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Genosensor for detection of four pneumoniae bacteria using gold nanostructured screen-printed carbon electrodes as transducers

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ABSTRACT

This work describes the development of different formats of hybridization-based genosensors for pneumonia using disposable gold nanostructured screen-printed carbon electrodes. Gold nanoparticles, which have been formed in situ by applying a constant current intensity during a fixed time, act as an immobilization and transduction surface.

Immobilization takes place through thiol–gold interaction in a relatively fast way, or through the well-known biotin–streptavidin interaction.

Analytical parameters such as linearity range, reproducibility and detection limit of the genosensor were evaluated. The best results are obtained when the probe is attached to the electrode surface through the streptavidin–biotin interaction and fluorescein is used as label. In this case an anti-fluorescein antibody labelled with alkaline phosphatase is used to obtain the analytical signal.

1. Introduction

Mycoplasma pneumoniae has been associated with a variety of clinical manifestations, including those involving the respiratory tract. It is a significant cause of respiratory tract infections in humans and an important pathogen in acute respiratory illnesses in children and adults, accounting for as many as 20–40% of all cases of community-acquired pneumonia [1,2]. However, these types of infections are not only associated to this bacterium; there are four main bacteria associated to this pathology: *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, *Legionella pneumophila*, and *Streptococcus pneumoniae*, and the treatment differs depending on the causing bacteria [3–5].

Biosensors have become a very important area of analytical chemistry, since the majority of current and future analytical requirements are being solved by simple and sensitive devices [6].

Nucleic acid detection is becoming relevant in clinical diagnosis, because a DNA/RNA test not only addresses the question of whether a patient is infected with a particular pathogen or not but also that is very useful for treatment monitoring, since elimination of pathogen nucleic acids indicates successful handling. This is why the development of electrical DNA hybridization biosensors has attracted considerable research efforts in the last years [7]. Such DNA sensing applications require high sensitivity through amplified transduction of the hybridization reaction. Electrochemical devices offer elegant routes for interfacing at molecular level, DNA recognition and signal transduction elements, and are uniquely qualified for meeting the low-cost, low-volume, and power requirements of decentralized DNA diagnostics [8,9].

Nanostructured materials have received broad attention due to their distinguished performance in catalytic, optic, and electronic devices among others. These materials frequently display unusual physical and chemical properties, usually different from those of the corresponding bulk materials and also tunable by changing parameters like size, shape, or surface chemistry of these nanostructures [10]. The unique properties of nanoscale materials offer excellent prospects for interfacing biological recognition events with electronic signal transduction and for designing a new generation of bioelectronic devices exhibiting novel functions [9].

Due to their astonishing properties, nanoparticles present numerous possibilities for applications in nanotechnology, but assembly of nanoparticles in regular patterns on surfaces and interfaces is required, generating new nanostructures. With this aim, electrochemical and wet-chemical methods are demonstrated to be effective approaches to make metal nanostructures under control without addition of a reducing agent or protecting agent [11,12].

Gold nanoparticles (AuNPs), have received a special attention due to their unique properties concerning immobilization of biomolecules retaining their biological activity, as efficient conducting interfaces with electrocatalytic ability and high surface-to-volume ratio that makes them a powerful tool to

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modify electrode materials and to construct robust and sensitive biosensors which can find application in many fields of interest [13], infusing new vigour into electrochemistry [14,15].

The AuNPs modified electrochemical sensing interfaces offer elegant ways for interfacing DNA recognition events with electrochemical signal transduction, and for amplifying the resulting electrical response. AuNPs-based amplification schemes reported have led to improved sensitivity of bioelectronic assays by several orders of magnitude. Several alternative ways of electrochemically detecting DNA are possible [8], however, several works report the use of enzymatic labels. The increase in sensitivity, due to their inherent amplification, makes possible to avoid PCR amplification [16–18]. However, this sensitivity must be complemented with specificity, which is reached by the adequate selection of probe and target sequences. One of the most used enzymatic labels for the development of DNA hybridization assays is alkaline phosphatase (AP). In our research group a methodology has been developed in which the substrate (3-indoxyl phosphate), produces a compound able to reduce silver ions in solution into a metallic deposit, which is located where the enzymatic label AP is attached. The deposited silver is electrochemically stripped into solution and measured by anodic stripping voltammetry. Compared with the direct voltammetric detection of indigo carmine, the anodic stripping voltammetry of silver ions is 14-fold more sensitive [19].

The use of several types of electrodes as electrochemical transducers for DNA hybridization sensors has recently been summarized [20]. Gold has always been an appropriate material for the electrochemical detection of substances, as well as allowing different formats. Moreover gold also offers the relevant possibility of generating self-assembled DNA monolayers through thiol groups [21] due to the strong binding between sulphur and gold, which may be considered almost covalent [22].

In this paper, a DNA hybridization assay with enzymatic electrochemical detection was carried out on a gold nanostructured screen-printed carbon electrode (SPCnAuE), which allows working with small volumes, and has been proved to obtain greater sensitivity than that obtained with non-nanostructured surfaces [23]. Although thick gold substrates are reported in the literature for enzymatic DNA detection (screen-printed gold electrodes [24], 2 μm thick film gold electrodes [25], or gold disk electrodes [26]), gold nanoparticles have been unusually used as electrochemical transducers [23], in despite of their widespread use as DNA labels due to the electrochemical properties of gold nanoparticles [27].

2. Materials and methods

2.1. Apparatus and electrodes

Voltammetric measurements were performed with a μAutolab Type II potentiostat (EcoChemie B.V., Ultrecht, The Netherlands) controlled by the Autolab GPES software version 4.9. All measurements were carried out at room temperature.

Screen-printed carbon electrodes (SPCEs) were purchased from DropSens (Oviedo, Spain). These electrodes incorporate a conventional three-electrode configuration, printed on ceramic substrates (3.4 cm × 1.0 cm). Both working (disk-shaped 4 mm diameter) and counter electrodes are made of carbon inks, whereas pseudo-reference electrode and electric contacts are made of silver. An insulating layer was printed over the electrode system, leaving uncovered the electric contacts and a working area which constitutes the reservoir of the electrochemical cell, with an actual volume of 50 μL.

A JEOL JSM-6100 scanning electron microscope (20 kV, Japan) was used to characterize the working electrodes.

2.2. Reagents and solutions

Synthetic oligonucleotides were obtained from Isogen (Barcelona, Spain). The target sequence employed corresponds to a portion of the genotype of four bacteria responsible of community-acquired pneumonia.

Mycoplasma pneumoniae (*MP*)
Target: biotin/fluorescein
–5′-TTG-GCA-AAG-TTA-TGG-AAA-CAT-AAT-GGA-GGT-TAA-CCG-AGT-G-3′
Probe: SH–$(CH_2)_3$/biotin
–5′-CAC-TCG-GTT-AAC-CTC-CAT-TAT-GTT-TCC-ATA-ACT-TTG-CCA-A-3′
Legionella pneumophila (*LP*)
Target: biotin/fluorescein –5′-AGT-GAA-TTT-TGC-AGA-GAT-GCA-TTA-GTG-CCT-TCG-GGA-ACA-CTG-AT-3′
Probe: SH–$(CH_2)_3$/biotin – 5′-ATC-AGT-GTT-CCC-GAA-GGC-ACT-AAT-GCA-TCT-CTG-CAA-AAT-TCA-CT-3′
Chlamidophila pneumoniae (*CP*)
Target: biotin/fluorescein – 5′-ATT-TGA-CAA-CTG-TAG-AAA-TAC-AGC-TTT-CCG-CAA-GGA-CAG-ATA-C-3′
Probe: SH–$(CH_2)_3$/biotin –
5′-GTA-TCT-GTC-CTT-GCG-GAA-AGC-TGT-ATT-TCT-ACA-GTT-GTC-AAA-T-3′
Streptococcus pneumoniae (*SP*)
Target: biotin/fluorescein – 5′-CTC-TGA-CCG-CTC-TAG-AGA-TAG-AGT-TTT-CCT-TCG-GGA-CAG-AGG-TG-3′
Probe: SH–$(CH_2)_3$/biotin – 5′-CAC-CTC-TGT-CCC-GAA-GGA-AAA-CTC-TAT-CTC-TAG-AGC-GGT-CAG-AG-3′

Probes were immobilized on the gold nanostructured surface and, depending on the study, were labelled with a thiol group, separated from the first base by an aliphatic linker of three carbons, or by a biotin group. Target oligonucleotides were labelled with a biotin or fluorescein group.

Oligonucleotide solutions were prepared in 0.1 M Tris–HNO_3 pH 8.0 buffer solution. Aliquots were prepared and maintained at −20 °C. Working solutions were stored at 4 °C. Hybridization took place in a 2× SSC (saline sodium citrate) buffer, i.e. a 30 mM sodium citrate buffer with 300 mM sodium nitrate containing 1% BSA, pH 7.2.

3-Indoxyl phosphate (3-IP) was purchased from Biosynth (Switzerland).

Bovine serum albumin (BSA, fraction V), casein from bovine milk (90%), magnesium nitrate, silver nitrate, trizma base, anti-fluorescein antibody labelled with alkaline phosphatase (Ab-AP), streptavidin (St) and streptavidin labelled to alkaline phosphatase (S-AP) were supplied by Sigma.

A mixture solution of 5.6 mM 3-IP and 0.4 mM silver nitrate were prepared daily in 0.1 M Tris–HNO_3 pH 9.8 buffer containing 20 mM $Mg(NO_3)_2$ and stored in opaque tubes at 4 °C.

Solutions of casein were prepared daily in 0.1 M Tris–HNO_3 pH 7.2 buffer.

Aliquots of S-AP were prepared in 0.1 M Tris–HNO_3 pH 7.2 buffer, containing 2 mM $Mg(NO_3)_2$, and aliquots of St were prepared in 0.1 M Tris–HNO_3 pH 7.2 buffer. Both were maintained at −20 °C. Working solutions were prepared in the same buffer and conserved at 4 °C.

Dilutions from Ab-AP were daily prepared in 0.1 M Tris–HNO_3 pH 7.2 buffer, containing 2 mM $Mg(NO_3)_2$.

Standard gold (III) tetrachloro complex was purchased from Merck (1.000 ± 0.002 g of tetrachloroaurate (III) in 500 mL 1 M HCl) Dilutions from this standard solution were prepared in 0.1 M HCl.

Hydrochloric acid (37%), sodium citrate and sodium nitrate were purchased from Merck.

All other chemicals employed were of analytical reagent grade. Ultrapure water obtained with a Milli-RO 3 plus/Milli-Q plus 185 purification system from Millipore Ibérica S.A. (Madrid, Spain) was used throughout this work.