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Enzymatic amplification-free nucleic acid hybridisation sensing on nanostructured thick-film electrodes by using covalently attached methylene blue

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ABSTRACT

Amplification-free (referring to enzymatic amplification step) detection methodologies are increasing in biosensor development due to the need of faster and simpler protocols. However, for maintaining sensitivity without this step, highly detectable molecules or very sensitive detection techniques are required. The nanostructuration of transducer surfaces with carbon nanotubes (CNTs), gold nanoparticles (AuNPs) or both in nanohybrid configurations has been employed in this work for DNA hybridisation sensing purposes. Methylene blue (MB), covalently attached to single stranded DNA, (ssDNA) was incubated with a complementary sequence immobilized on nanostructured screen-printed electrodes (AuSPEs). Although CNTs can increase notoriously the signal of the marker, adsorptive properties should also be considered when bioassays are performed because non-specific adsorption (NSA) phenomena are magnified. In this work, strategies for decreasing NSA were thoroughly evaluated for the detection of *Mycoplasma pneumoniae* (MP) on CNTs-nanostructured screen-printed electrodes. Among them, the employ of UV-radiation or long incubation times (72 h) allowed obtaining higher signals for the complementary strand with respect to the non-complementary one. The use of CNTs/AuNPs nanohybrids, together with the use of streptavidin–biotin (ST–B) interaction allows the higher differentiation (with a 3.5 ratio) in the genosensing of *M. pneumoniae*.

1. Introduction

Amplification-free (with no enzymatic reaction steps) detection methodologies are currently having a great development as biosensing detection strategies mainly due to the simplification of the procedures and the reduction of analysis time and costs [1]. Enzymes are active proteins that constitute the best example of amplification approaches, since one molecule can covert a high number of substrate molecules into a detectable product. This is the reason why they have been widely employed as labels for indirect detection methods of affinity assays [2], with almost infinite possibilities such as redox cycling [3], bienzymatic approaches [4] or product signal amplification [5]. In the special case of DNA sensors, one of the strands has to be labeled to an enzyme. Although equilibrium measurements are possible, in order to decrease analysis time, kinetic measurements are commonly made and a strict control of conditions has to be followed, usually including a reaction stopping step [6]. Non-enzymatic based approaches will produce faster speed of response and also lower costs since expensive bioreagents are avoided. Direct monitoring of the analyte through a reporter molecule is possible in different ways, mainly by differential interaction with the single and double strands or by covalent attachment of the indicator to the DNA oligonucleotide.

Methylene blue (MB), 3,7-bis(dimethylamino)phenazathionium chloride is a biolabel that has been used as an indicator of the hybridization event [7–17] through the electrostatic interaction between cationic MB and anionic DNA [11,12], by intercalation of MB in the DNA double helix between alternating G–C base sequences [13,14] and by covalent bound to the end of the single-stranded DNA target (ssDNA) [15,16], the least exploited of all. An immobilized stem-loop strand with MB attached at the end is employed in the first strategy, in such a way that the signal decreases after hybridization and subsequent opening of the strand [15]. An unlabeled strand is immobilized in the second strategy, that proposes both, direct and competitive assays, with MB covalently labeled to a complementary strand [16]. Gold disk [15] and thin film [16] non-disposable electrodes are employed. The covalent attachment avoids the step of washing away the

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excess of label because the labeled strand that did not interact, it is eliminated after the hybridization event. Moreover, the complementary covalently labeled DNA strand contains all-in-one the hybridization and detection agents, simplifying the procedure.

Since the discovery of carbon nanotubes (CNTs) [18] intensive studies have been made, receiving a great deal of attention for their properties, especially high specific surface area, electronic conductivity, chemical and electrochemical stability and one dimensional tubular-structure [19]. Other nanostructures that have aroused great interest owing to unique electric, magnetic, optical and catalytic properties are gold nanoparticles (AuNPs), with applications in several areas [20,21]. These properties differ from those of the bulk materials and mainly depend on the particle size and morphology [22]. Combination of both in AuNPs/CNTs nanohybrids generates a new kind of composite materials which successfully integrate the unique properties of two class materials and exhibit some new functions caused by the cooperative effects between them. Therefore, nanohybrids have shown very attractive applications in many fields. Since the first report about the assembly of Au nanoparticles on carbon nanotubes [23] the number of literatures escalates at an enormously increasing rate each year.

Mycoplasma pneumoniae (*MP*) is a very small bacterium that has been associated to a variety of clinical infections in humans, including those involving the respiratory tract. It is considered an important pathogen responsible for acute respiratory illnesses in children and adults. Approximately 10% of the cases of community-acquired pneumonia that occur endemically, and up to 50% of the cases that occur in epidemic periods are caused by *M. pneumoniae* [24]. Its diagnosis still relies on classical methods of culture and serology. Bacteria culture is time consuming, labor-intensive and expensive with only 23–64% of serologically cases successfully diagnosed [25]. These methods are easier to perform but are generally non-specific [26], and insensitive [27].

In this work, an enzyme-free electrochemical sensor strategy was proposed. With this aim, methylene blue (MB) covalently attached to ssDNA was incubated with complementary ssDNA on nanostructured screen-printed gold electrodes (AuSPEs) and the electrochemical detection of the hybridization event was then carried out. A non-complementary sequence corresponding to *Chlamydophila pneumoniae*, a widely distributed pathogen that also causes infections of the respiratory tract was employed for studying non-specific adsorptions. This bacterium is responsible, apart from sinusitis, for pharyngitis, bronchitis and community-acquired pneumonia, the most important clinical manifestation [28] in elderly and debilitated patients as well as in healthy adults. This is the first time, to the best of our knowledge, that an electrochemical genosensor based on the detection of MB as electroactive biolabel on AuSPEs modified with either carbon nanotubes (CNTs), gold nanoparticles (AuNPs) or both (in a nanohybrid configuration) is performed.

2. Experimental

2.1. Reagents and solutions

Synthetic oligonucleotides were obtained from Isogen (Barcelona, Spain) and from BioTez (Berlin, Germany). The target sequence employed corresponds to a portion of the genotype of one of the bacteria responsible of community acquired pneumonia, *M. pneumoniae* (Myc). The probe sequences, complementary (SH-Myc) and non-complementary (SH-Chla, *Chlamydophila pneumoniae*), are labelled in the 5′-end with a thiol group separated from the first base by an aliphatic linker of three carbons. The sequences employed in this work were

MB labeled ssDNA (40-mer)	**MB-Myc**	5′-MB-TTG GCA AAG TTA TGG AAA CAT AAT GGA GGT TAA CCG AGT G-3′
Complementary ssDNA (40-mer)	Without modification, **Myc**	5′-CAC TCG GTT AAC CTC CAT TAT GTT TCC ATA ACT TTG CCA A-3′
	Thiolated, **SH-Myc**	5′-SH-$(CH_2)_3$-CAC TCG GTT AAC CTC CAT TAT GTT TCC ATA ACT TTG CCA A-3′
	Biotinylated, **B-Myc**	5′-B-CAC TCG GTT AAC CTC CAT TAT GTT TCC ATA ACT TTG CCA A-3′
Non-complementary ssDNA (40-mer)	Without modification, **Chla**	5′-GTA TCT GTC CTT GCG GAA AGC TGT ATT TCT ACA GTT GTC AAA T-3′
	Thiolated, **SH-Chla**	5′-SH-$(CH_2)_3$-GTA TCT GTC CTT GCG GAA AGC TGT ATT TCT ACA GTT GTC AAA T-3′
	Biotinylated, **B-Chla**	5′-B-GTA TCT GTC CTT GCG GAA AGC TGT ATT TCT ACA GTT GTC AAA T-3′

Oligonucleotide solutions were prepared in 0.1 M Tris–HNO_3 pH 8.0 buffer solution, 1 mM EDTA. 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. Tris–hydroxymethyl–aminomethane hydrochloride (Trizma), streptavidin (ST, molecular weight 66 kDa) and bovine serum albumin (BSA, fraction V) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nitric acid (65% purity), sodium citrate and sodium nitrate were obtained from Merck (Darmstadt, Germany). EDTA (Ethylenediaminetetraacetic acid) was provided by Panreac (Barcelona, Spain).

Amine-functionalised carbon nanotubes (MWCNT-NH_2) were obtained from Belgium Nanocyl (Auvelais, Belgium). CNTs were produced *via* the catalytic carbon vapor deposition (CCVD) process. They have 9.5 nm of average diameter, < 1 μm of average length, > 95% in carbon purity and < 0.5% of functionalization. Their dispersion was carried out in a Nafion®/ethanol solution [29]. Standard gold (III) complex (1.000 ± 0.001 mg of tetrachloroaurate (III) in 500 mL of 1 M HCl) was purchased from Merck. Dilutions from this standard solution were prepared in 0.1 M HCl. Nafion® (perfluorinated ion-exchange resin, 5 wt% solution in a mixture of lower aliphatic alcohols and water) was purchased from Sigma-Aldrich and ethanol and hydrochloric acid (37% purity) were obtained from Merck.

Casein from bovine milk, gelatin from cold water fish skin, poly-ethylene glycol (PEG), 1-hexanethiol, 6-mercapto-1-hexanol (MCH) and 4-mercapto-1-butanol (MCB) were obtained from Sigma-Aldrich and Tween 20 was provided by Merck.

Water was purified employing a Milli-Q direct QS system from Millipore (Bedford, MA, USA).

2.2. Apparatus

Voltammetric and chronopotentiometric measurements were performed with an Autolab PGSTAT 10 (ECO Chemie) potentiostat interfaced to a Pentium 120 computer system and controlled by Autolab GPES software version 4.8. A JEOL JSM-5600 Scanning Electron Microscope (30 kV) was used to characterize the working gold electrodes modified with carbon nanotubes dispersions. An Elma ultrasonic bath, a Nahita centrifuge with interchangeable car, a Mettler Toledo (AB54) balance, a Crison Micro-pH 2001 pH-meter, a