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AFM, CLSM and EIS characterization of the immobilization of antibodies on indium–tin oxide electrode and their capture of *Legionella pneumophila*

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ABSTRACT

The microscopic surface molecular structures and properties of monoclonal anti-*Legionella pneumophila* antibodies on an indium–tin oxide (ITO) electrode surface were studied to elaborate an electrochemical immunosensor for *Legionella pneumophila* detection. A monoclonal anti-*Legionella pneumophila* antibody (MAb) has been immobilized onto an ITO electrode via covalent chemical bonds between antibodies amino-group and the ring of (3-Glycidoxypropyl) trimethoxysilane (GPTMS).

The functionalization of the immunosensor was characterized by atomic force microscopy (AFM), water contact angle measurement, cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) in the presence of $[Fe(CN)_6]^{3-/4-}$ as a redox probe. Specific binding of *Legionella pneumophila* sgp 1 cells onto the antibody-modified ITO electrode was shown by confocal laser scanning microscopy (CLSM) imaging and EIS. AFM images evidenced the dense and relatively homogeneous morphology on the ITO surface. The formation of the complex epoxysilane-antibodies acting as barriers for the electron transfer between the electrode surface and the redox species in the solution induced a significant increase in the charge transfer resistance (R_{ct}) compared to all the electric elements.

A linear relationship between the change in charge transfer resistance ($\Delta R_{ct=}R_{ct}$ after immunoreactions – R_{ct} control) and the logarithmic concentration value of *L. pneumophila* was observed in the range of 5×10^1–5×10^4 CFU mL^{-1} with a limit of detection 5×10^1 CFU mL^{-1}.

The present study has demonstrated the successful deposition of an anti-*L. pneumophila* antibodies on an indium–tin oxide surface, opening its subsequent use as immuno-captor for the specific detection of *L. pneumophila* in environmental samples.

1. Introduction

Legionella are ubiquitous in natural and man-made water ecosystems [1]. *Legionella pneumophila* is the major causative agent of legionnaires' disease, a pneumonia-like illness with a case/fatality ratio of 20% [2,3]. Transmission to humans from the environment occurs through contaminated aerosols [4]. Detection of *Legionella* in environmental samples can be achieved through standardized methods: cultivation (AFNOR T90-431 or ISO 11731) and PCR (AFNOR T90-471). Both methods are hampered either by their low sensitivity and the time needed to complete the analysis (cultivation) or by their cost and inefficiency to discriminate between live and dead bacteria (PCR). Several attempts have been made to purpose other detection tools such as flow cytometry for instance in a wish to identify viable but not culturable forms of *L. pneumophila* [5,6] but this method remains technically demanding. The use of specific antibodies (immunosensors) may be seen as alternative tools for *Legionella* detection. Such methods have been proposed to increase the recovery rates from complex plurimicrobial samples [7], or to extend the detection range to other *Legionella* species [8], a few antibodies being available for such purposes.

Antibodies, on these sensors, are usually immobilized through organic covalent bonds on various oxide surfaces such as SiO_2, Al_2O_3, SnO_2, and TiO_2 [9]. Immobilization of biomolecules such as

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antibodies has been recently expended to ITO [10]. Although ITO surfaces are stable, transparent and conductive making them suitable for the design of several applications [11], the reproducibility of the results is strongly correlated to the cleaning and functionalization process [12,13].

Depending on transducers, detection of *Legionella* using electrochemical impedance spectroscopy [14,15], surface plasmon resonance or optical waveguide light mode spectroscopy [16–19] are still hampered by a low sensitivity probably due to the use of low affinity antibodies [14–19] or by the fact that so far only DNA detection was achieved [20] but not direct sensing of *Legionella* bacteria cells. In addition, discriminating between living and dead bacteria by choosing a redox probe that can freely transverse across the bacterial cell membrane and probe the cell activity may be useful [20].

Here, we characterized a rapid, miniaturized low-cost electrochemical system that uses an indium–tin oxide (ITO) electrode in combination with a specific monoclonal antibody previously tested for its very high efficiency in recovering *L. pneumophila serogroup 1* strains from environmental samples [21].

2. Material and methods

2.1. Chemicals, antibodies and ITO electrodes

An epoxysilane compound, (3-Glycidoxypropyl)trimethoxysilane, was purchased from Aldrich (St. Louis MO). Carbon tetrachloride USP-NF was from Panreac Quimica SA Barcelona (Espana). Potassium ferrocyanide trihydrate ($K_4Fe(CN)_6 \cdot 3H_2O$), potassium ferricyanide ($K_3Fe(CN)_6$), disodium hydrogen orthophosphate and potassium dihydrogen orthophosphate were received from Sigma-Aldrich (France). Absolute ethanol from Carlo Erba (France), Hydrogen peroxide (30%) and ammonium hydroxide (28%) were obtained from Prolabo (France). All reagents were of analytical grade and ultrapure water (resistance 18.2 MΩ cm^{-1}) produced by a Millipore Milli-Q system (MilliQ academic, Millipore).

A mouse monoclonal antibody (MAb), DP-Lp1-O2, raised against a *Legionella*- specific epitope of the LPS protein and taken from the Dresden *Legionella* MAb panel [22] was used. The DP-Lp1-O2 antibody does not recognize all the *L. pneumophila* serogroups [23] but we have previously shown its ability to detect a large number of *L. pneumophila* serogroup 1 strains [21] although no detection of non-*Legionella* species including those frequently co-existing with *L. pneumophila* in water samples [24] could be observed when using it for ELISA [22] or immuno-magnetic separation (IMS) purposes [21].

Phosphate buffer saline solution (0.16 mol L^{-1}, pH 7.2) was prepared with Na_2HPO_4 and KH_2PO_4. Tin-doped indium oxide on glass (ITO) which has a surface resistance $\leq 200\ \Omega$, was cut into small pieces of pre-defined geometric area with dimensions 1 cm × 1 cm and 1 mm thickness, used as working electrodes.

2.2. Preparation of calibrated suspensions of Legionella

Calibrated suspensions were made using a wild *L. pneumophila* strain isolated from an environmental sample (water) in Tunisia. It was stored at −20 °C in Cryobank tubes (Mast Diagnostic, Amiens, France). The strain was grown for 3 days on BCYEα medium (buffered charcoal yeast extract agar) at 37 °C before being used in the experiments.

All suspensions were prepared by resuspending *Legionella* colonies into sterile saline buffer (0.9% NaCl). The optical density (OD) of the bacterial culture was measured at 600 nm and serial dilutions (spiked samples) were used to study the interaction of the bacterium with the electrode. Real numbers of bacteria in each dilution were obtained by plating some aliquots on BCYE agar media (in duplicates). Bacterial numbers were expressed as of Colony Forming Units (CFU) per mL.

2.3. Electrode modification and immobilization of the antibodies

Prior to use, the ITO coated glass were ultrasonically cleaned using acetone, absolute ethanol, and distilled water for 15 min each, and dried with nitrogen flow. Then it was immersed into a basic solution 5 M of KOH for 1 h and rinsed thoroughly with copious amounts of ultra-pure water. The electrodes were also cleaned chemically by immersion in RCA solution (NH_4OH (28%), H_2O_2 (30%), H_2O; 1:4:20 v/v) at 60 °C for 30 min followed by rinsing with ultra-pure water and dried with nitrogen flow. A Piranha solution (3:7 v/v of H_2O_2 and H_2SO_4) was applied for 1 min. Finally, the electrodes were washed extensively with water, absolute ethanol and dried under a stream of nitrogen before the silanization step.

Silanization was conducted by immersing the cleaned ITO electrode in 2% epoxysilane (20 mL CCl_4/400 μL silane) solution overnight at room temperature. Electrodes were then rinsed with absolute ethanol and with pure carbon tetrachloride to remove unbound epoxysilane from the surface and the modified electrodes were then dried 2 h in an oven at 120 °C.

Then, 20 μL of monoclonal antibody (2.36 μg μL^{-1}) were added on the ITO electrode, incubated 2 h at 4 °C and washed with the PBS (0.16 M, pH 7.2). To block the non-specific binding sites on the electrode surface, 20 μL of BSA 1% were added onto the electrode surface, incubated 1 h at 4 °C and washed with PBS. The monoclonal antibody modified electrode (ITO-Epoxy-MAb) was stored for further use at 4 °C. The way to assemble the epoxysilane monolayer and the immobilization of the anti-*L. pneumophila* antibodies is shown in Fig. 1.

2.4. Electrochemical impedance spectroscopy (EIS) measurements

Impedance measurements were performed using the Volta Lab 40 (PGZ 301) impedance analyzer (Radiometer Analytical SA, Villeurbanne, France) with a conventional electrochemical setup. The three-electrode consists of the ITO as working electrode, a platinum counter electrode, and a saturated Calomel $Hg/Hg_2Cl_2/KCl$ (+0.248 V vs SHE) as the reference electrode. The impedance spectra were recorded in the frequency range from 0.1 Hz to 100 kHz at the formal potential of the $[Fe(CN)_6]^{3-/4-}$ redox couple 200 mV. The amplitude of the alternating voltage was 10 mV. The measurements were carried out at room temperature (22 ± 3 °C) in a Faraday cage in order to improve the signal-to-noise ratio and repeated three times in order to ensure result reproducibility. The impedance data were represented in the complex impedance plot (Nyquist plot). The data of electrochemical impedance measurements were simulated with an equivalent circuit to understand the role of each electrical component in this circuit. The electric equivalent circuit bare electrode could be interpreted by the Randle's equivalent circuit model in which: R_s, Z_W, R_{ct}, and C_{dl} represent respectively the ohmic resistance, the Warburg impedance resulting from diffusion of ions from bulk electrolyte to the electrode interface, the charge transfer resistance of the redox probe and the double layer capacitance. The two components in the electric equivalent circuit of an electrochemical cell, R_s and Z_w, represent bulk properties of the electrolyte solution and diffusion features of the redox probe in solution. These components are not affected by chemical transformations occurring at the electrode surface. The other two components R_{ct} and C_{dl}, depend on the dielectric and insulating features at the electrode/electrolyte.

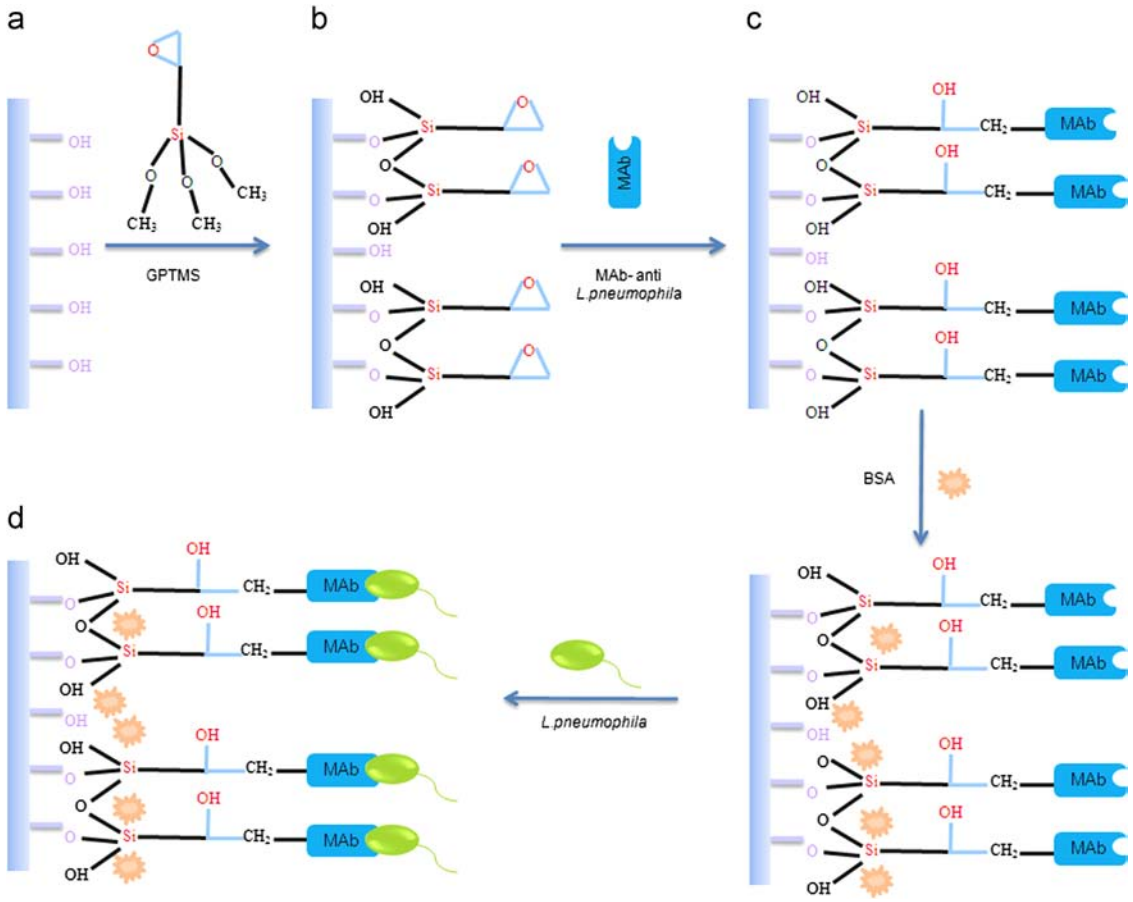

Fig. 1. Schematic illustration of each steps of surface modification: (a) 3-(Glycidoxypropyl) trimethoxysilane SAM (2% epoxysilane/CCl_4, v/v), (b) covalent immobilization MAb-anti *L. pneumophila* on modified electrode (20 μl of a 2.36 μg μL^{-1}), (c) BSA chemical blocking agent and (d) specific binding of *Legionella pneumophila*.

2.5. Atomic force microscopy (AFM)

The surface topologies of the modified electrode were imaged using Atomic Force Microscopy (AFM) Nanoscope V dimension 3100 (Digital Instruments) in tapping mode. The Root Mean Square (RMS) was used to characterize the surface in terms of irregularity and height distribution. In our work, AFM roughness profiles were measured on five different zones of the surface and the average value roughness was determined. AFM imaging was made at room temperature in constant force. Deflection and topographic mode images were scanned simultaneously at a fixed scan rate of 1 line/s. All electrodes were washed thoroughly using water and dried with nitrogen before testing in air.

2.6. Confocal laser scanning microscopy (CLSM)

Confocal images and analysis were performed at the Center of Confocal Microscopy Multiphotonic of the University of Saint-Etienne (France). We used a TCS-SP2 confocal microscope from Leica Microsystems (Leica®, Heidelberg, Germany), attached to an inverted microscope with oil immersion objective (HCX APO L U-V-I 63 × 1.2NA), and fitted with Acousto-Optical Beam Splitter (AOBS) covering a 350–850 nm wavelength range.

The system was equipped with a set of lasers: argon (458, 476, 488 and 514 nm), helium-neon (573 nm), helium (633 nm) lasers for classical CLSM (confocal laser scanning microscopy). For our purposes, pictures were made using 488 nm argon laser.

2.7. Sessile drop experiment

Contact angle were measured using a GBX-Digidrop Scientific Instrument (Romans, France). The measurements were conducted to investigate the hydrophilic/hydrophobic nature of the electrode surface before and after each step of surface modification by the static sessile drop method. The drop image was stored by a video camera and an image analysis system calculated the contact angle (θ) from the shape of the drop.

3. Results and discussion

3.1. AFM images of the epoxysilane monolayer and antibody

In order to characterize the surface modification and to obtain information on its architecture, AFM measurements were performed. Differences in the topography of the ITO surface can be observed before (data not shown) and after cleaning (see Fig. 2A). The decrease of the RMS value from 17 to 1.3 nm was attributed to the homogeneous and flat surface of the bare electrode and to the effectiveness of the cleaning method. This structure is consistent with the STM nanograph of a bare ITO surface reported by Ng et al. [25]. Fixation of epoxysilane films on ITO electrode (Fig. 2B) was significantly different from the topography obtained on bare ITO electrode after cleaning (see Fig. 2A). This feature indicated that the formation of SAM was accomplished by adsorption from carbon tetrachloride solution with 2% silane on the electrode surface.

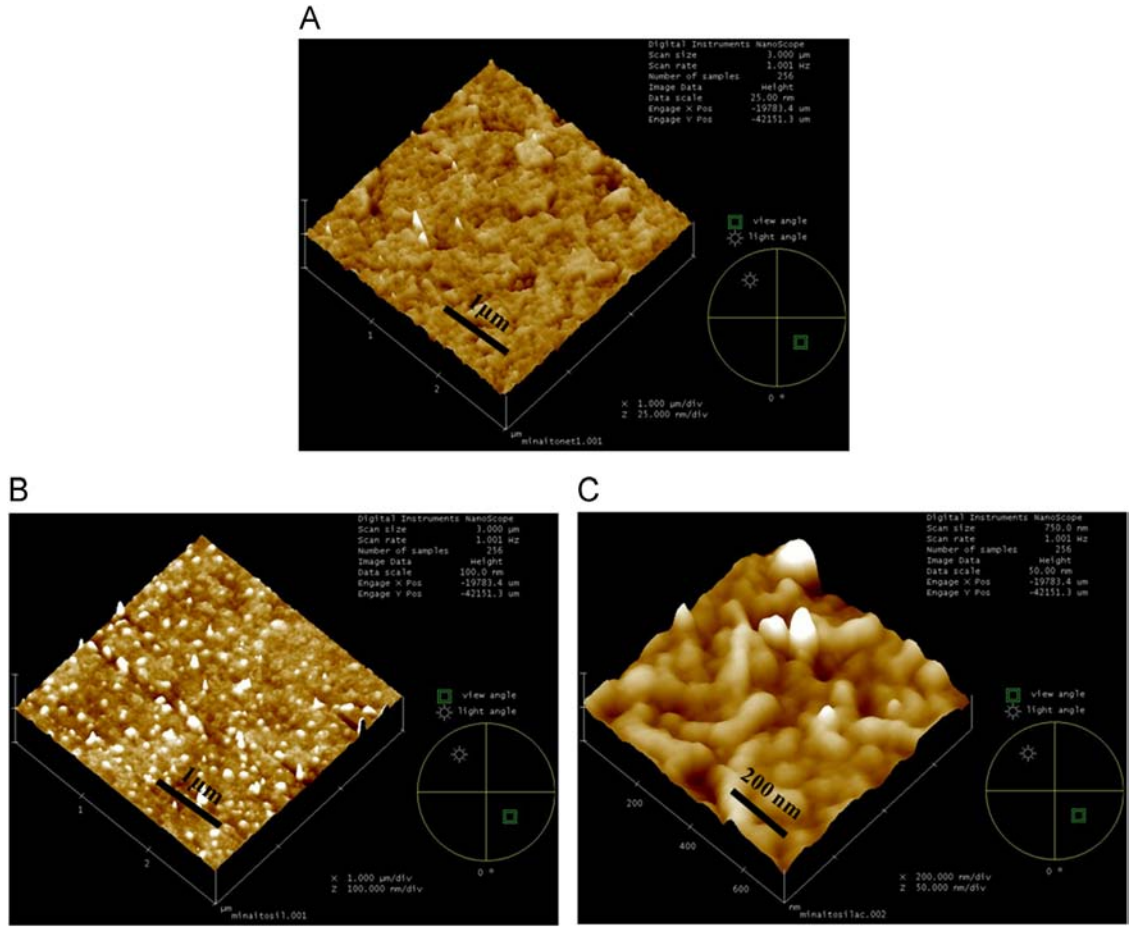

Fig. 2. AFM images of (A) bare ITO electrode after cleaning. (B) GPTMS-modified electrode and (C) MAb anti-*Legionella pneumophila* immobilization.

The monolayer composed was dense, complete, relatively smooth, roughly homogeneous and with only a few aggregates (RMS=3.13 nm). This result is consistent with the report on epoxy-terminated self-assembled monolayers on single crystal silicon and ITO surfaces [10,26]. Other studies have confirmed a non-smoothing GPTMS monolayer surface by the RMS value (RMS=3.5 nm) [27].

The AFM topography image of the SAM after treatment by antibodies exhibited a quite different aspect, consisting in distributed round globular particles that may correspond to the MAb bound onto the epoxysilane layer (see Fig. 2C). The antibody molecules did not assemble as the epoxysilane molecules in a monolayer, because some aggregates can still be observed on the surface and then induce an RMS increase (RMS=6.7 nm). It could be explained by the short length of the alkyl chain of GPTMS.

3.2. CLSM images of the specific binding of L. pneumophila sgp 1 cells

In order to verify the *L. pneumophila* attachment on the electrode surface, observation of the sensor surface was performed using a Confocal Laser Scanning Microscope (CLSM). After the electrode preparation (see paragraph 2.3), suspensions of *L. pneumophila* (5×10^6 CFU mL^{-1}) were incubated during 1 h at room temperature in order to evaluate their capability to bind to detection antibodies. The biosensor was then washed using PBS and dried. A CLSM image of *L. pneumophila* cells successfully captured on the sensor surface is shown in Fig. 3.

3.3. Contact angle

A low contact angle of $<5^\circ\pm3^\circ$ (Table 1) initially obtained for the bare ITO-glass plate after cleaning was related to surface super-hydrophilic hydroxyl groups. The contact angle increased from $57.6^\circ\pm3^\circ$ for the GPTMS modified ITO electrode to $65.5^\circ\pm3^\circ$ after the covalent immobilization of the antibodies. This increase could be explained by the presence of hydrophobic alkyl chains of GPTMS molecules that reduce the hydroxyl groups of the bare ITO-glass after the silanization step and hydrophobic nature of antibodies. These results were in accordance with those obtained by Luzinov et al. who show 51° under the same conditions but with a 1% epoxysilane concentration [26].

3.4. Electrochemical impedance spectroscopy of the epoxysilane monolayer and antibody

Fig. 4 shows the impedance response of the $[Fe(CN)_6]^{3-/4-}$ redox probe on the bare ITO electrode (a curve), the SAM modified ITO electrode (b curve) and the antibody dipped electrode (c curve). This increase in the charge transfer resistance (R_{ct}, the diameter of semicircles) was observed following silanization and then antibody binding.

It indicates that the presence of the epoxysilane monolayer formed a barrier that prevented direct approach of the redox species to the electrode surface. This observation is consistent with the result in the literature that the deposition of the SAM of alkylsiloxane on ITO surface led to a large increase in charge-

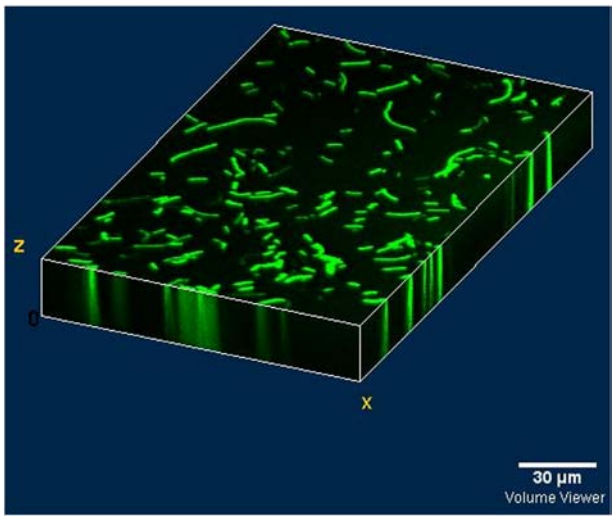

Fig. 3. 3D CLSM images of the ITO-Epoxy-MAb electrode after *Legionella pneumophila* deposition (5×10^6 CFU mL^{-1}).

Table 1
Contact angles of coating samples.

Materials	Contact angle
Bare ITO	$< 5^\circ \pm 3^\circ$
ITO-Epoxysilane	$58^\circ \pm 3^\circ$
ITO-Epoxysilane-MAb	$65^\circ \pm 3^\circ$

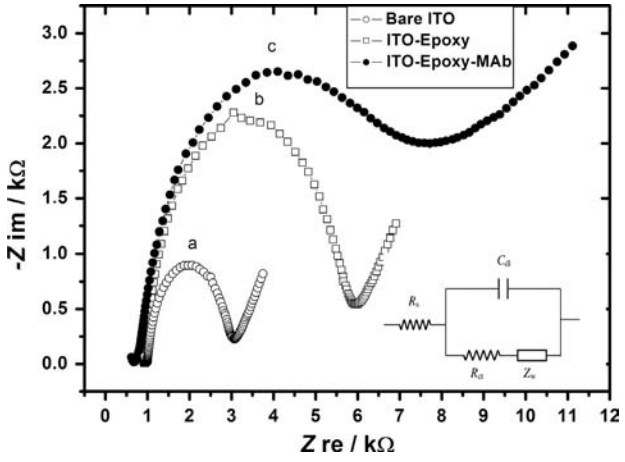

Fig. 4. Nyquist diagrams for impedance measurements corresponding to (a) bare ITO, (b) ITO-Epoxy and (c) ITO-Epoxy-MAb electrode. The inset shows the equivalent circuit.

transfer resistance [28]. This higher charge-transfer resistance value suggests that the resultant monolayer films are compact, highly ordered with very low defects [29].

Similarly, an increase in charge transfer resistance between the monolayer-modified electrode and the antibody-immobilized electrode was observed. This was also the greatest change among all these electrical elements, suggesting that antibody layer created an additional barrier and further prevented the access of the redox probe to the electrode surface. The magnitude of the changes in the charge transfer resistance indicates that the antibody blocked the electron transfer more effectively than the epoxysilane monolayer.

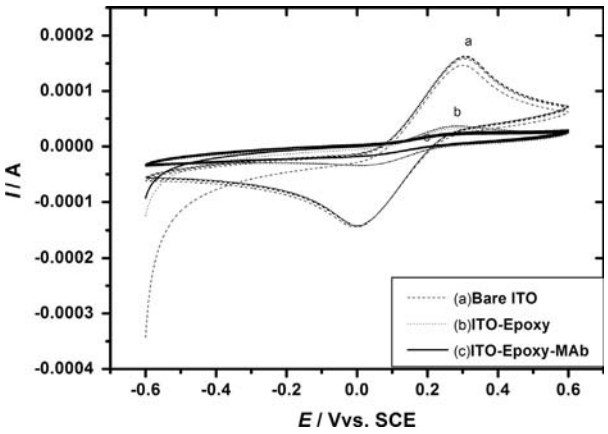

Fig. 5. Cyclic voltammograms of three repetitive scans of (a) bare ITO, (b) ITO-Epoxy and (c) ITO-Epoxy-MAb electrode, in PBS solution (0.16 mol L^{-1} pH=7.2) containing 3 mmol L^{-1} $[Fe(CN)_6]^{3-/4-}$. The scan rate is 0.1 V s^{-1}.

3.5. Cyclic voltammetry of the epoxysilane monolayer and antibody

Cyclic voltammetry using $[Fe(CN)_6]^{3-/4-}$ as redox couple is a suitable technique to investigate and monitor the various stages of the biosensor construction on ITO electrode [30]. Fig. 5 shows the superimposed cyclic voltammograms of $[Fe(CN)_6]^{3-/4-}$ recorded for bare ITO electrode (a curve), silane (b curve) and antibody (c curve) modified ITO electrode. It is shown that the epoxysilane monolayer and the antibody act as barriers for the electron transfer between the electrode surface and the redox species in the solution, resulting in most significant decreases in the current. These results corroborate those found by electrochemical impedance spectroscopy.

3.6. Detection of bacteria

The impedimetric biosensor was used for the detection of *L. pneumophila* in spiked samples. Fig. 6A shows the Faradaic impedance spectra, presented as Nyquist plots (-Zim vs Zre) of the anti- *Legionella pneumophila* antibody-modified ITO electrode (a curve) after binding of *L. pneumophila* at different concentrations: without bacteria (a curve), 5×10^1 CFU mL^{-1} (b curve), 5×10^2 CFU mL^{-1} (c curve), 5×10^3 CFU mL^{-1} (d curve), 5×10^4 CFU mL^{-1} (e curve) and 5×10^5 CFU mL^{-1} (f curve). The electrode's charge transfer resistance was determined for each bacterial concentration by fitting the measured impedance onto the electrodes using the Randle's equivalent circuit (see inset Fig. 4). The results showed the gradually increase in the R_{ct}, (the diameter of semicircles) due to the bacteria cells bounds and may gave rise to an additional barrier between the ITO electrode surface and the redox species $[Fe(CN)_6]^{3-/4-}$ in the solution leading to an electron transfer blockage. This result is consistent with the findings obtained by Ehret et al. [31]. Fig. 6B shows a linear relationship between the variation of charge transfer resistance ΔR_{tc} and different logarithmic concentrations of *L. pneumophila* in the range of 5×10^1–5×10^5 CFU mL^{-1} with a correlation coefficient of 0.994. The lowest concentration of *L. pneumophila* which could be unambiguously detected during this study was 5×10^1 CFU mL^{-1}. This relatively good sensitivity, in comparison to the literature, is likely to be due to the use of an antibody of particular affinity for the targeted bacteria in complex pluri-contaminated environmental samples (water samples) as previously shown by Allegra et al. [21]. This detection threshold is of lower value when compared to those obtained using other immunosensors either using electrochemical impedance spectroscopy detection [14,15,31], surface plasmon resonance detection or optical waveguide light

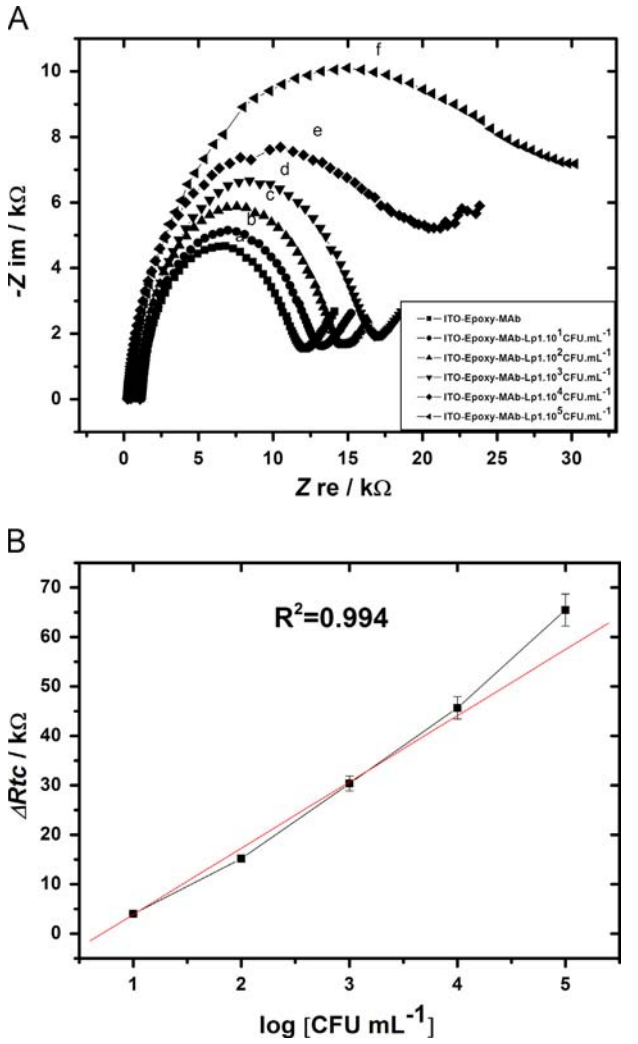

Fig. 6. (A) Impedance Nyquist plot for different *Legionella pneumophila* concentrations in presence of 3 mmol L^{-1} $[Fe(CN)_6]^{3-/4-}$ for (a) ITO-Epoxy-MAb (0 CFU mL^{-1}), (b) 5×10^1, (c) 5×10^2, (d) 5×10^3, (e) 5×10^4 and (f) 5×10^5 CFU mL^{-1}; (B) Variation of the fitted ΔR_{ct} with the logarithmic concentration of *Legionella pneumophila*.

mode spectroscopy [19–23]. Quite similar to us is the detection threshold (2×10^2 CFU mL^{-1}) achieved by Li et al. [14] using an immunochip prepared by covalently immobilizing fluorophore-conjugated *L. pneumophila* antibodies on gold chip.

Recently, Lei and Leung [32] have reported the design of an ITO-based interdigitated microelectrode array for the detection of *L. pneumophila* serogroup 5. Bacterial concentrations in samples could be estimated in a range between 10^5 and 10^8 CFU mL^{-1}. But, for low bacterial concentration ($<10^4$ CFU mL^{-1}), the authors did not find statistically significant difference in impedance measurements.

None of the non-*Legionella* bacteria that are frequently co-isolated with *L. pneumophila* in environmental samples could be detected by our antibody. In a previous study, DP-Lp1-O2 MAb was shown to not cross-react with non-*Legionella* bacteria [21]. When various concentrations of *Pseudomonas aeruginosa* (for instance) together with *L. pneumophila* were tested for their recovery though IMS (immunomagnetic separation) testing, the percentage recovery of *L. pneumophila* by DP-Lp1-O2 was not influenced by the proportion of *P. aeruginosa* [21]. In addition, when testing the capability of the DP-Lp1-O2 MAbs on real highly contaminated environmental samples (biofilms, sanitary hot water, cooling tower water) using IMS, the percentage recovery of *L. pneumophila* was not influenced by the proportion of interfering bacteria present in the samples [21].

Although specificity of the anti-*Legionella* antibody used in this study was previously reported [21], it was also tested against different bacteria (*P. aeruginosa* and *Staphylococus aureus*) at high concentration (10^5 CFU mL^{-1}). Our results confirmed that no apparent binding between the monoclonal anti-*Legionella pneumophila* antibody immobilized on the functionalized ITO surface and *Pseudomonas* or *Staphylococcus* was observed as no variation of R_{tc} measurement could be measured (data not shown).

4. Conclusion

Our goal was to first characterize the immobilization of an anti-*Legionella pneumophila* antibody on an indium–tin oxide electrode and to evaluate its potential for the detection of *L. pneumophila* in spiked samples. Characterization of the immunosensor using AFM, contact angle measurements, CV and EIS demonstrated its appropriate design. These methods confirmed the functionalized ITO electrode by both epoxysilane and antibodies.

The detection threshold reproducibly achieved in spiked samples was 5×10^1 CFU mL^{-1}. This relatively good sensitivity is likely to be due to the use of the exclusive antibodies selected from the Dresden panel [22,23] which has already proven its strong efficiency when testing the capability of an immunomagnetic separation assay on highly contaminated environmental samples [21]. Quantitative changes in electric parameters demonstrated that the epoxysilane monolayer and the antibody immobilization act as barriers for the electron transfer between the electrode surface and the redox species in the solution, resulting in most significant increases in the charge transfer resistance compared to all other impedance components. When compared to previously described immunosensors aimed at detecting *L. pneumophila* in environmental samples, our assay is easy to use, non-time consuming and does not require labels or specific sample preparation procedure. It benefits from a unique design through the use of a monoclonal antibody which has a particular affinity for the targeted bacteria and, provided that our preliminary results on spiked samples are confirmed in natural water samples, has the potential to be used as an alternative to current detection methods for *L. pneumophila*.

The addition of a recirculation sample device on the immunosensor may also increase its sensitivity. It may also be used to evaluate the risk of exposure to *L. pneumophila* with the potentiality to be used in various types of field applications (following aerosol sampling in a liquid medium for instance). For hot water, the miniaturization of the captor could allow its installation directly within water lines.

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