Talanta 142 (2015) 11–19

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

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

Raquel García-González, Agustín Costa-García, M. Teresa Fernández-Abedul *

Departamento de Química Física y Analítica, Universidad de Oviedo, 33006 Oviedo, Asturias, Spain

ARTICLE INFO

Article history:
Received 22 September 2014
Received in revised form
11 March 2015
Accepted 15 March 2015
Available online 24 March 2015

Keywords:
Amplification-free biosensors
Nanohybrids
Nanostructured gold screen-printed electrodes
Nucleic acid strands detection
Methylene blue

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

* Corresponding author. Tel.: +34 985102968; fax: +34 985103125.
E-mail address: mtfernandeza@uniovi.es (M.T. Fernández-Abedul).

http://dx.doi.org/10.1016/j.talanta.2015.03.028
0039-9140/

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

magnetic stirrer Asincro (J.P. Selecta), a Sanyo refrigerator and a Sanyo (MIR-162) incubator were also used. Finally, a UV lamp (MLW 160 W) was obtained from Philips.

2.3. Voltammetric measurements

In all cases, voltammograms were recorded in buffer solution. Different techniques were employed: cyclic (v=0.25 V s^{-1}), square wave (f=50 Hz, A=0.05 V, s=0.008 V) and differential pulse (A=0.05 V, s=0.008 V) voltammetry, scanning the potential from 0.0 to −0.7 V. Due to their low cost and the time needed for effective regeneration, electrodes were considered as single-use devices. Therefore, all measurements were recorded on 3 disposable electrodes and error bars are included in the corresponding graphics.

2.4. Gold screen printed electrodes (AuSPEs)

Gold screen-printed electrodes purchased from DropSens (Llanera, Spain) include a traditional three-electrode configuration printed on the same strip. The format of these SPEs includes a gold disk electrode (12.6 mm^2) as working electrode, a silver pseudo-reference electrode and a gold counter electrode with the same ink of the working electrode. All of them are screen-printed on a ceramic substrate (3.4 × 1.0 × 0.05 cm^3) and subjected to high-temperature curing (AuSPE-AT). An insulating layer serves to delimit the working area and electric contacts. The production characteristics of commercial SPEs are regarded by the manufacturers as proprietary information. A specific connector supplied by DropSens allows their connection to the potentiostat.

2.5. Dispersion of carbon nanotubes

Most of the applications of CNTs and SPEs require a pre-solubilization for obtaining a homogeneous dispersion. Achieving this step is not easy because CNTs are not dispersed in many solvents. The CNTs weight/solvent volume ratio and the dispersion procedure are very important. In this case, an ultrasonic bath and a centrifuge with interchangeable car for 1.5 and 5 mL were employed for dispersion, following a procedure previously optimized [30]. Briefly, 1 mg of MWCNTs-NH_2 was dissolved in 1 mL of 0.5% Nafion® (in EtOH) and 4 cycles (2 h under sonication and 10 min of centrifugation) were applied. After each cycle the precipitate is discarded and the supernatant is subjected to a new cycle. Finally, a completely homogeneous dispersion was obtained.

2.6. Nanostructuration of SPEs with CNTs, AuNPs and nanohybrids formation on electrode surface

Nanostructuration of electrodes with CNTs was carried out by evaporation at room temperature of a drop of CNTs dispersion deposited on the working electrode. The procedure was previously described by our research group [31]. Similarly, the electrochemical generation of gold nanostructures on the electrode surface was performed for modifying its surface. The procedure was reported, for the detection of lead using underpotential deposition [32]. To obtain gold nanoparticles on the electrode surface, an aliquot of 40 µL of a solution of $AuCl_4^-$ in 0.1 M HCl was dropped onto the SPEs and a constant current was applied for a fixed time to ensure good reproducibility. Different current intensities and deposition times were tested. A chronopotentiogram was recorded during the electrodeposition of gold. After the electrodeposition of gold, AuNPs–AuSPEs were generously rinsed with water.

The modification of the screen-printed electrodes with nanohybrid structures was previously studied in our group [33]. AuSPEs were modified with 4 µL of 0.1 mg mL^{-1} MWCNTs dispersion and the solution was left to dry at room temperature until its complete evaporation. Then, the modified electrode was carefully washed with water and dried at room temperature. The coating process was followed by *in situ* electrochemical deposition of gold nanoparticles (AuNPs) by applying a constant current intensity for a time in an acidic solution of $AuCl_4^-$. The resulting AuNPs–MWCNTs–AuSPEs were rinsed with water and were ready to use.

2.7. DNA-assay formats

Two different assay formats that employed single stranded DNA probes were tested. In both cases, the hybridization assay is performed on CNTs nanostructured AuSPEs. In the conventional assay, Fig. 1, an amount of thiolated DNA probe sequence is deposited on the electrode surface and maintained at 37 °C for 30 min. After washing with buffer solution, incubation with the MB-labelled complementary DNA target takes place for 60 min at room temperature. The electrochemical measurement is carried out in a 40 µL-drop of buffer solution after washing the electrode surface with this solution.

In the second format, two electrochemical measurements must be recorded. After the immobilization of the MB-labelled DNA sequence and a washing with buffer solution, the first electrochemical measurement is performed. Further incubation with the DNA probe sequence takes place for 60 min at 37 °C. After this hybridization step and the corresponding washing, the second electrochemical measurement is performed. A net signal is obtained as a difference between both signals.

In both formats and in order to avoid non-specific adsorptions, after the immobilization of the DNA probe, a blocking step is

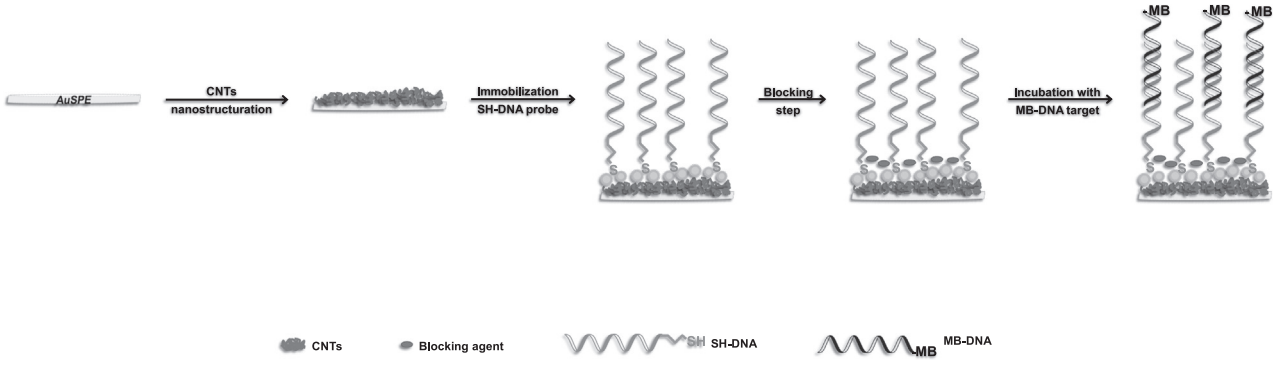

Fig. 1. Scheme of the conventional hybridization assay employed.

carried out. This was performed using different physical strategies or chemical agents that prevent or minimize non-covalent interactions.

Apart from taking advantage of the interaction Au–S, the biotin–streptavidin affinity interaction was also employed as an immobilization strategy. This was carried out using AuSPEs that were nanostructured with CNTs and AuNPs. After the exhaustive washing step with water and drying under a N_2 stream, a 10 μL-drop of 10^{-7} M streptavidin (in Tris–H_2SO_4 pH 7.2) was deposited on the WE overnight at 4 °C. Then, a 40 μL-drop of B-Myc (complementary strand) or B-Chla (non-complementary strand) in 2 × SSC buffer solution were placed on the electrode surface at room temperature for 30 min. The incubation with the target DNA is performed by placing 40 μL-drop of B-Myc at 37 °C for 1 h. A washing step is performed with the next buffer solution between each step. Finally, electrochemical measurements (DPV) were performed in a deoxygenated buffer solution.

3. Results and discussion

Methylene blue is a molecule that presents a well-defined two-electron redox process [34] with cathodic (conversion to leucomethylene blue (LB)) and anodic (reoxidation to MB) peaks. The electrochemical behavior of this molecule converts it into a suitable candidate for electrochemical labeling. Since a covalent attachment does not produce a high label density (in this case one DNA strand wear one MB molecule), the transducer has to favor electron transfer. Although modification of electrodes implies a slowdown in the process due to the inclusion of new stages, nanostructuration of SPEs can be performed in order to improve their sensitivity [35]. It has been demonstrated that nanostructuration with MWCNTs-NH_2 allows a very substantial increase in the analytical signal of MB covalently attached to single stranded DNA [36]. In this case, a DNA sensor based in the hybridization of immobilized probes with a MB labeled strand (target) is intended.

Screen-printed electrodes are mass-produced low-cost miniaturized electrochemical cells and then, they allow paralellization of assays. Therefore, probe immobilization as well as the rest of the steps required in a specific study could be made in parallel. Later, after hybridization and detection, electrodes are disposed.

3.1. Evaluation of DNA concentrations.

The study of DNA concentrations (probe and target sequences) was carried out on CNTs nanostructured AuSPEs, with SWV as the electrochemical measurement technique. With this aim, concentrations of 10, 50, 100, 500 and 1000 nM of probe DNA strands were incubated for 60 min with 10, 50, 100 and 500 nM target MB labeled DNA sequences. The results obtained, reported in Fig. 2 show higher analytical signals for the immobilization of 500 nM probe strand. At the same time, when this strand is immobilized, the peak current increases for lower target DNA concentrations, in such a way that the combination 500 nM/10 nM gave the highest intensity.

3.2. Blocking step

Blocking the surface after the immobilization step is one of the most important to minimize and control non-specific adsorptions (NSA). The bars diagrams corresponding to DPV and SWV signals after the incubation of the MB labeled strand for 30 min on a CNT modified surface with and without immobilized complementary strand and further washing are included in the Supplementary material section (Fig. S1). Even though the adsorption of MB-ssDNA

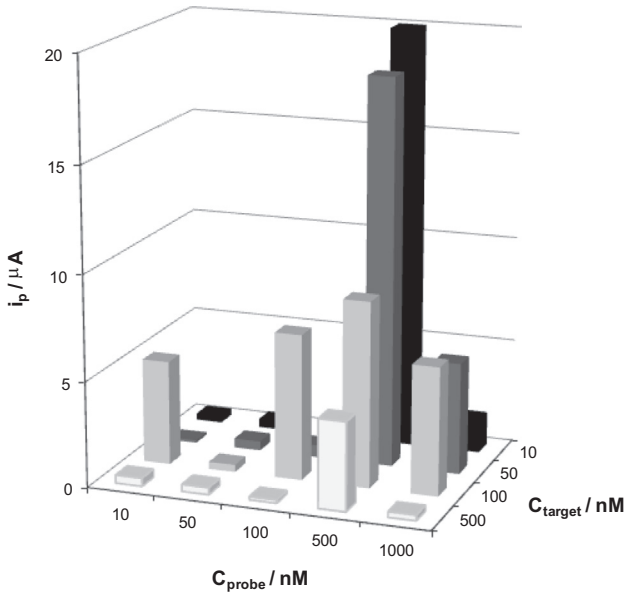

Fig. 2. Optimization of probe and DNA target concentrations. Conditions: CNTs–AuSPEs as surface transducer and 30 min for immobilization and hybridization steps.

on nanostructured electrodes is known [36], it was expected that the specific interaction with the complementary strand should increase the amount of captured MB-strand. However, it can be seen that NSA occur in a great extension, being lower the signal after the specific interaction and constituting the 84.2% and 76.5% (for DPV and SWV, respectively) of the NSA. This adsorption-controlled process of MB occurs on CNTs-nanostructured electrodes. However, a notorious increase of the MB signal is produced when compared to bare electrodes [36]. In order to take advantage of this enhancement but avoiding at the same time NSA phenomena, different strategies were checked. Several agents can be employed as potential blockers of these interactions.

3.2.1. Chemical blocking agents

The most common strategy for avoiding non-specific adsorptions is the addition of a blocking agent (commonly a protein) that fills void spaces of the electrode surface. In many cases, when several steps are included in the bioassay, surfactants are included in the washings for breaking weak interactions, usually between antibodies. In our case, biomolecules such as proteins and also DNA were checked, and also polymers such as the cationic exchanger Nafion® and PEG. On the other hand, and taking into account that a gold electrode is employed as substrate, one of the types of molecules more employed for modifying the surface and properties of gold electrodes are alkanethiols, being in this case important not to block the electron transfer.

3.2.1.1. Biomolecules. Proteins as bovine serum albumin (BSA) [6,37] or casein [38–40] are common blocking agents in bioanalysis and adsorb well onto gold surfaces, due to their amino and thiol groups. Aqueous solutions of BSA and casein were tested as blocking agents. With this aim, a 15 μL-drop of a 2% solution was deposited on CNTs nanostructured WEs and maintained for 30 min before washing. Then, a MB-labelled DNA strand was immobilized for 60 min at room temperature. Finally, a washing was performed before the electrochemical measurement is recorded. Results obtained are presented in the bar diagram of Fig. S2, included in the Supplementary material section. As can be seen here, the BSA blocking power is lower than this of casein. Thus, smaller currents

were obtained in the case of casein, *i.e.* -8 ± 1 and -6 ± 1 μA for BSA and casein respectively. Even though, the currents recorded after the blocking step are high compared with those obtained without any blocking (only a 20% and 40% of decrease in the signal is obtained for BSA and casein, respectively) and therefore, other agents have to be introduced. A mixture of 0.8% gelatin including a 0.05% of the surfactant Tween 20 [41] was also checked, but not appropriate blocking effect was produced.

On the other hand, another biomolecule, a DNA strand could be used as blocking agent and its use has been reported. With the aim of knowing if this approach can be used for eliminating the signal of non-specific MB, thiolated *Mycobacterium* (complementary) and *Chlamydophila* (non-complementary) *pneumoniae* DNA strands, both of the same length, were immobilized together on the electrode surface for 60 min. In this way, the incubation of MB-ssDNA (MB-Myc) will interact only with the specific strands and no adsorption will occur on the CNTs/gold surface, now occupied also by the non-complementary strand. However, as shown in the bar diagram of Fig. 3A, even when the blocking strand is employed a differentiation between Myc and Chla strands is not possible.

3.2.1.2. Polymers. The important blocking effect observed when AuSPEs were modified with a Nafion®/ethanol solution [29] employed for CNTs dispersion was here used as possible blocking agent of the surface before the hybridization assay. This polymer produces a total blockage of the electrode surface and no signal is observed for MB after modification. However, when CNTs are added on this blocking layer, the signal is totally recovered. Similarly, the modification with CNTs dispersion on Nafion®/ethanol does not produce this blocking.

After immobilization of the DNA probe (SH-Myc) and washing, a 4 μL-drop of 0.5% Nafion®/ethanol solution was deposited until evaporation of the solvent. The intensities of the signals recorded after interaction with the MB-labeled *Mycobacterium* strand are shown in the bar diagram of Fig. 3B. As it can be seen, a blocking of the electron transfer occurred, so when the nanostructured electrode with immobilized DNA is modified with a Nafion® membrane, lower intensities were recorded for both, complementary and non-complementary strands. However, discrimination from the background was higher, indicating a decrease in the NSA. In SWV these currents were -2.1 ± 0.5 and -0.9 ± 0.5 μA for the complementary and non-complementary strands, respectively ($S/B=2.3$). However, the analytical signal should be as high as possible and blocking of the MB-electron transfer for the specific interaction is not desired.

The use of a different polymer, PEG, as blocking agent in biosensors is reported in the bibliography [42]. Amphiphilic molecules, as proteins, can interact with surfaces *via* a variety of intermolecular forces (van der Waals, electrostatic and Lewis acid–base) and also *via* entropically driven effects such as hydrophobic interactions and conformational changes. The end-result of these effects is an apparent irreversibility of the adsorption process [43], leading to increased background signal in the sensor. The inclusion of polar (neutral) molecules minimizes protein interaction with surfaces and therefore, their adsorption. In our case, a 2% PEG aqueous solution was employed as blocking agent before the hybridization assay. Photographies of three electrodes without blocking step (Fig. 3C-1) and with PEG as blocking agent (Fig. 3C-2) are presented. Loss of CNTs and immobilized DNA strands occurs and can be clearly seen. Incompatibility between PEG and the hybridization assay here proposed indicates that another blocking agent should be employed.

3.2.1.3. Thiols. Thiols are a group of compounds that contain sulphur atoms and therefore present a high affinity for gold. Solutions of mercaptohexanol (MCH) [44,45] and 1-hexanethiol [46] are usually employed for generating self-assembled monolayers (SAMs) [47]. There is a compromise between length of the chain and blocking effect. Higher lengths could be difficult for electronic transfer [48] and the smaller ones result to be extremely volatile [6]. The use of MCH in the formation of SAMs to control the structure of DNA monolayers on gold surfaces was reported by Levicky et al. [49]. The negatively charged $-$OH head group of the MCH repels the negatively charged SH-ssDNA and prevent the non-specific DNA interactions and orients the flat lying SH-ssDNA strands in perpendicular configuration to give high freedom for the target coiling to form double stranded DNA (dsDNA) [50]. Moreover, assembly of MCH created a compact layer, which efficiently prevents the non-specific adsorption of the complementary or non-complementary sequences on the gold surface [45]. The adsorption kinetics of alkanethiols on a gold surface is often described as a two-step process [51]. Initially, there is a fast (few minutes) growth of the film thickness to 80–90% of the final value, followed by a slower process in which both the thickness and wettability approach an equilibrium value in approximately 10–20 h. As it depends on the composition and concentration, lower time (3 h) has been reported for a mercaptoundecanoic acid SAM (40 mM) [52]. The alkanethiol immobilization leads also to a decrease in the charging currents [6,48], obtaining well-defined signals when compared with other common blocking agents. MCB is a less exploited thiol blocking agent, that was used by Lisdat et al. [17] for reducing non-specific DNA binding in the development of a label-free DNA sensor. In this work, 1-hexanethiol and MCB were tested.

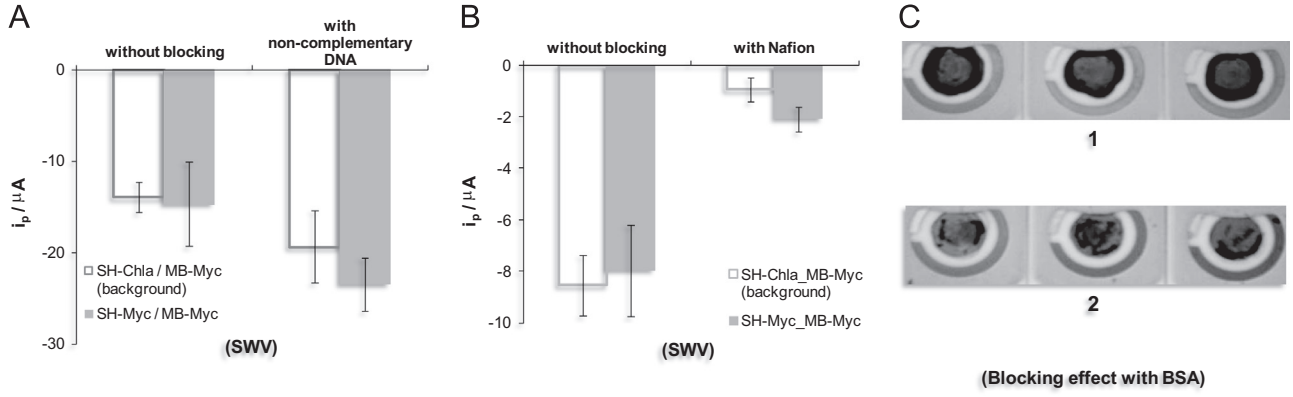

Fig. 3. Effect of biomolecules and polymers as blocking agents. Bars diagram corresponding to the hybridization assay development after surface blocking with non-complementary DNA (Conditions: CNTs–AuSPEs as surface transducer, 30 min as blocking time and 60 min as immobilization time) (A) and with 4 μL-drop of 0.5% Nafion®/ethanol solution (B). Photographs of the effect associated to the blocking with PEG (C). C1 without blocking step and, C2 with a 2% of PEG.

A 15-μL drop is deposited on the WE until its evaporation (10 min), taking into account the kinetics of alkanethiol adsorption on gold [53]. In the case of 1-hexanethiol (Fig. 4A) no blocking effects were observed (only 41.7% blocking capacity was calculated). The bar diagram shows also an unusual effect. Lower currents were recorded without blocking step and when 1-hexanethiol was used as blocking agent, higher intensities were obtained.

MCB was also checked but employing different conditions (80 μL for 3 h) based on a previously described work [17]. Fig. 4A shows the bar diagram from SWV obtained, with peak currents higher than those by DPV (not shown). Values are -42 ± 1 and -39 ± 4 μA (in DPV, with and without blocking respectively) and -133.1 ± 0.9 and -110 ± 14 μA (in SWV, with and without blocking respectively), indicating a blocking capacity lower than 14.5%.

3.2.2. Physical blocking strategies

It has already been reported in the literature photodesorption phenomenon from carbon nanotubes and this strongly depends on the wavelength of light [54], the details of which lead to a fundamental understanding of how light stimulates molecular desorption from CNTs, which can be applied to nanotube-based molecular electronics, miniature chemical sensors, and optoelectronic devices. In our case, UV-light isolation was checked in order to minimize non-specific adsorptions. In this case, nanostructured electrodes with immobilized probe strands were insolated for 10 min with a UV-lamp (λ=340–380 nm). Then, the hybridization event was conducted normally and the electrochemical signal was recorded by DPV and SWV with results shown in Figs. S3 (included in the Supplementary material section) and 4B, respectively. Similar peak currents were obtained for both complementary and non-complementary strands in DPV (-6.0 ± 0.8 and -6.5 ± 0.2 μA, respectively) and a small differentiation could be seen by SWV (-29 ± 3 and -36.0 ± 0.9 μA, respectively). In this case, the signal is 19.4% higher for the complementary strand, which means a blocking capacity of 19.4%. Higher differences should be desirable for the bioassay and conditions were more carefully studied.

3.2.3. Assay conditions

One of the first parameters that has to be considered in bioassays is the incubation time. This is very important in eliminating the cross reactivity in immunoassays in which a competition between structurally similar molecules trying to bind to an antibody occurs. The kinetics of antigen–antibody reactions is such that, in most cases, cross reactivity decreases with incubation time and is minimal when equilibrium is reached. The time necessary to reach equilibrium varies considerably depending on reaction conditions, but most non-specific interactions could be decreased with time [55]. In these cases, the desire for rapid assays and increase in productivity must be carefully weighed against the possibility of decreased specificity. In our case, the DNA probe immobilization time was studied in order to minimize non-specific adsorptions. This time was dramatically increased up to 72 h, in order to observe its effect. The increase in the surface coverage values with immobilization times until 72 h was previously observed in the rapid screening of mutated genes [56], with increase in the peak current after hybridization with the complementary sequence. This could be due to the length of the probe, as strands would need more time not only for attachment but also for reorganization on the electrode surface. In our case, results obtained (Fig. 4B) show that the immobilization of the probe sequence under these conditions partly prevents the generation of non-specific adsorptions and currents obtained with non-complementary probe are much lower than those for the complementary one. It seems that the ratio signal/background is good, especially when SWV as electrochemical technique was used (value of 3.17). In this case, the signal for the non-complementary strand is almost similar (-24 ± 3 μA) but the signal for the complementary one is greatly magnified, indicating that equilibrium times are high.

The joint immobilization of CNTs and DNA probe strands was tested too and compared with the assay performed in two steps: nanostructuration of gold surface and probe immobilization. Thus, there will be a competition between CNTs and DNA for its linkage to the gold electrode surface. This joint immobilization simplifies the hybridization assay because the inclusion of elements for biorrecognition and transducer modification takes place in one step. The washing step in-between is then eliminated. With this aim, a 4 μL of a CNTs dispersion and 5 or 40 μL of DNA sequences (SH-Myc and SH-Chla) were deposited on the working electrode and the evaporation was performed at 37 °C for 30 min. Volumes of 5 or 40 μL were chosen in order to compare the effect of the presence of the DNA strand only in WE or in the electrochemical cell. Results were compared with those obtained for the two-step format. Several designs were checked. They are collected in Table 1 together with the peak currents obtained. In view of these, we can conclude that higher currents were obtained for the two steps assay, *i.e.* nanostructuration electrodes and DNA strand immobilization in separate stages, and for 40 μL of DNA strand.

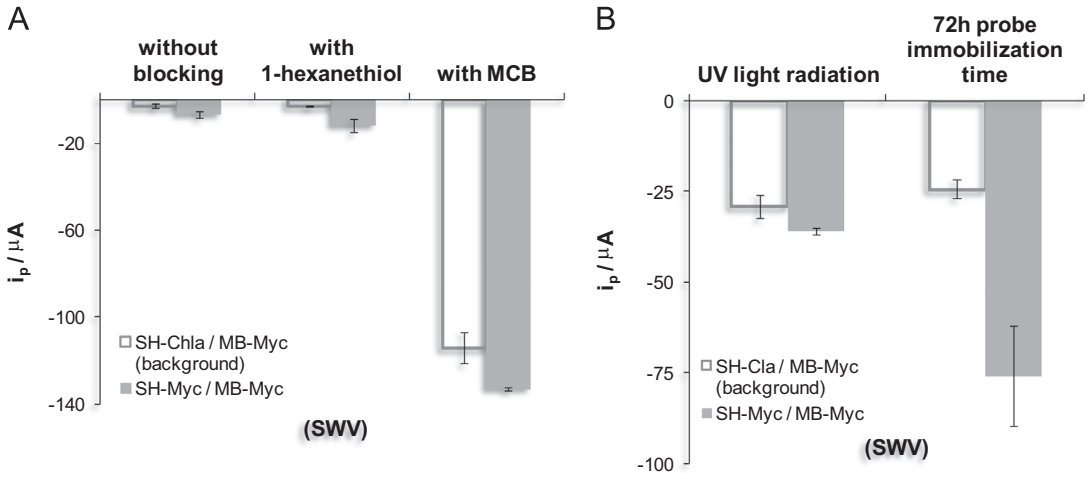

Fig. 4. Effect of thiols and physical blocking strategies. Bars diagram corresponding to the hybridization assay development without blocking and after surface blocking with 1 mM 1-hexanethiol and 1 mM MCB (A), after the treatment with UV-light radiation (B) or with 72 h as immobilization time (C).

Table 1
Analytical signals obtained for MB-labeled DNA on CNTs nanostructured electrodes.

Format	Electrode nanostructuration	DNA immobilization	Electrochemical measurement (i_p/μA)		
			CV	DPV	SWV
1 (2 Steps)	4 μL CNTs, 70 °C 1 h	5 μL MB-Myc 37 °C 30 min	-0.22 ± 0.03	-0.71 ± 0.06	-3.7 ± 0.2
2 (2 Steps)	4 μL CNTs, 70 °C 1 h	40 μL MB-Myc 37 °C 30 min	-3.1 ± 0.3	-5.8 ± 0.3	-20 ± 1
3 (1 Step)	4 μL CNTs + 5 μL MB-Myc 37 °C 30 min		-0.40 ± 0.02	-0.90 ± 0.04	-9.0 ± 0.6
4 (1 Step)	4 μL CNTs + 40 μL MB-Myc 37 °C 30 min		-0.29 ± 0.03	-1.01 ± 0.09	-10.4 ± 0.8

The analytical uses of CNTs can be classified based on their adsorptive, electronic or other properties [57] in such a way that electroanalytical applications are commonly included in the second approach. However, these methodologies take advantage from both electronic and sorption properties [58] (actually adsorptive stripping voltammetry on CNTs-modified electrodes takes advantage of sorption phenomena [59]). These are very important in the development of biosensors where apart from covalent, non-covalent interactions with the large active surface of CNTs can facilitate the adsorption of analytes. In our case, since a stepped bioassay takes place before transduction, only the electronic properties are desired. With the aim of avoiding CNTs adsorptive properties as well as non-specific interactions, addition of CNTs is performed after the hybridization event. Due to this premise hybridization assay was realized on AuSPEs and CNTs solution was added later in order to enhance the sensitivity. However, since the electrode surface after hybridization assay is wet, the drop of CNTs, partly organic, diffused throughout the electrochemical cell. When this happens, the interconnection between the three electrodes of the potentiostatic system occurs and stops working properly. However, even when adhesive stickers were used to better define the working electrode area, very broad peaks with low intensities were obtained and sometimes the drop spilled across the electrochemical cell. Also as this work is tedious and irreproducible this type of modification was discarded.

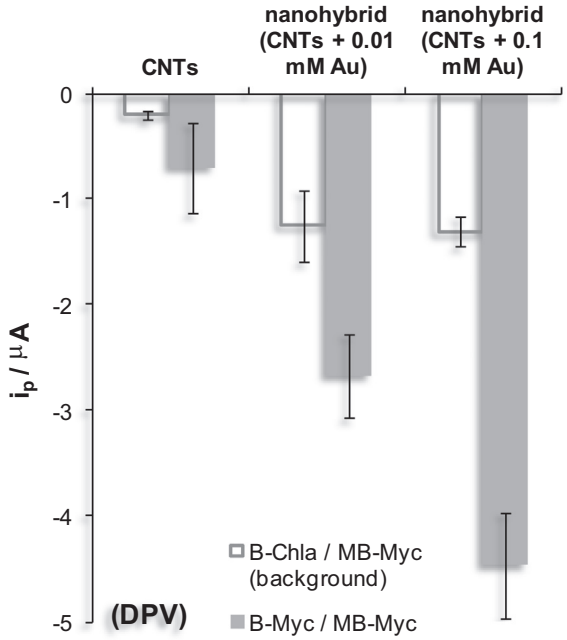

Fig. 5. Bars diagram corresponding to the nanohybrid-based assay ([ST] = 10^{-7} M).

3.3. Nanohybrid-based assay

In order to decrease the MB adsorption on CNTs and taking advantage of nanostructuration, a hybrid approach (with CNTs and AuNPs) was checked. The introduction of gold nanoparticles onto the electrochemical interfaces has infused new vigor to electrochemistry and different strategies were designed for enhancing analytical selectivity and sensitivity [60]. They have received considerable attention due to their relative high surface area-to-volume ratio and excellent catalytic activity. On the other hand, nanohybrid materials (*e.g.* carbon and gold) have been reported as very appropriate transducer surfaces for electrochemical sensing [61].

In this case, a design was proposed based on the streptavidin (ST)–biotin (B) interaction, a biological reaction model. It is also likely that ST, being a protein, acts in part as a blocking agent for avoiding NSA. Several conditions were checked and results obtained from three of them were collected in Fig. 5. When CNTs–AuSPEs were used, similar intensities were obtained from background and from hybridization assay with complementary probe DNA (data not shown). When AuNPs–AuSPEs were used, recorded intensities were extremely low (data not shown). However, the nanohybrid structure formed with CNTs and 0.01 mM Au^{3+} presents a slight difference between the two assays. Recorded intensities were -1.2 ± 0.3 and -2.7 ± 0.4 μA from background and the hybridization assay. The last experience presents the higher difference between the two signals, *i.e.* -1.3 ± 0.1 and -4.5 ± 0.5 μA from background and hybridization assay. It can be seen that the signal due to the non-specific adsorptions is maintained meanwhile the signal increases by using higher gold concentration and therefore higher nanoparticle density. The nanohybrid structured from CNTs and *in situ* AuNPs electrochemical generation with 0.1 mM Au^{3+} supposes a blocking capacity (complementary/non-complementary ratio) of 3.5. Apart from selectivity, sensitivity was also considered. The limits of detection (LOD) and quantitation (LOQ) were calculated on the basis of *S*/*N* equal to 3 and 10 for LOD and LOQ, respectively. Values of 1.6 and 2.3 nM were obtained for the limits of detection and quantitation, respectively. Since all the steps of the different procedures are performed successively in parallel, neither stability nor regeneration was considered in this work. Regeneration takes time, not only due to the activity of regeneration itself but also for the necessity of performing studies sequentially. Additionally, effectivity is difficult to achieve when biomolecules are considered. However, for simple determination of molecules that does not adsorb on electrodes, reuse is also possible [62]. In this case, since biomolecules are involved, they are always used as disposable.

In Table 2 a summary of the results obtained for each approach is presented. In all the cases, since the complementary/non-complementary ration is higher than 1, a blocking capacity is observed, being higher for the last assay, and very low for the employ of MCB, UV radiation or non-complementary DNA. Very good values are obtained also for long incubation times, demonstrating a magnification of the specific interaction. However, long analysis times are not desirable. The use of nanohybrid structuration and the immobilization through ST–B interaction allowed attaining slightly higher blockings.

Table 2
Summary of results obtained with each blocking agent or strategy.

Blocking	Signal increase over the blocked (%)	Non-complementary signals (μA)	Complementary signals (μA)	Complementary/non-complementary signals	Comments
Nonblocking	–	-14 ± 2	-15 ± 5	1.1	–
Non-complementary DNA	37.5	-19 ± 4	-24 ± 3	1.3	No effective blocking
Nafion®	57.1	-0.9 ± 0.5	-2.1 ± 0.5	2.3	Blocking of the electron transfer even for complementary strand
1-Hexanethiol	41.7	-7 ± 1	-12 ± 3	1.7	No effective blocking
MCB	14.4	-106 ± 3	-133.1 ± 0.9	1.2	No effective blocking and very high intensities for non-complementary assay
UV radiation	19.4	-29 ± 3	-36.0 ± 0.9	1.2	No effective blocking
72 h immobilization	68.4	-24 ± 3	-80 ± 14	3.3	Acceptable blocking effect but analysis times too high
Nanohybrids	–	-1.3 ± 0.1	-4.5 ± 0.5	3.5	Acceptable blocking effect

4. Conclusions

In this work, a hybridization assay was developed with a thiolated probe and single-stranded DNA of *M. pneumoniae* with methylene blue as electrochemical biolabel, covalently attached in an enzymatic amplification-free electrochemical design. The assay was carried out with nanostructured AuSPEs as transducer surface. CNTs, AuNPs and nanohybrid of both were checked. MB-DNA is adsorbed onto CNTs and therefore an exhaustive study of possible strategies and agents to avoid non-specific adsorptions was thoroughly made. Several blocking strategies and agents were probed. An acceptable blocking is obtained after increasing the immobilization time to 72 h. The electrochemical generation of AuNPs on CNTs–AuSPEs to form nanohybrids as transducer surface together with the immobilization through the biotin–streptavidin interaction decreases considerably the NSA without increasing analysis time.

In this case, the ratio between the complementary/non-complementary signal was 3.5 for nanohybrids of CNTs and AuNPs obtained from 0.1 mM Au^{3+}.

Acknowledgments

This work has been supported by the Ministry of Science and Innovation (MICINN) under Project CTQ2011-25814.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2015.03.028.

References

[1] X. Liu, Q. Huo, J. Immunol. Methods 349 (2009) 38–44.
[2] C.-H. Kim, L.-P. Lee, J.-R. Min, M.-W. Lim, S.-H. Jeong, Biosens. Bioelectron. 51 (2014) 426–430.
[3] A. Walter, J. Wu, G.-U. Flechsig, D.A. Haake, J. Wang, Anal. Chim. Acta 689 (2011) 29–33.
[4] C.-H. Zhou, Y.-M. Long, B.-P. Qi, D.-W. Pang, Z.-L. Zhang, Electrochem. Commun. 31 (2013) 129–132.
[5] J. Su, H. Zhang, B. Jiang, H. Zheng, Y. Chai, R. Yuan, Y. Xiang, Biosens. Bioelectron. 29 (2011) 184–188.
[6] P. Abad-Valle, M.T. Fernández-Abedul, A. Costa-García, Biosens. Bioelectron. 20 (2005) 2251–2260.
[7] K. Kerman, D. Ozkan, P. Kara, B. Meric, J.J. Gooding, M. Ozsoz, Anal. Chim. Acta 462 (2002) 39–47.
[8] Y. Wang, A. Zhou, J. Photochem. Photobiol. A 190 (2007) 121–127.
[9] E. Farjami, L. Clima, K.V. Gothelf, E.E. Ferapontova, Analyst 135 (2010) 1443–1448.
[10] S.F. Baranovskii, P.A. Bolotin, M.P. Evstigneev, D.N. Chernyshev, J. Appl. Spectrosc. 75 (2008) 251–260.
[11] D. Pau, X. Zuo, Y. Wan, L. Wang, J. Zhang, S. Song, C. Fan, Sensors 7 (2007) 2671–2680.
[12] E. Tuite, B. Norden, J. Am. Chem. Soc. 116 (1994) 7548–7556.
[13] R. Rohs, H. Sklenar, R. Lavery, B. Roder, J. Am. Chem. Soc. 122 (2000) 2860–2866.
[14] S.O. Kelley, J.K. Barton, N.M. Jackson, M.G. Hill, Bioconjugate Chem. 8 (1997) 31–37.
[15] A.A. Lubin, R.Y. Lai, B.R. Baker, A.J. Heeger, K.W. Plaxco, Anal. Chem. 78 (2006) 5671–5677.
[16] O. Pänke, A. Kirbs, F. Lisdat, Biosens. Bioelectron. 22 (2007) 2656–2662.
[17] J. Kafka, O. Pänke, B. Abendroth, F. Lisdat, Electrochim. Acta 53 (2008) 7467–7474.
[18] S. Iijima, Nature 354 (1991) 56.
[19] K.P. Gong, F. Du, Z.H. Xia, M. Durstock, L.M. Dai, Science 323 (2009) 760.
[20] V. Georgakilas, D. Gournis, V. Tzitzios, L. Pasquato, D.M. Guldi, M. Prato, J. Mater. Chem. 17 (2007) 2679.
[21] P.D. Cozzoli, T. Pellegrino, L. Manna, Chem. Soc. Rev. 35 (2006) 1195.
[22] G. Hodes, Adv. Mater. 19 (2007) 639.
[23] X. Li, Y. Liu, L. Fu, L. Cao, D. Wei, G. Yu, D. Zhu, Carbon 44 (2006) 3113–3148.
[24] H.M. Foy, G.E. Kenny, M.K. Cooney, I.D. Allan, J. Infect. Dis. 139 (1979) 681–687.
[25] J. Williamson, B.P. Marmion, D.A. Worswick, T.-W. Kok, G. Tannock, R. Herd, R.J. Harris, Epidemiol. Infect. 109 (1992) 519–537.
[26] M. Sillis, Rev. Med. Microbiol. 4 (1993) 24–31.
[27] J.W. Dorigo-Zetsma, S.A.J. Zaat, P.M.E. Wertheim-van Dillen, L. Spanjaard, J. Rijntjes, G. van Waveren, J.S. Jensen, A.F. Angulo, J. Dankert, J. Clin. Microbiol. 37 (1999) 14–17.
[28] M. Heinemann, W.V. Kern, D. Bunjes, R. Marre, A. Essig, Clin. Infect. Dis. 31 (2000) 181–184.
[29] R. García-González, M.T. Fernández-Abedul, A. Costa-García, Talanta 107 (2013) 376–381.
[30] P. Fanjul-Bolado, P. Queipo, P.J. Lamas-Ardisana, A. Costa-García, Talanta 74 (2007) 427–433.
[31] R. García-González, A. Fernández-la Villa, A. Costa-García, M.T. Fernández-Abedul, Sens. Actuators B 181 (2013) 353–360.
[32] G. Martínez-Paredes, M.B. González-García, A. Costa-García, Electrochim. Acta 54 (2009) 4801–4808.
[33] M.M.P.D. Neves, M.B. González-García, C. Delerue-Matos, A. Costa-García, Electroanalysis 23 (2011) 63–71.
[34] E.M. Boon, D.M. Ceres, T.G. Drummond, M.G. Hill, J.K. Barton, Nat. Biotechnol. 18 (2000) 1096–1100.
[35] R. García-González, A. Costa-García, M.T. Fernández-Abedul, Sens. Actuators B 202 (2014) 129–136.
[36] R. García-González, A. Costa-García, M.T. Fernández-Abedul, Sens. Actuators B 191 (2014) 784–790.
[37] M. Díaz-González, M.B. González-García, A. Costa-García, Sens. Actuators B 113 (2006) 1005–1011.
[38] M.M.P.S. Neves, M.B. González-García, A. Santos-Silva, A. Costa-García, Sens. Actuators B 163 (2012) 253–259.
[39] V. Escamilla-Gómez, D. Hernández-Santos, M.B. González-García, J. M. Pingarrón-Carrazón, A. Costa-García, Biosens. Bioelectron. 24 (2009) 2678–2683.

[40] G. Martínez-Paredes, M.B. González-García, A. Costa-García, Sens. Actuators B 149 (2010) 329–335.
[41] Z.C. Sánchez-Acevedo, J. Riu, F.X. Rius, Biosens. Bioelectron. 24 (2009) 2842–2846.
[42] J.F. Masson, M.T. Battaglia, J.M. Davidson, Y.-C. Kim, A.M.C. Prakash, S. Beaudoin, K.S. Booksh, Talanta 67 (2005) 918–925.
[43] W. Kusnezow, J.D. Hoheisel, J. Mol. Recognit. 16 (2003) 165–176.
[44] H.K. Suda, D.Y. Petrovykh, M.J. Tarlov, L.J. Whitman, J. Am. Chem. Soc. 125 (2003) 9014–9015.
[45] I. Palchetti, S. Laschi, G. Marrazza, M. Mascini, Anal. Chem. 79 (2007) 7206–7213.
[46] P. Abad-Valle, M.T. Fernández-Abedul, A. Costa-García, Biosens. Bioelectron. 22 (2007) 1642–1650.
[47] S. Dulay, P. Lozano-Sánchez, E. Iwuoha, I. Katakis, C.K. O'Sullivan, Biosens. Bioelectron. 26 (2011) 3852–3856.
[48] M. Akram, M.C. Stuart, K.Y.D. Wong, Anal. Chim. Acta 504 (2004) 243–251.
[49] R. Levicky, T. Herne, M. Tarlov, S. Satija, J. Am. Chem. Soc. 120 (1998) 9787–9792.
[50] V. Dharuman, Jong Hoon Hahn, Sens. Actuators B 127 (2007) 536–544.
[51] C.D. Bain, E.B. Troughton, Y.-T. Tao, J. Evall, G. Whitesides, R.G. Nuzzo, J. Am. Chem. Soc. 111 (1989) 321–335.
[52] S. Campuzano, R. Gálvez, M. Pedrero, F.J.M. de Villlena, J.M. Pingarrón, Electroanal. Chem. 526 (2002) 92–100.
[53] W. Pan, C.J. Durning, N.J. Turro, Langmuir 12 (1996) 4469–4473.
[54] R.J. Chen, N.R. Franklin, J. Kong, J. Cao, T.W. Tombler, Y. Zhang, H. Dai, Appl. Phys. Lett. 79 (2001) 2258–2260.
[55] E.P. Diamandis, T.K. Christopoulos, Immunoassay, Academic Press, London (1996) 172.
[56] T. García, M.G. Fernández-Barrena, M. Revenga-Parra, A. Núñez, E. Casero, F. Pariente, J. Prieto, E. Lorenzo, Anal. Bioanal. Chem. 398 (2010) 1385–1393.
[57] M. Valcárcel, S. Cardenas, B.M. Simonet, Anal. Chem. 79 (2007) 4788–4797.
[58] M.T. Fernández-Abedul, A. Costa-García, Anal. Bioanal. Chem. 390 (2008) 293–298.
[59] M. Trojanowicz, Trends Anal. Chem. 25 (2006) 480–489.
[60] S. Guo, E. Wang, Anal. Chim. Acta 5989 (2007) 181–192.
[61] M.M.P. da Silva Neves, M.B. González-García, C. Delerue-Matos, A. Costa-García, Electroanalysis 23 (2011) 63–71.
[62] R. García-González, M.T. Fernández-Abedul, A. Pernía, A. Costa-García, Electrochim. Acta 53 (2008) 3242–3249.