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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*

Mina Souiri [a], Nesrine Blel [a,b], Dejla Sboui [a,c], Lotfi Mhamdi [a], Thibaut Epalle [c], Ridha Mzoughi [b], Serge Riffard [c,*,1], Ali Othmane [a,1]

[a] Laboratory Advanced Materials and Interfaces, Faculty of Medicine, 5019 Monastir, Tunisia
[b] Regional Laboratory of Hygiene, University Hospital Farhat Hached, 4000 Sousse, Tunisia
[c] Groupe Immunité des Muqueuses et Agents Pathogènes (GIMAP), EA 3064, SFR 143, University of Lyon, F42023, France

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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

* Correspondence to: Groupe Immunité des Muqueuses et Agents Pathogènes (GIMAP), EA 3064, SFR 143, Faculté de Médecine J. Lisfranc, Université Jean Monnet, 15 rue Ambroise Paré, F42023, France. Tel.: +33 4 77 42 14 67; fax: +33 4 77 42 14 86.
E-mail address: serge.riffard@univ-st-etienne.fr (S. Riffard).
[1] Contributed equally to the work.

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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.