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# Impedimetric genosensors employing COOH-modified carbon nanotube screen-printed electrodes

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ABSTRACT

Screen-printed electrodes modified with carboxyl functionalised multi-walled carbon nanotubes were used as platforms for impedimetric genosensing of oligonucleotide sequences specific for transgenic insect resistant Bt maize. After covalent immobilization of aminated DNA probe using carbodiimide chemistry, the impedance measurement was performed in a solution containing the redox marker ferrocyanide/ferricyanide. A complementary oligomer (target) was then added, its hybridization was promoted and the measurement performed as before. The change of interfacial charge transfer resistance between the solution and the electrode surface, experimented by the redox marker at the applied potential, was recorded to confirm the hybrid formation. Non-complementary DNA sequences containing a different number of base mismatches were also employed in the experiments in order to test specificity. A signal amplification protocol was then performed, using a biotinylated complementary target to capture streptavidin modified gold nanoparticles, thus increasing the final impedimetric signal (LOD improved from 72 to 22 fmol, maintaining a good reproducibility, in fact RSD < 12.8% in all examined cases). In order to visualize the presence and distribution of gold nanoparticles, a silver enhancement treatment was applied to electrodes already modified with DNA–nanoparticles conjugate, allowing direct observation by scanning electron microscopy.



## 1. Introduction

Electrochemical impedance spectroscopy (EIS) is a rapidly developing technique for the transduction of biosensing events at the surface of an electrode (Berggren et al., 1999). Due to its effectiveness to directly probe the interfacial properties (capacitance, electron transfer resistance) of modified electrodes (Gabrielli, 1990; Patolsky et al., 1998), EIS is becoming an attractive electrochemical tool for numerous applications such as immuno (Willner and Willner, 1999; Farace et al., 2002; Huang et al., 2006) and genosensing (Tlili et al., 2005; Peng et al., 2007; Xu et al., 2004), enzyme activity determination (Kharitonov et al., 2000; Saum et al., 1998), studies of corrosion (Mansfeld et al., 1997; Rout, 2007) and surface phenomena (Millan et al., 1994). In the more recent years, EIS (MacDonald, 1987; Bard and Faulkner, 2000) was widely used for the detection of DNA hybridization, occurring at a sensor surface (Breggen et al., 2000; Katz and Willner, 2003; Lisdat and Shafer, 2008).

Carbon nanotubes (CNTs) have attracted increasing interest of many researchers due to their remarkable tensile strength, high resistance, flexibility and other unique structural, mechanical, electrical and physicochemical properties (Khabashesku et al., 2005; Hu et al., 2005; Treachy et al., 1996) (high specific surface area, electrocatalysis effects reflected on increased signal currents and decreased overpotentials, versatility for biofunctionalization). Besides these brilliant properties, also the possibility to functionalize them directly with different kinds of biomolecules (Daniel et al., 2007; Vairavapandian et al., 2008) and their biocompatibility make CNTs extremely attractive for electrochemical sensing. Numerous platforms employing different kinds of CNTs alone or combined with gold nanoparticles were recently fabricated and characterized (Gong et al., 2008; Vairavapandian et al., 2008; Yun et al., 2008). Electrodes modified with CNTs have been employed for the improved detection of either inorganic or biological molecules (Strano, 2004; Wang et al., 2004a,b). CNTs have been recently used as transducers for enhanced electrical detection of DNA hybridization (Wang et al., 2003, 2004a,b). CNTs were also used for biosensing in combination with nanoparticles in order to improve the final response (Wang et al., 2003; Male et al., 2004).

DNA biosensor technologies are rapidly developing as an alternative to the classical genic assays, due to several features such as low cost, rapid analysis, simplicity and possibility of multiplexing and miniaturization (Cattrall, 1997). Genosensors (or DNA biosensors) are devices that combine a transducer with a single-stranded DNA (ssDNA) called DNA probe, acting as a recognition element. These devices make use of hybridization event to detect a target

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DNA sequence (Yang et al., 1997). The determination of nucleic acid fragments from humans, animals, plants, bacteria and viruses is the departure point to solve different problems: investigation about food and water contamination caused by micro organisms, detection of genetic disorders, identification of species, tissue matching, forensic applications, etc. (Drummond et al., 2003; Righetti and Gelfi, 1997).

One of the most recent and requested DNA-sensor applications is the detection of genetically modified organisms (GMOs) in foods. A GMO is referred to a living organism whose genome has been modified by the introduction of an exogenous gene able to express an additional protein that confers new characteristics. An intense debate is in progress, which is the ethical use of transgenic crops to improve agricultural productivity in food production. The main transgenic crops are soybean, maize and cotton, and the major incorporated traits include herbicide tolerance and resistance to virus, antibiotics and insects (Mariotti et al., 2002; Minunni et al., 2001). The potentially increasing number of GMO-derived commercialized products, present at the moment in the food market has led many countries to require regulation and labeling of grains and foodstuff containing GMOs. For this reason, there is a strong interest in the development of reliable and rapid analytical methods for GMO detection and quantification (Erickson, 2000; Hubner et al., 1999). At present, analytical methods for GMOs can detect both the new protein expressed by the genetically modified DNA sequence or the sequence itself. The detection of the former is generally based on enzymatic immunoassay analysis (Lipp et al., 2000; Stave, 2002). However, since food processing often leads to protein denaturation, the analysis of DNA, which is much highly stable than proteins, is preferred for screening of both raw ingredients and processed products. DNA recognition is based on the hybridization of target DNA sequence with GMO-specific probes that are immobilized on the surface of the sensor (Kalogianni et al., 2006). At the moment, various transduction principles have been reported, such as protocols based on the use of quartz crystal microbalance (Minunni et al., 2001; Mannelli et al., 2003), surface plasmon resonance (Giakoumaki et al., 2003; Feriotto et al., 2002) or electrochemical transduction based on the use of electroactive compounds (Minunni et al., 2001).

In this work we report the impedimetric detection of transgenic insect resistant Bt maize DNA, by the use of carbon nanotubes as sensing platform. Carbon nanotubes used were modified with carboxylic groups, which allowed the covalent immobilization of the oligonucleotide sequence under study. The impedimetric technique, employed for the first time with this kind of electrodes, allowed either the detection of complementary DNA sequences or their quantification. Moreover, with the use of a signal amplification protocol based on the use of streptavidin-modified gold nanoparticles (strept-AuNPs), the detection of different number of mismatches was attainable.

## 2. Experimental

### 2.1. Materials

Potassium ferricyanide $K_3[Fe(CN)_6]$, potassium ferrocyanide $K_4[Fe(CN)_6]$, streptavidin-gold nanoparticles (Ref. S9059), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), sodium dodecylsulphate (SDS), iron (III) nitrate nonahydrate, were purchased from Sigma (St. Louis, MI). N-Hydroxysulphosuccinimide sodium salt (sulpho-NHS), poly(ethylene glycol) (PEG) was purchased from Fluka (Buchs, Switzerland). Hydroxylamine hydrochloride was purchased from Merck (Darmstadt, Germany). LI Silver Enhancement Kit was obtained from Nanoprobes (Yaphank, NY). Other reagents were commercially available and were all of analytical reagent grade. All solutions were made up using doubly distilled water. The following buffer was employed: 0.1 M PBS (0.1 M NaCl, 0.01 M sodium phosphate buffer, pH 7.0).

The different oligonucleotides used in the study were prepared by TIB-MOLBIOL (Berlin, Germany). Their sequences and modifications are the following: probe-$NH_2$: 5′ $NH_2$-CCGCTGTATC ACAAGGGCTG GTAC 3′; complementary target: 5′ GTACCAGCCC TTGT-GATACA GCGG 3′; target-bio: 5′ BIO-GTACCAGCCC TTGTGATACA GCGG 3′; target with one mismatch: 5′ GTACCAGCCC TTGT**C**ATACA GCGG 3′; target with two mismatches: 5′ GTACCAG**G**CC TTGTGATAC**C** GCGG 3′; target with three mismatches: 5′ GTACC**T**GCCC TTGTG**C**TACA G**A**GG 3′; non-complementary target: 5′ AAAAAAAAAA AAAAAAAAAA AAAA 3′.

Stock solutions of the oligonucleotides were diluted with sterilized and deionised water, separated in fractions and stored at a temperature of −20 °C. When required, a single fraction was defrosted and used.

Screen-printed electrodes (SPEs) modified with carboxyl functionalised multi-walled carbon nanotubes (MWCNT-COOH, Ref. 110-CNT) were obtained from Drop Sens (Oviedo, Spain).

### 2.2. Apparatus

AC impedance measurements were performed with an IM6e Impedance Measurement Unit (BAS-Zahner, Germany). Thales software was used for the acquisition of the data and the control of the experiments. A three electrode cell was used to perform impedance measurements; working electrode (4 mm diameter) was made of –COOH modified carbon nanotubes, counter electrode was made of carbon, whereas reference electrode and electric contacts were made of silver.

A scanning electron microscope (SEM, Hitachi S-570, Tokyo, Japan) was used to visualize strept-AuNPs on electrode surface.

### 2.3. Biosensing protocol

#### 2.3.1. Electrochemical pre-treatment

The various SPE electrodes were electrochemically pre-treated (Musameh et al., 2005; McCreery, 2008) prior to voltammetric measurement (see 'Supporting information' section for experimental details).

#### 2.3.2. Immobilization of DNA probe

Modification of the MWCNT-COOH modified screen-printed electrodes was performed in different steps: (i) 15 μL of 0.05 M EDC and 0.03 M sulpho-NHS in PBS buffer, pH 7, were deposited onto the electrode surface for 15 min in order to activate carboxylic acid groups; (ii) the electrodes were then thoroughly rinsed with PBS; (iii) 15 μL of 3 pmol of probe-$NH_2$ solution in PBS, pH 7 (see 'Supporting information' section for optimization of probe concentration), were then deposited onto the electrode surface and let stood overnight at room temperature under a wet environment; (iv) this was followed by a washing step with 0.05% SDS and 0.04 M hydroxylamine hydrochloride to remove non-specific adsorbed probe and deactivate any remaining carboxylic group (Shervedani and Bagherzadeh, 2008). Negative controls were performed bypassing the first activation step.

#### 2.3.3. Hybridization with DNA target

Before hybridization, a blocking step was performed in order to avoid non-specific adsorption of target oligonucleotide. The electrode surface was treated with a 0.04 M solution of PEG in PBS, pH 7 for 15 min and then rinsed with PBS.

Hybridization was performed incubating the $NH_2$-probe modified electrodes with 15 μL of 2 pmol of target oligonucleotide solution in PBS buffer (concentration optimized in Section 3.1.2). The hybridization was achieved at room temperature, for 30 min.