and Voltamaster 4) from Radiometer Analytical SA (Villeurbanne-France). A Pt plate (0.46 cm^2) and a saturated calomel electrode (SCE) were used as counter and reference electrodes, respectively. Functionalized gold electrodes act as working electrodes with an effective surface of 0.22 cm^2.

Impedance measurements were carried out in the absence of a redox probe in PBS at 20 °C over a frequency range from 100 mHz to 100 kHz, at a polarization potential of −0.6 V/SCE with a frequency modulation of 10 mV. The Z-view modeling program was used to analyze the impedance spectra. All electrochemical measurements were performed in a Faraday cage.

Pretreatment of Gold Substrate and Formation of the Self-Assembled Multilayer by SPR. Gold substrates were provided by the Institute of Semiconductor Physics (Kiev, Ukraine). With a thickness of 50 nm, thin gold films were prepared by vacuum vapor deposition of 99.999 pure gold at a deposition rate of 1.0−1.5 $nm \cdot s^{-1}$ onto a cleaned glass substrate (refractive index n = 1.61) precoated with a 1−1.5-nm intermediate adhesive Cr layer.

Before the formation of the mixed SAM, the gold substrates were cleaned for 1 min at room temperature in a water-diluted aqua regia mixture ($HCl/HNO_3/H_2O$) and in a water-diluted basic mixture ($H_2O_2/NH_4OH/H_2O$), 3:1:16 and 1:1:5 by volume concentration respectively. Between and after these treatments, the gold electrodes were rinsed thoroughly with ultrapure water, then rinsed with absolute ethanol/ and dried in a flow of pure nitrogen.

After cleaning, the gold substrates were immersed immediately into a mixed solution of biotin thiol and spacer alcohol thiol, at a concentration of 0.1 and 0.9 mM, respectively, in 50/50 ethanol/chloroform solution for 14 h. The gold electrodes functionalized with mixed SAMs were then removed, rinsed with the corresponding solvent for 5 min, dried under N_2 flow/ and fixed to the supporting prism of the SPR instrument (n = 1.61). Optical contact was realized using immersion oil (polyphenyl ether, n = 1.61). The mixed SAMs were first stabilized by injecting PBS (pH 7.0) at a flow rate of 0.2 mL/min for 30 min (step I). Subsequently, a volume of 300 μL of 1 μM BSA solution was added at a flow rate of 0.2 mL/min to prevent any further nonspecific adsorption on the mixed SAMs. After the beginning of the angle shift. the flow rate was decreased to the value of 0.04 mL/min and then stopped, and the SAM functionalized electrode was incubated in BSA solution for 30 min (step II). Next, the same protocol was used to inject 300 μL of 10 μM neutravidin with an incubation time of 40 min (step III) followed by adding 330 μL of 1 μM concentration of the biotinylated polyclonal anti-*E. coli* antibody with an incubation time of 40 min (step IV). After each step, the electrode was rinsed by injecting PBS solution (pH 7.0) at a flow rate of 0.2 mL/min for 10 min to remove the unbound biological compounds.

***E. coli* Detection.** After the elaboration of the multilayer by SPR, *E. coli* detection was conducted either by surface plasmon resonance or by electrochemical impedance spectroscopy (Step V).

For SPR measurements, 200 μL aliquots of different antigen concentrations (10, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8 cfu/mL cultures)

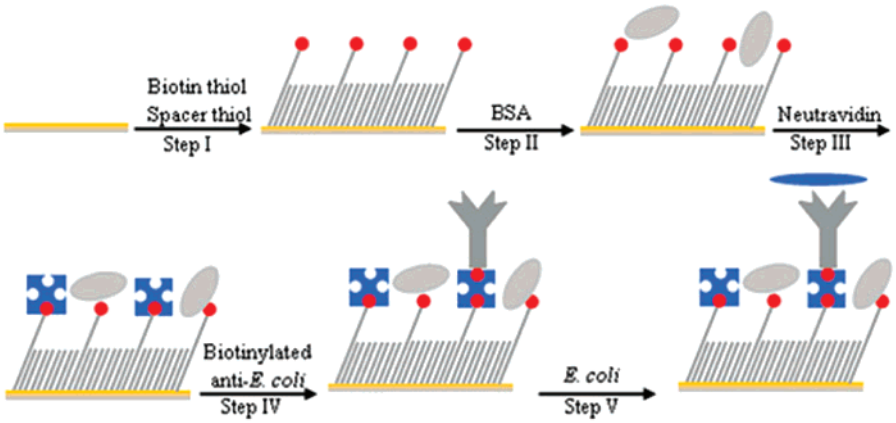

Figure 1. Schematic illustration of the binding reactions involved in this study: BSA blocking agent, neutravidin immobilization, biotin-labeled antibody assembly, and bacteria detection.

were passed over the self-assembled multilayer at a flow rate of 0.2 mL/min. After each concentration, the electrode surface was rinsed by injecting PBS solution (pH 7.0) at a flow rate of 0.2 mL/min for 5 min to remove the unbound antigen compounds.

For electrochemical impedance spectroscopy measurements, the functionalized self-assembled multilayer electrode was fixed onto a conventional electrochemical cell containing a three-electrode system. Different antigen concentrations (10, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8 cfu/mL cultures) were successively injected with an incubation time of 1 h for each. The electrode surface was then rinsed by a phosphate buffer solution pH 7.0, and the complex impedance was measured at the polarization potential of −0.6 V/SCE.

The schematic drawing of the multilayer architecture employed, composed of SAM/BSA/neutravidin/antibody/bacteria, is summarized in Figure 1.

Atomic Force Microscopy. Atomic force microscopy was used in order to characterize the clean bare gold substrate and to measure its roughness. A rectangular silicon AFM tip was employed (MikroMasch NSC18/AlBS, spring constant 0.15 N/m, resonant frequency 12 kHz), and images were recorded under ambient conditions with relative humidity around 30% using a commercial Dimension 3100 AFM (Veeco Instruments). The WSxM v4.0 software (Nanotec Electrónica) was used to treat and analyze AFM images.

A rectangular bare silicon AFM tip (MikroMasch CSC17/AlBS, spring constant 3.5 N/m, resonant frequency 75 kHz) was used for topographical measurements of *E. coli* on a functionalized gold electrode. Images were recorded and treated using the same instrument and software cited above.

RESULTS AND DISCUSSION

***E. coli* Detection by Immunoblot Analysis.** *E. coli* detection by immunoblot analysis was used to control the quality of the rabbit anti-*E. coli* polyclonal antibody−biotin conjugated used in the multilayer system. As shown in Figure 2, this polyclonal antibody allows the detection of several bacterial proteins in pure cultures *of E. coli* and in the antigen preparations. The detection limit of this immunoblot analysis is 10^4 cfu/mL of pure cultures of *E. coli*. No unspecific signal was observed when we tested the PBS medium used in the culture dilutions and a pure culture of *S. epidermidis* (10^6 cfu/mL).

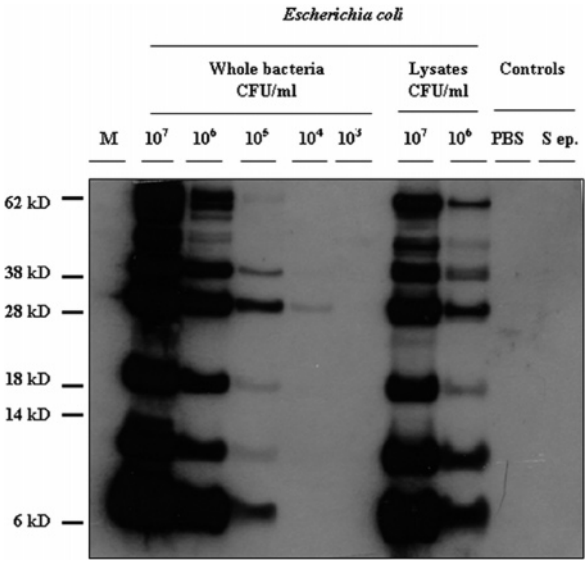

Figure 2. Detection of *E. coli* based on immunoblot analysis. Whole bacteria from the pure cultures *of E. coli* (10^7−10^3 cfu/mL) and antigens from the bacterial lysates (10^7 and 10^6 cfu/mL) were analyzed under reducing conditions after electrophoresis on a 12% acrylamide gel, transfer to a nitrocellulose membrane and immunodetection with a rabbit anti-*E. coli* polyclonal antibody−biotin conjugated. PBS and pure cultures of *S. epidermidis* (10^6 cfu/mL) were used as specificity controls. The size markers were prestained proteins (see Blue, Invitrogen).

Formation of the Self-Assembled Multilayer Monitored by Surface Plasmon Resonance. SPR is a nondestructive analysis technique that presents the important advantage of being capable of monitoring reactions in real time. The immobilization protocol described in the Experimental Section was adopted to build the self-assembled multilayer on a gold electrode. The immobilization processes of BSA, neutravidin, and biotinylated anti-*E. coli* were recorded as sequential increases in the resonance angle as shown in Figure 3. The increase of the resonance angle degree of 0.17° after injecting BSA indicates that this agent is fixed on the functionalized electrode surface and is blocking the nonspecific binding sites on the mixed thiol SAMs layer. BSA is often used in traditional immunoassays to block uncoated surface sites and to reduce nonspecific adsorptions.[29] After an incubation time of 30 min, PBS rinsing causes no variation in the resonance angle

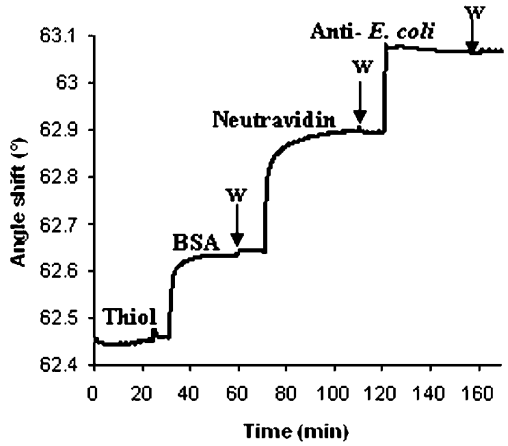

Figure 3. SPR response to the sequential reaction steps of 1 μM BSA, 10 μM neutravidin, and 1 μM anti-*E. coli.*

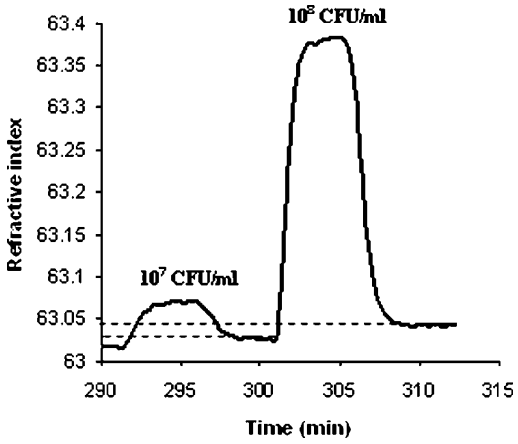

Figure 4. SPR response for increasing concentrations of *E. coli.* Detection limit 10^7 cfu/mL.

degree, showing the stability of the adsorbed BSA layer. Exposing the functionalized surface to a 10 μM neutravidin solution led to a rapid increase in the resonance angle followed by a second slower increase phase. The second phase is considered to be associated with a nonspecific aggregation of neutravidin molecules, because the buffer rinsing was able to gradually decrease the signal accumulated in that phase. The SPR angle shift upon neutravidin binding to the surface was measured to be $\Delta\theta = \sim 0.25°$. The injection of 1 μM biotinylated anti-*E. coli* solution induced another quick jump in the baseline to a higher level with an angle variation of 0.17°. This quick jump indicates a strong and highly specific binding of the biotinylated antibody via a biotin−neutravidin linkage.

The surface coverage of Γ proteins in units of mass/area (ng/mm^2) can be estimated using the corresponding refractive index increment, dn/dc:[30]

$$\Gamma = (\mathrm{d}n/\mathrm{d}c)^{-1} \quad (\Delta\theta/K)$$

where $\Delta\theta$ is the angle shift in arc seconds such that 1 arc second corresponds to 1/3600 degree and K is the conversion constant equal to 5300 s of arc/nm. Since the refractive index increment does not vary essentially with the kind of protein,[31] we assume the mean value of 0.188 cm^3/g for all proteins used in this study. Therefore, the neutravidin binding amount in the biotin thiol system is 0.9 ng/mm^2, whereas the subsequent biotinylated anti-*E. coli* coverage is 0.61 ng/mm^2. Taking into account the molecular weights $MW_{neutravidin} \approx 60\,000$ and $MW_{biotin-Ab} \approx 150\,000$, we convert the surface coverage mass into units of molecules per square centimeter. These calculations assume a value of 0.9×10^{12} and 0.25×10^{12} molecules/cm^2 for the neutravidin and the biotinylated antibody, respectively, leading to a neutravidin/biotin−antibody binding ratio of ~3.6. This ratio exceeds that between the molecular masses of the antibody and the neutravidin (150 kDa/60 kDa = 2.5). In fact, the combination of electrostatic repulsion and steric hindrance may prevent efficient coupling of one molecule of the antibody onto 2.5 molecules of neutravidin.

From the repeated experiments, we found that the building up of the self-assembled multilayer is a robust procedure, leading to a reproducible BSA blocking step (standard error mean (SEM) ≈ 0.007) followed by a neutravidin immobilization (SEM ≈ 0.009) and subsequent biotin-labeled specific antibody assembly (SEM ≈ 0.008) with $n = 6$.

***E. coli* Detection by SPR.** The sensitivity of detection of *E. coli* was determined by running an assay of different concentrations of bacteria ranging from 10 to 10^8 cfu/mL. The SPR response was determined by the difference between the signal at the injection of sample and the signal at the end of washing. It can be seen from Figure 4 that no response was obtained for an *E. coli* concentration ranging from 10 to 10^6 cfu/mL. A signal of 0.01° and 0.026° was obtained for the concentrations of 10^7 and 10^8 cfu/mL, respectively. Therefore, the detection limit of this SPR biosensor is 10^7 cfu/mL. Subrananian et al. and Taylor et al. have reported in the literature a detection limit of 10^6 cfu/mL for the whole *E. coli* O157:H7; however, to improve the sensitivity of the immunosensor, they investigated, respectively, the sandwich assay and the use of different preparation methods of *E. coli*.[24,32]

***E. coli* Detection by EIS.** Nonfaradic impedance measurements were carried out in PBS (pH 7.0) in the frequency range from 100 mHz to 100 kHz at a polarization potential of −0.6 V/SCE. As mentioned before, self-assembled multilayers were monitored by surface plamson resonance and *E. coli* detection was performed by electrochemical impedance spectroscopy.

In order to evaluate the recognition properties of the system, in terms of sensitivity, we exposed the gold electrode with self-assembled multilayers to various concentrations of *E. coli*. The corresponding Nyquist plots of impedance spectra in PBS pH 7.0 are shown in Figure 5A. A significant difference in the Nyquist impedance spectra is observed upon the step-by-step addition of increasing concentration of *E. coli*.

(29) Peterfi, Z.; Kocsis, B. *J. Immunoassays* **2000**, *21*, 341−354.
(30) Snopok, B. A.; Kostyukevych, K. V.; Rengevych, O. V.; Shirshov, Y. M.; Venger, E. F. *Semicond. Phys. Quant. Electron. Optoelectron.* **1998**, *1*, 121−134.
(31) Clerc, D.; Lukosz, W. *Sens. Actuators, B* **1993**, *11*, 461−465.
(32) Taylor, A. D.; Yu, Q.; Chen, S.; Homola, J.; Jiang, S. *Sens. Actuators, B* **2005**, *107*, 202−208.
(33) Ruan, C.; Yang, L.; Li, Y. *Anal. Chem.* **2002**, *74*, 4814−4820.
(34) Chen, H.; Heng, C. K.; Puiu, P. D.; Zhou, X. D.; Lee, A. C.; Lim, T. M.; Tan, S. N. *Anal. Chim. Acta* **2005**, *554*, 52−59.
(35) Yang, L.; Li, Y. *Biosens. Bioelectron.* **2005**, *20*, 1407−1416.
(36) Yang, L.; Li, Y.; Erf, G. F. *Anal. Chem.* **2004**, *76*, 1107−1113.
(37) Zhang, D.; Chen, S.; Qin, L.; Li, R.; Wang, P.; Li, Y. *IEEE Eng. Med. Biol. Soc.* **2005**, *7*, 7111−7113.
(38) Varshney, M.; Li, Y. *Biosens. Bioelectron.* **2007**, *22*, 2408−2414.

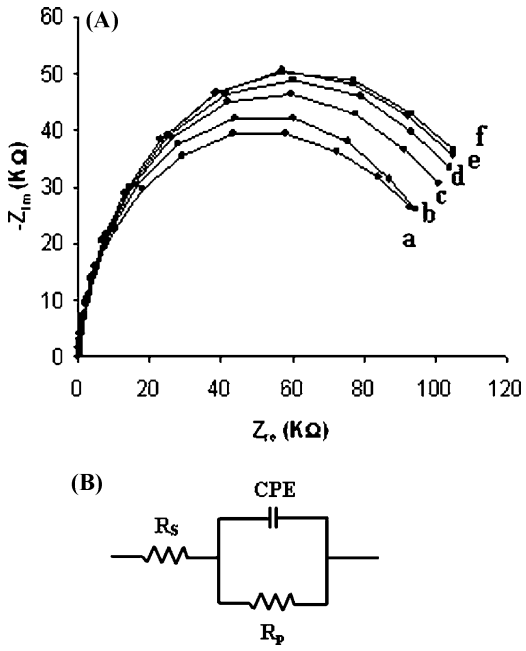

Figure 5. Electrochemical detection of *E. coli* (A) Nyquist plots of impedance spectra taken in PBS solution at a polarization potential of −0.6 V vs SCE in the frequency range from 100 mHz to 100 kHz with a frequency modulation of 10 mV: (a) functionalized SAM/BSA/neutravidin/antibody electrode; (b) 10, (c) 10^2, (d) 10^3, (e) 10^4, and (f) 10^5 cfu/mL. (B) Equivalent electrical circuit diagram used to fit the impedance spectra.

Table 1. Fitting Values of the Equivalent Circuit Elements Taken by Zplot/Zview Software for *E. coli* Detection

E. coli concn (cfu/mL)	R_s (Ω)	CPE_1-T (μF/cm^2)	α	R_p (Ω)
0	99.88	2.978	0.93	94 281
10	174.1	3.084	0.93	99 056
10^2	194.3	3.291	0.92	107 720
10^3	212.4	3.349	0.93	112 720
10^4	166.3	3.552	0.93	114 860
10^5	212.1	3.508	0.93	115 640

The data of electrochemical impedance measurements can be simulated with an equivalent model using the commercially available software Zplot/Zview (Scribner Associates Inc.). The equivalent circuit, which consists of the ohmic resistance of the electrolyte (R_s), the polarization resistance (R_p)m and the constant phase element (CPE), was proposed to interpret the impedance measurements (Figure 5B). The quality of fitting to the equivalent circuit was evaluated by an acceptable error value. The diameter of the semicircle represents the polarization resistance (R_p). CPE is used instead of a pure capacitance because it takes into account the topological imperfections of the biolayer at the electrode surface. The CPE is given by

$$Z = A(j\omega)^{-\alpha} \quad (1)$$

where A and α are experimental parameters ($0 \le \alpha \le 1$). Three special cases depending on the α parameter can be considered: if $\alpha \approx 0$, Z represents an ideal resistance and $A = R$; if $\alpha \approx 1$, then Z corresponds to an ideal capacitor where $A = 1/C$, and if $\alpha \approx 0.5$, the circuit element is termed the Warburg impedance, where $A = W$, which is associated with a diffusion process.

The fitting values are presented in Table 1. The interfacial polarization resistance increases as the concentrations of *E. coli* cells increase, implying that the higher content of *E. coli* cells was linked to the interface and generated a higher blocking effect. The system tends to be saturated above the concentrations of 10^4 and 10^5 cfu/mL.

It can be seen from Table 1 that applying a concentration of 10 cfu/mL onto the antibody-modified gold electrode results in an increase in polarization resistance of 4775 Ω, indicating that a concentration of 10 cfu/mL could be detected using this immunosensor. The comparison of this result to the detection limits of targeted bacteria for some biosensors based on EIS techniques reported in the literature (Table 2) allows us to conclude that our system is more suitable for bacteria detection. To our knowledge, this is the first impedancemetric biosensor elaborated via mixed SAM (biotin-thiol/OH-thiol) for detection of blood bacterial contamination. The mixed SAM enables the rapid interaction of biotin−neutravidin allowing the formation of well-organized layers and a good orientation of the biotinylated antibodies.[27] A linear relationship between the polarization resistance and decimal logarithmic value of *E. coli* concentrations was found ranging from

Table 2. Characteristics of Some Bacterial Biosensors Reported in the Literature

refs	immobilization method	detection limit	linear range
Radke et al.[22]	antibody immobilization via heterobifunctional cross-linkers	10^4 cfu/mL	10^4−10^7 cfu/mL
Ruan et al.[33]	epoxysilane monolayer on ITO substrate for chemical anchoring of antibodies	6×10^3 cells/mL	6.10^4−6.10^7 cells/mL
Chen et al.[34]	alkanethiolate SAM on gold substrate for chemical anchoring of yeast cells	10^2 cfu/mL	10^2−10^8 cfu/mL
Yang and Li[35]	epoxysilane monolayer on ITO substrate for chemical anchoring of antibodies	detection of 6×10^5 cells/mL	
Yang et al.[36]	immobilizing anti-*E. coli* antibodies onto an indium−tin oxide interdigitated array microelectrode	6×10^6 cfu/mL	4.36×10^5−4.36×10^8 cfu/mL
Zhang et al.[37]	epoxysilane monolayer on ITO substrate for chemical anchoring of antibodies	4.215×10^3 cfu/mL	4.215×10^3−4.215×10^6 cfu/mL
Varshney and Li[38]	interdigitated array microelectrode coupled with magnetic nanoparticle−antibody conjugates	7×10^4 cfu/mL	

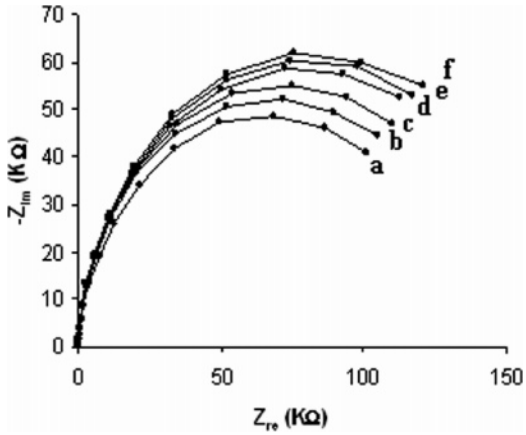

Figure 6. Nyquist plots of impedance spectra taken in PBS solution at a polarization potential of −0.6 V vs SCE in the frequency range from 100 mHz to 100 kHz with a frequency modulation of 10 mV for lysed *E. coli* detection (a) functionalized SAM/BSA/neutravidin/antibody electrode; (b) 10^3, (c) 10^4, (d) 10^5, (e) 10^6, and (f) 10^7 cfu/mL.

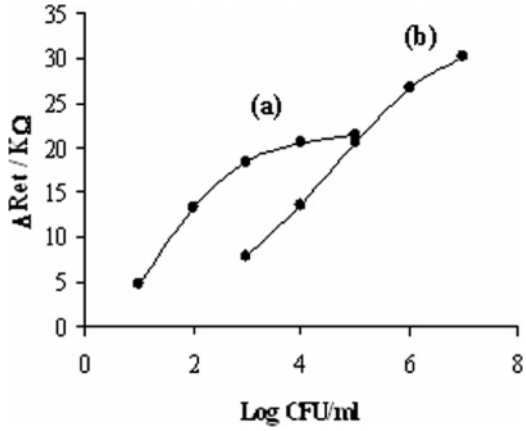

Figure 7. Relationship between the polarization resistance and logarithmic value of *E. coli* concentrations for (a) whole bacteria and (b) lysed bacteria.

10 to 10^3 cfu/mL with a slope of 6.832 and a correlation coefficient of 0.9766 (Figure 7a).

The changes in resistance are calculated following the equation

$$\Delta R = R_{\mathrm{Ab-Ag}} - R_{\mathrm{Ab}}$$

where R_{Ab} is the value of the resistance when the biotinylated anti-*E. coli* is immobilized on the electrode and $R_{\mathrm{Ab-Ag}}$ is the value of the resistance after adding *E. coli* bacteria.

It has been reported in the literature that the preparation methods of *E. coli* affect their detection limits and their sensitivities. Since electrochemical impedance spectroscopy is much more sensitive to *E. coli* detection than the SPR measurements, it will be used for the detection of lysed *E. coli*.

Lysed *E. coli* Detection by EIS. The same protocol employed before was used for the detection of lysed *E. coli*. Therefore, increasing concentrations of lysed bacteria were added to the SAMs-modified gold electrode, and corresponding Nyquist plots of impedance spectra in PBS (pH 7.0) are presented in Figure 6. No variation was obtained for concentrations of 10 and 10^2 cfu/mL, but an increase of 7820 Ω in the polarization resistance was observed for 10^3 cfu/mL. The fitting values obtained after modeling by using the equivalent circuit of Figure 5B are presented in Table 3. The fitting quality was evaluated by an acceptable error value. A linear relationship between the polarization resistance and decimal logarithmic value of lysed *E. coli* concentrations was found to range from 10^3 to 10^7 cfu/mL with a slope of 5.782 and a correlation coefficient of 0.9885 (Figure 7b). The highest detection limit of the lysed bacteria compared to the whole bacteria can be due to the RIPA buffer (pH 8.0), which could affect the structure conformation, limiting therefore the recognition properties.

Table 3. Fitting Values of the Equivalent Circuit Elements Taken by Zplot/Zview Software for Lysed *E. coli* Detection

E. coli lysed concn (cfu/mL)	R_s (Ω)	CPE_1-T ($\mu F/cm^2$)	A	R_p (Ω)
0	99.95	3.909	0.92	112 430
10^3	187.2	3.913	0.93	120 250
10^4	213.1	3.768	0.93	126 060
10^5	194.1	4.030	0.92	133 090
10^6	218.9	3.997	0.92	139 090
10^7	216	3.955	0.92	142 520

Specificity Control. The specificity test is conducted by using a Gram-positive bacterial model *S. epidermidis* (CIP 68.21). No change in polarization resistance was noticed for the concentration of 10 cfu/mL. The variations obtained for other concentrations in comparison with *E. coli* detection are presented in the diagram of Figure 8. This immunosensor can be used to detect *E. coli* in the presence of small concentrations of Gram-positive bacteria. The application of a biotinylated monoclonal antibody for bacteria detection can play an important role in decreasing the nonspecific adsorption.

***E. coli* Characterization by AFM.** Clean bare gold substrate was characterized prior its functionalization by measuring its roughness in different places leading to a statistical root-mean-square value of 0.97 ± 0.22 nm.

The *E. coli* topographic image is given in Figure 9. Two bacteria are clearly seen in the 3.2 × 3.2 μm^2 AFM image. A horizontal cross section reveals an approximate *E. coli* thickness

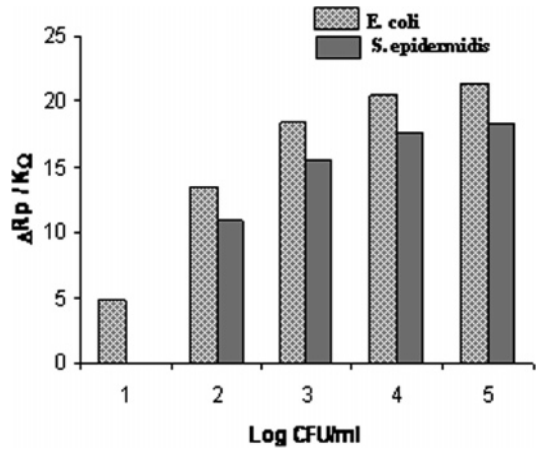

Figure 8. Specificity diagram to compare the impedance detection signal between *E. coli* and *S. epidermidis* bacteria.

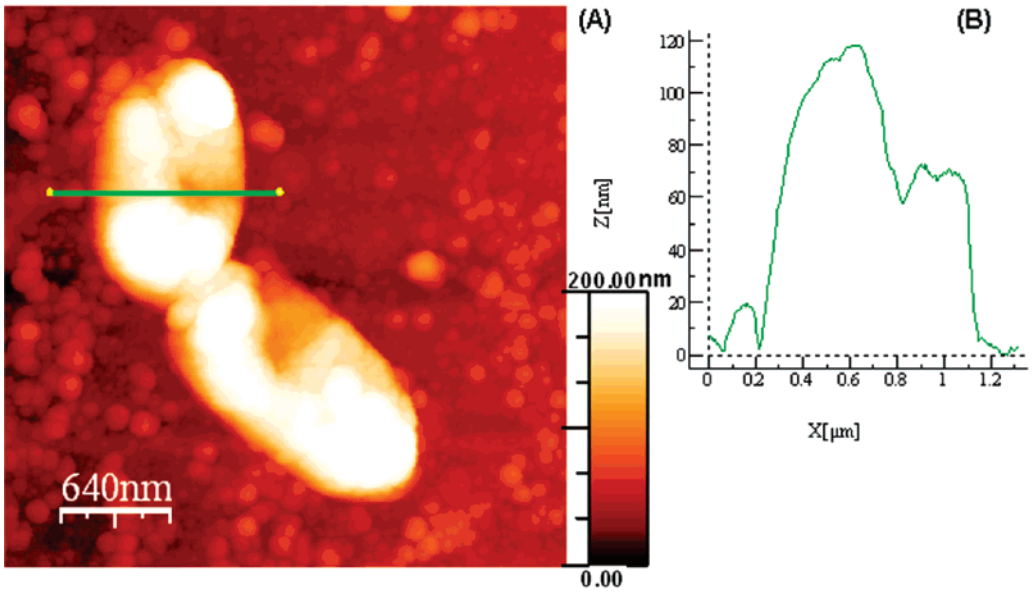

Figure 9. Topographic AFM image (3.2 μm $\times$ 3.2 μm) obtained in tapping mode: after *E. coli* immobilization (A); horizontal cross section (B).

of ~120 nm, neglecting the gold roughness; we can also distinguish the crystallized salt of the phosphate buffer solution.

CONCLUSION

The present study has demonstrated the successful deposition of mixed SAMs of biotin-thiol and OH-thiol on a gold electrode, enabling the subsequent immobilization of biotinylated anti-*E. coli* via a strong biotin−neutravidin interaction. In future work, a monoclonal antibody will be applied to decrease the nonspecific adsorption as much as possible and detection of bacteria in real blood samples will be performed.

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