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Biosensors based on carbon nanotube field effect transistors (CNTFETs) for detecting pathogenic microorganisms

A dissertation presented by

Raquel Amanda Villamizar Gallardo

to receive the degree of Doctor with the mention of Doctor Europeus
by the Universitat Rovira i Virgili

UNIVERSITAT ROVIRA I VIRGILI

Department of Analytical and Organic Chemistry

Tarragona, Spain. 2009

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Supervised by: Dr. Alicia Maroto

Prof. F. Xavier Rius

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DEPARTAMENT DE QUÍMICA ANALÍTICA
I QUÍMICA ORGÀNICA

Campus Sescelades
Marcel·lí Domingo, s/n
43007 Tarragona
Tel. 34 977 55 81 37
Fax 34 977 55 95 63
e-mail: secqaqo@quimica.urv.es

Dr. Alicia Maroto, Lecturer at the École Supérieure de Chimie Organique et Minérale (ESCOM), Compiègne, France and Dr. F. Xavier Rius, Professor at the Department of Analytical Chemistry and Organic Chemistry at the Universitat Rovira i Virgili,

CERTIFY:

The Doctoral Thesis entitled: "**Biosensors based on carbon nanotube field effect transistors (CNTFETs) for detecting pathogenic microorganisms**", submitted by **Raquel Amanda Villamizar Gallardo** to obtain the Doctor degree with European mention by the Universitat Rovira i Virgili, has been carried out under our supervision, at the Department of Analytical Chemistry and Organic Chemistry at the Universitat Rovira i Virgili, and all the results reported in this thesis were obtained from experiments performed by the above mentioned student.

Tarragona, November, 2009

Dr. Alicia Maroto **Prof. F. Xavier Rius**

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"To my dear husband Oscar and my divine force Ozy"

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Table of contents

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Summary

Microorganisms are present in a variety of sources, including food, water, animals, environment as well as the human body. They can be harmless or harmful. The latter is also called pathogenic and their detection is extremely important due to health and safety reasons.

It is well known that food contaminated with bacteria can produce a number of foodborne diseases. As a consequence, thousands of euros are invested each year in medical treatments trying to keep the population healthy. There are more than 250 known foodborne diseases. For example, outbreaks of salmonellosis have increased in many countries in the last decades being Salmonella Infantis one of the most important etiological agents associated with this enteric disease. Moreover, due to the wide distribution of the microorganisms, they can also contaminate foods in the field as well as during the storage stage. In that sense, filamentous fungi are one of the etiological agents responsible for most post-harvest food spoilage producing quality losses and economic devaluation.

On the other hand, the invasive fungal infections due to yeast have risen considerably in recent years. Candidiasis is the so-called disease produced by *Candida albicans.* This is an opportunistic infection that affects immunocompromised patients requiring costly treatment with advanced medicine.

Several methods have been proposed so far to detect pathogenic microorganisms. Conventional culture is highly selective and sensitive but they also require several days to yield the results. To simplify and automate the identification of both bacteria and fungi rapid biochemical kits have been developed. Although the results obtained with these kits are comparable to the traditional biochemical tests they also need 1 or 2 days to obtain results. Enzyme-linked immunosorbent assays (ELISA) can be applied for the direct identification of pathogenic microorganisms in real samples. This immuno-based method has been widely used in both food and the medical sector with high sensitivity. Nevertheless, the main disadvantage of this method is that it can also be time-consuming because a pre-enrichment of the

sample is often required in order to achieve low limits of detection. As a consequence, many researchers have addressed their efforts towards the development of alternative methods to allow the rapid detection of pathogens.

Molecular biology-based methods, specifically polymerase chain reaction (PCR) and real-time PCR are nowadays the most common tools used for pathogen detection. They are highly sensitive and allow the quantification of the target. In addition, microarray platforms of DNA have been developed in order to analyse hundreds of targets simultaneously. However, this technique is costly and reagent-consuming.

The introduction of biosensors has brought new alternatives in pathogenic detection. Biosensors are the most used tools in pathogenic detection after PCR, culture methods and ELISA. They provide rapid results after the sample has been taken. However, their real application lies in achieving selectivities and sensitivities comparable to the established methods and at low cost.

Since carbon nanotubes (CNTs) were discovered by Iijima, many papers have reported their unique electronic and optical properties which, together with their size, make these nanostructures interesting materials in the development of biosensing platforms. Their very high capacity for charge transfer between heterogeneous phases makes them suitable as components in electrochemical sensors. The electrical conductivity of the CNTs is highly sensitive to changes in their chemical environment and, as a result, they have been successfully applied in the study of molecular recognition processes.

An approach for the direct electrical detection of biomolecules integrates CNTs as transducer elements within a field-effect transistor (FET) configuration. The main advantages of this kind of configuration lies in that the conducting channel is usually located on the surface of the substrate and as a result, they are extremely sensitive to any change in the surrounding environment. Moreover, CNTFET devices can operate at room temperature and in ambient conditions.

At the beginning of this research (2006) electrochemical CNTFETs based on single walled carbon nanotubes had not been applied to detect bacteria or fungi. Only the interaction between CNTs and bacteria had been explored, but without sensing purposes. Therefore, this thesis reports the first CNTFET devices applied to the detection of pathogenic microorganisms. First, the background and the introduction containing the state of the art are presented covering relevant investigations made in the last years. Next, the main analytical methods are described. These descriptions involve detailed information of all procedures, analytical tools and materials used throughout this research work.

In the following chapters, the application of the CNTFETs for the determination of bacteria, yeast and moulds is presented throughout the scientific articles published along the development of the thesis. Briefly, the first device developed was applied to the detection of *Salmonella* Infantis in a simple matrix (0.85 % saline solution) and it was proven for first time, that this kind of sensor was able to detect, at least, 100 cfu/mL of the bacteria in just one hour with high selectivity. Subsequently, we enlarged the application field to other types of microorganisms: *Candida albicans.* In this study we improved not only the detection limit of the devices to 50 cfu/mL but also we proved the selectivity of the CNTFETs against possible interference that can be present in real samples like serum proteins. Finally, the devices were applied to the detection of the mould *Aspegillus flavus* in real samples. In this assay the response time was 30 minutes and a high sensitivity (10 µg of *A. flavus* / 25 g of rice) was obtained.

As the final chapters, general conclusions extracted from the overall work and annexes are reported. It can be stated that nanomaterials displaying extraordinary properties like carbon nanotubes can be combined with biological entities to obtain highly sensitive and selective biosensors able to detect bacteria, yeasts and moulds in a very short time. In future work, other performance parameters such as, long term stability, robustness and reusability must be studied further and contrasted with standard methods before thinking of the commercialization of the devices.

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Resumen

Los microorganismos están presentes en una gran variedad de orígenes, incluyendo alimentos, agua, animales, medio ambiente también como en el propio cuerpo humano. Estos pueden ser beneficiosos o perjudiciales. Los microorganismos perjudiciales reciben el nombre de patógenos y su detección es de gran importancia por razones de salud y seguridad.

Es bien conocido que los alimentos contaminados con bacterias pueden producir cierto número de enfermedades. Como consecuencia de esto, miles de euros se invierten cada año en tratamientos médicos para mantener la salud de la población. Existen más de 250 enfermedades transmitidas por alimentos. En las últimas décadas se ha incrementado por ejemplo, la incidencia de brotes de salmonelosis en muchos países, siendo *Salmonella* Infantis uno de los agentes etiológicos más importantes asociados con la producción de esta enfermedad entérica. Debido a la amplia distribución de los microorganismos, estos pueden llegar también a contaminar alimentos durante su cultivo como durante la fase de almacenamiento. En este sentido, los hongos filamentosos son en gran parte los agentes etiológicos responsables del deterioro de alimentos después de la cosecha produciendo pérdidas en la calidad y devaluación económica.

Por otra parte, las infecciones fúngicas invasivas producidas por levaduras han aumentado considerablemente en los últimos años. Candidiasis, es la enfermedad producida por *Candida albicans.* Esta es una de las infecciones más comunes que afectan pacientes inmunocomprometidos requiriendo tratamientos de elevado coste.

Se han propuesto varios métodos hasta la fecha para la detección de microorganismos patógenos. El cultivo es el método de referencia utilizado para la detección y cuantificación de bacterias. Tiene la ventaja de ser altamente selectivo y sensible pero tiene el inconveniente de requerir varios días para obtener un resultado. Para simplificar y automatizar la identificación de bacterias y hongos se han desarrollado kits bioquímicos rápidos. Aunque los resultados obtenidos

usando esta clase de kits son comparables a las pruebas bioquímicas tradicionales, también 1 o 2 días son requeridos para la obtención de resultados. El enzimoinmunoensayo ("Enzyme Linked Immunosorbent Assay", ELISA) es un método immunológico de gran sensibilidad que se utiliza ampliamente para detectar y cuantificar microorganismos patógenos, tanto en el sector médico como en el alimentario. Sin embargo, su principal desventaja es que a veces el tiempo de análisis puede aumentar considerablemente, específicamente cuando se realizan etapas de pre-enriquecimiento de la muestra para disminuir el límite de detección. Como consecuencia, muchos investigadores han dirigido sus esfuerzos hacia el desarrollo de métodos más rápidos.

Los métodos basados en el uso de la biología molecular, específicamente la reacción en cadena de la polimerasa (PCR) y la PCR en tiempo real, son hoy en día las herramientas más comúnmente usadas para la detección de patógenos. Estas técnicas son altamente sensibles y permiten la cuantificación del patógeno. Adicionalmente, se han desarrollado chips con plataformas de DNA para analizar cientos de patógenos simultáneamente. Sin embargo, esta técnica es costosa y requiere el uso de muchos reactivos.

La introducción de los biosensores ha contribuído a generar nuevas alternativas para la detección de patógenos. Los biosensores son las herramientas más usadas en la detección de patógenos después de la PCR, los métodos convencionales y el ELISA. Tienen la ventaja de proporcionar respuestas rápidas entre la toma de muestra y la obtención de los resultados. No obstante, el reto para su aplicación en muestras reales radica en alcanzar selectividades y sensibilidades comparables a los métodos convencionales ya establecidos y a un costo económico reducido.

Desde que Iijima descubrió los nanotubos de carbono (CNTs) se han publicado numerosos trabajos sobre sus excelentes propiedades electrónicas y ópticas, las cuales, en conjunción con su tamaño, hacen de estas nanoestructuras materiales interesantes en el desarrollo de plataformas de biodetección. Los CNTs presentan una gran capacidad de transferencia de carga entre estructuras heterogéneas. Ello

les confiere una gran utilidad en la elaboración de sensores de tipo electroquímico. Su conductividad eléctrica varía de forma muy acusada con cambios en su ambiente químico y, como resultado, se han aplicado con éxito en el estudio de procesos de reconocimiento molecular.

Una metodología para la detección directa de biomoléculas integra los CNTs como elementos transductores dentro de una configuración de transistor de efecto campo (FET). Las principales ventajas de esta clase de configuraciones radican en que el canal conductor se localiza sobre la superficie del substrato y, como resultado, es altamente sensible a cualquier cambio en el medio ambiente. Además, los CNTFETs pueden operar a temperatura y, humedad ambientales.

Al inicio de esta tesis (2006), todavía no se habían aplicado los CNTFETs basados en nanotubos de carbono monocapa a la detección de bacterias y hongos. Sólo se había estudiado la interacción entre los CNTs y bacterias, pero sin el objetivo de detección. Por tanto, esta tesis aporta los primeros CNTFETs aplicados a la detección de microorganismos patógenos. En primer lugar, se presentan los antecedentes y la introducción, donde se realiza una revisión crítica y actualizada de los métodos e investigaciones más relevantes para detectar microorganismos patógenos. Posteriormente, se incluye un capítulo con la información detallada de todos los procedimientos experimentales, herramientas analíticas y materiales utilizados a lo largo del trabajo de investigación.

En los siguientes capítulos, se presenta la aplicación de CNTFETs en la determinación de bacterias, mohos y levaduras mediante artículos científicos publicados a lo largo del desarrollo de la tesis. Brevemente, el primer dispositivo desarrollado se aplicó a la detección de *Salmonella* Infantis en una matriz simple (solución salina 0.85 %) y se comprobó por primera vez que esta clase de sensores eran capaces de detectar al menos 100 ufc/mL de la bacteria en tan solo una hora con alta selectividad. Seguidamente, se amplió el campo de aplicación a otro tipo de microorganismo, *Candida albicans.* En este estudio, se mejoró no sólo el límite de detección de los dispositivos a 50 ufc/mL sino que también se mejoró la selectividad de los CNTFETs frente a posibles interferentes que pueden estar

presentes en muestras reales, tales como proteínas séricas. Finalmente, se aplicaron los dispositivos a la detección del moho *Aspergillus flavus* en muestras reales. En este ensayo, el tiempo de respuesta fue de 30 minutos y se obtuvo una buena sensiblidad (10 µg de *A. flavus* / 25 g de arroz).

Como parte final de la tesis, se presentan las conclusiones generales extraídas a lo largo del trabajo completo junto con los anexos. Puede concluirse que, gracias a las propiedades únicas de los nanotubos de carbono, dichos nanomateriales pueden combinarse con entidades biológicas (como los anticuerpos) para obtener biosensores altamente sensibles y selectivos capaces de detectar bacterias, levaduras y mohos en un tiempo de análisis muy reducido. Como trabajo futuro, se deberán estudiar otros parámetros de calidad de los dispositivos tales como la estabilidad a lo largo del tiempo, la robustez o su reutilización con el fin de contrastarlos con los métodos estándar antes de poder iniciar la comercialización de este tipo de sensores.

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Chapter 1

Introduction

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1.1. Background

The incidence of diseases caused by foodborne pathogens and opportunistic yeasts and moulds has increased in the last years. Enteric diseases are normally related to bad consumer habits and bad manufacturing processing. Nowadays, fast-food is replacing traditional meals. A high volume of food is being processed every day and distributed to thousands of consumers and if it is contaminated, there exists a high potential risk of mass epidemics. In Spain (July, 2005), a batch of contaminated pre-cooked chicken resulted in a *Salmonella* outbreak causing 2500 sick people and at least one death by salmonellosis (Lazcka, O. et al, 2007). This bacterium can be found in a variety of sources, including food, water, animals and the environment causing serious morbidity, especially among infants and immunocompromised people (Marcus, R. 2008).

On the other hand, the incidence of invasive fungal infections due to yeast and mould has risen considerably in recent years. Mycoses produced by opportunistic fungi constitutes one of the most important infections in human beings. These fungi can be levaduriform or filamentous. Levaduriform fungi involves etiologic agents, such as; *Candida* spp., *Cryptococcus neoformans*, *Trichosporon* spp. and *Saccharomyces* spp.(Pontón, J. and del Palacio, A. 2007) where the main pathogen is *Candida albicans*. This yeast is the fourth leading cause of bloodstream infections in the USA; moreover, it causes life-threatening systemic infections in premature infants, surgical patients, chemotherapy patients, as well as other patients with weakened immune systems with a mortality rate of over 30 %. The strong prevalence of this yeast has been related to the excessive use of antifungal treatments. As a consequence, this yeast has developed drug resistance (Asleson, C.M. et al, 2001).

Filamentous fungi are becoming recognised as important human pathogens but they are also responsible for most post-harvest food spoilage. *Aspergillus flavus* follows *Candida* as the second most frequent fungi in opportunistic mycoses. In addition it is one of the most significant fungi that contributes to the spoilage of grain producing quality losses during storage and therefore, it is responsible for the

economic devaluation of the grain due to mycotoxin contamination (Gordon, S. H. et al, 1999).

Several methods have been proposed so far to detect pathogenic microorganisms. The conventional culture is a standard method used in both the food and medical sectors. It relies on specific microbiological media to isolate and enumerate viable cells. They contain both specific substrates and inhibitors that only allow the growth of the targeted strains (Lazcka, O. et al, 2007). They are very sensitive and inexpensive and can give both qualitative and quantitative information regarding the microorganisms present in a sample. However, the main disadvantage of traditional methods is that they are labour intensive and require several days to give results while the microorganism is able to multiply and produce visible colonies (de Boer, E. and Beumer, R.R**.** 1999). Moreover, biochemical tests are normally required to confirm the species of the microorganism.

Rapid biochemical kits have been developed to simplify and automate the identification of individual microorganisms. They are available on the market for both bacteria and fungi (e.g. API systems, BBL-Crystal) and the results obtained are comparable to the traditional biochemical tests (de Boer, E. and Beumer, R.R**.** 1999). However, an incubation period of 24 to 48 h is necessary previous to the interpretation of the biochemical reactions.

Since conventional cultures are time consuming, chromogenic and fluorogenic substrates have been incorporated into the culture media as an alternative method that allow both enumeration and identification of the microorganism directly on the media avoiding further biochemical tests. This method is based on the ability of the microorganism to produce specific enzymes or metabolites able to react with the fluorogenic or chromogenic substrates. As a result, different colour colonies are generated making it possible to differentiate between genera and even species (de Boer, E. and Beumer, R.R**.** 1999.; Eraso, E. et al, 2006). Nevertheless, a disadvantage is that some substrates are expensive. On the other hand, in some media there exists the possibility of fluorescence diffusion over the agar surface making difficult the colony differentiation. In addition false positive outcomes can be

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obtained due to the presence of the specific enzymes in raw foods(de Boer, E. and Beumer, R.R**.** 1999). Figure 1-1 shows the conventional and rapid methods used to identify *Candida* spp.

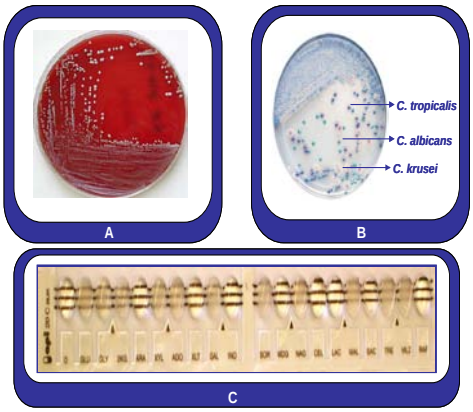

Figure 1-1. A). Conventional culture (*Candida* in blood agar). B). Conventional culture using chromogenic medium (BBLCHROMagar that allows the presumptive identification of different species of *Candida*). C). Rapid biochemical test (API 20 C AUX for identification of yeast).

Methods based on immunoassays have been developed in order to obtain a direct identification of the microorganism present in a sample. In 1971, Engvall and Perlmann reported the first application of the enzyme-linked immunosorbent assays (ELISA) for detecting IgG in rabbit serum with alkaline phosphatase as the reported label (Lequin, R. M. 2005). Since then, this method has been widely used in both food and the medical sectors. It relies on the specific binding of an antibody to an antigen. This method combines the specificity of antibodies which are usually immobilized on solid supports, like polystyrene tubes or microtiter plates, with the sensitivity of conjugated enzymes (Lazcka, O. et al, 2007). By using antibody-enzyme conjugates it is possible to measure the antigen or antibody concentration (figure 1-2 shows a scheme of the different ELISA assays). It normally has a limit of detection for pathogens ranging from 10^3 to 10^5 cfu/mL. However, by using pre-enrichment procedures of the sample, it has been possible to detect up to 2 cfu/mL (Kumar, S. et al, 2008). Therefore, the main disadvantage of this method is that, at

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least 16-24 h of pre-enrichment are required to reach a low limit of detection. In addition, this is an indirect method that requires the use of specific labels.

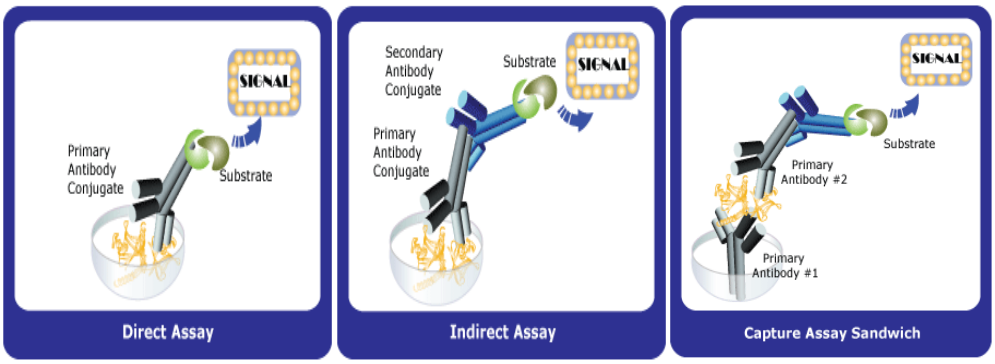

Figure 1-2. Diagram of the different types of ELISA. (Adapted from http://www.genwaybio.com/gw_file.php?fid=6056)

Molecular biology-based methods for detection and characterisation of pathogens have been developed in the last decades. Polymerase chain reaction (PCR) is, according to Lazcka et al, (Lazcka, O. et al, 2007), the most common tool used for pathogen detection. It was developed in the mid-80's by Mullis *et al* (Mullis, K. et al, 1986), and is based on the isolation, amplification and quantification of a short DNA sequence including the targeted pathogen's genetic material in just a few hours (Lazcka, O. et al, 2007). Nowadays, microarray platforms of DNA allow the analysis of thousands of targets at the same time with high sensitivity and specificity (Mullis, K. et al, 1986). PCR is without a doubt highly specific and has been proven to be accurate. Nevertheless, it is not a widely used test due to the difficulty to establish a comparison between the results obtained throughout the different investigations performed (Leaw, S.N. et al, 2007). Moreover, this technique is also costly and time-consuming due to the pre-processing steps of culturing bacterial cells and extracting the DNA before the amplification procedure (Steven, H. and Martin, L. 2007). Real time-PCR allows results to be obtained in a short time; however, this mechanism is based on the fluorescent emission. Therefore, specific dyes are required to label samples (Lazcka, O. et al, 2007). Figure 1-3 shows a representative scheme of the PCR process.

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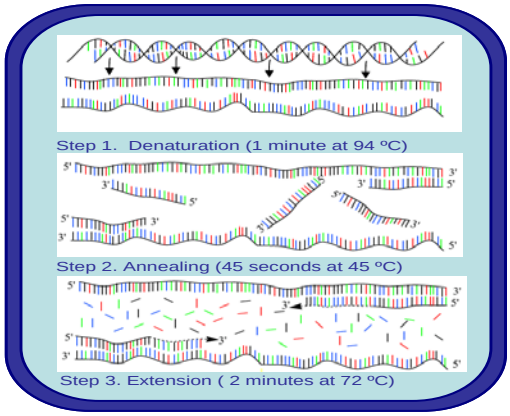

Figure 1-3. Principle of the PCR. Denaturation, annealing and extension steps are repeated for at least 35 cycles and about 68 billion copies of the selected gene can be obtained (Adapted from reference http://users.ugent.be/~avierstr/principles/pcr.html).

Since 1962 when the technology of biosensors started to expand, there have been many attempts to develop simple and reliable devices with analytical applications. Biosensors are the method most used to detect pathogenic microorganisms after PCR, conventional methods and ELISA (Lazcka, O. et al, 2007). Mass of sensors is a kind of biosensor frequently based on ultrasonic technology that utilizes films deposited on the surface of the piezoelectric device to sense chemicals and biological analytes. This is the case of the quartz crystal microbalance (QCM). On the other hand, microcantilevers have also been developed to detect the presence of bacteria and fungi. These sensors are based on the recording of resonance frequency changes as a consequence of mass changes on the transducer surface. They can also detect changes in the deflection, quality factor (Q-factor), and amplitude due to adsorption or changes in the environment (Yan, X. et al, 2006). Normally, piezoelectric detectors are used to determine the change in the frequency while the deflection of the cantilevers can be detected using optical detectors (Gautshchi, G. 2002).

In order to be used as biosensors, mass sensors must be functionalised with adequate molecular receptors, such as; antibodies, enzymes or peptides allowing the identification of specific targets. For instance, the use of antibody-modified-microcantilevers has made it possible to reach sensitivities on the order of

attograms where a single bacteria has been detected (Ilic, B. et al, 2001). Figure 1-4 shows a scheme of a functionalised cantilever and the immobilized bacteria. On the other hand, the use of microcantilevers permits observing microbial growth process in situ. Nugaeva *et al* (Nugaeva, N. et al, 2005), functionalised microcantilevers with proteins like concanavaline A (con A), fibronectine (Fn) or anti-*Aspergillus niger* antibodies to monitor the growth of *Saccharomyces cerevisiae* and the mould *A. niger* respectively. Only 4 hours were required to observe the germination process.

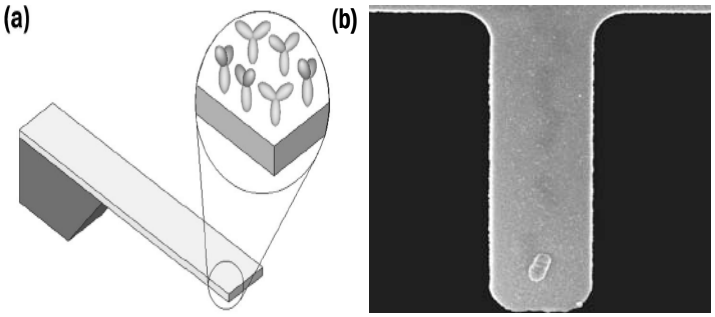

Figure 1-4. Scheme of a functionalised cantilever showing (a) antibody immobilization (b) SEM image of a single *E. coli* O157:H7 cell bound to the immobilized antibody layer on top of the oscillator (Adapted from Ilic, B. et al, 2001).

Mass sensors have several advantages over other sensor technologies, including small sized, high sensitivity, short response times and the possibility to build microarrays to detect multiple microorganisms on a single chip. However, the main disadvantage of these devices, is, for example, in the case of the piezoelectric detectors; the level of intrinsic noise reduces the resolution and the sensitivity. In addition, if optical detectors are employed, the main disadvantage is the amount of time required for the calibration of the equipment. Moreover, the measurement can be affected by the optical density when the analysis is carried out in liquid samples (Carrascosa, L.G. et al, 2006). Finally, adsorption phenomena often hinder high levels of selectivity and the instruments required are rather costly.

Another type of biosensor employed to detect pathogenic microorganisms, in this case frequently used in a homogeneous format, is based on the use of

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nanoparticles (NPs). NPs can be defined as small clusters of atoms with a diameter ranging from 5 to 100 nm., containing approximately 20 to 15000 atoms. They can be classified based on the electrical characteristics of the semiconductor or metallic material used (Liu, W.T. 2006). The development of NPs has been extensively pursued in recent years due to their unique optical, electronic, and magnetic properties (Yang, H. et al, 2008). Metallic NPs involve, among others, gold and silver nanoparticles. These NPs have absorption throughout most of the visible region. Colloidal gold spheres have a characteristic red colour, while silver spheres are yellow. The colour is related to the surface plasmon oscillation; a process that involves a collective oscillation of the electrons in the conduction bands. The oscillation is usually in the visible region for gold and silver giving rise to the strong surface plasmon resonance absorption. The resonance condition is determined by the shape, size and dielectric constants of both the metal and the surrounding material (Eustis, S. and El-Sayed, M. A. 2006).

The optical properties of the noble metal NPs lead to many uses, such as sensing and imaging techniques. It is well known that gold NPs display a red colour when they are well-dispersed in solution. However, a blue colour appears when they are aggregated (Eustis, S. and El-Sayed, M. A. 2006). Different sensors have taken advantage of this phenomenon. This detection system of gold NPs offers several advantages; like the use of visible light instead of using flourescent light emission and excitation, reducing the cost (Liu, W.T. 2006). In addition, the optical properties of the gold NPs do not undergo change when they are conjugated to biomolecules and the biomolecules attached to their surface preserve their biological activity (Hernández, D. et al, 2002) being useful as labels when optical techniques are used as the detection system.

Silver NPs have received considerable attention due to their attractive physicochemical properties (Elechiguerra, J.L. et al, 2005). These NPs compared to other metal NPs exhibit a larger area for adsorption which makes them more sensitive and more suitable for the detection of microorganisms. In addition they also display a surface plasmon resonance (SPR) band which enables following the detection process by observing the change in the spectral position of the SPR band. Using this approach, Kalele *et al* (Kalele, S.A. et al, 2006) used silver nanoshells

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functionalized with anti-*E.coli* antibodies to detect this bacterium in drinking water. Figure 1-5 shows a schematic representation of the functionalisation and detection process. The technique allowed determining in less than a minute; small (1 to 5 cells/mL) or high (10^9 cells/mL) concentrations of the bacteria were seen by observing the reduction in the SPR-band intensity.

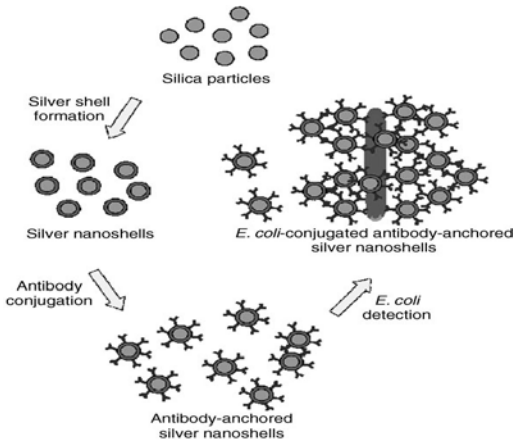

Figure 1-5. Diagram of the functionalised silver nanoshells for the detection of *E. coli* by Kalele *et al* (Kalele, S.A. et al, 2006).

On the other hand, semiconductor NPs like quantum dots (QDs) have been the subject of intensive investigation because of their unique photoluminescent properties and potential applications. QDs are small three-dimensional groupings of atoms in which the electron motion is "confined" by potential barriers in all three dimensions. In QDs there are discreet electronic energy levels in which the spacing of these electronic energy levels can be precisely chosen through the variation of their size. The electronic and optical properties of a semiconductor arise primarily through the quantum mechanical scattering of the valence electrons by the atomic cores (Alivisatos, P. 2004). QDs have significant advantages over traditional fluorescent dyes, including better stability, stronger fluorescent intensity, and different colours, that are adjusted by controlling the size of the dots. They display a high photostability and, therefore, they exhibit more resistance to photobleaching than organic dyes. QDs with different sizes have a wide absorption spectrum and a narrow emission spectrum. This allows simultaneous detection of multiple microorganisms (Rotem, E. et al, 2006).

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Liu Y *et al* (Liu, Y. et al, 2007), reported the use of CdSe/ZnS core/shell dendron nanocrystals functionalised with antibodies against *Escherichia coli* O157:H7 and hepatitis B that were able to detect 2.3 cfu/mL for *E. coli* O157: H7 and 5 ng/mL for the hepatitis B surface Ag (HBsAg). The detection process was carried out in only 30 minutes and no pre-treatment of the sample was required.

Magnetic nanoparticles coupled with QDs have also been used in bacterial detection. In this method, magnetic beads coated with anti-*E. coli* O157 antibodies were employed to selectively capture the target bacteria, and biotin-conjugated anti-*E. coli* antibodies were added to form sandwich immuno-complexes. After magnetic separation, the immuno-complexes were labeled with QDs via biotin-streptavidin conjugation. This was followed by a fluorescent measurement. The fluorescent emission was proportional to the initial cell concentration of *E. coli* O157:H7 in the range of 10^3-10^7 cfu/mL in less than 2 hours (Su, X.L. and Li, Y. 2004). However, nanoparticles have certain limitations. It is necessary to 'fine tune' the synthesis methodology to produce homogeneous sized nanostructures. Moreover, their small size and large surface area can easily lead to particle-particle aggregation, making physical handling of nanoparticles difficult in liquid and dry forms (Mohanraj, V.J. and Chen, Y. 2006). Additionally, in many cases, NPs only allow the detection but not the quantification of a microbial population.

Electrochemical sensors have also been developed with the aim of detecting microorganisms. These devices are mainly based on the observation of current or potential changes due to interactions occurring at the sensor sample matrix interface. Techniques are generally classified according to the observed parameter: current (amperometric), potential (potentiometric) or impedance (impedimetric) (Lazcka, O. et al, 2007). Chen *et al* (Chen, H. et al, 2005), used an electrochemical impedance biosensor for the rapid detection of *Saccharomyces cerevisiae.* The yeast cells were immobilized on a gold surface modified with an alkanethiolate SAM. A linear relationship between the electron-transfer resistance and the logarithmic value of yeast concentrations was found in the range between 10^2 and 10^8 cfu/mL. Dungchai *et al* (Dungchai, W. et al, 2008), developed an electrochemical metalloimmunoassay based on a copper-enhanced gold

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nanoparticle label for *S. typhi* determination in real samples. The detection limit enhanced by the authors was 98.9 cfu/mL. Obuchowska A (Obuchowska A. 2008), used cyclical voltammetry to quantify *Micrococcus luteus*, *Clostridium sporogenes* and *E. coli* JM105 in the exponential and stationary growth phases following adsorption of cellular components by screen-printed carbon electrodes (SPCEs). The detection limit of the devices had a range of 10^3 to 10^6 cfu/mL with a detection time of 15 minutes per measurement using lysate bacterial samples. The device can be miniaturized and is adaptable to a lab-on-a-chip device. Compared to optical methods, electrochemistry can be applied to turbid samples and the capital cost of equipment is much lower. On the other hand, electrochemical methods usually display slightly more limited selectivity and sensitivity than their optical counterparts (Lazcka, O. et al, 2007).

At the beginning of this research (2006) CNTFETs based on single walled carbon nanotubes had not been applied to detect bacteria or fungi. Only the interaction between CNTs and bacteria had been explored (Huang, T. S. et al, 2004.; Elkin T. et al, 2005.; Lin, Y. et al, 2006) but without sensing purposes. Figure 1-6 shows an image of bacteria entrapped in a network of SWCNTs.

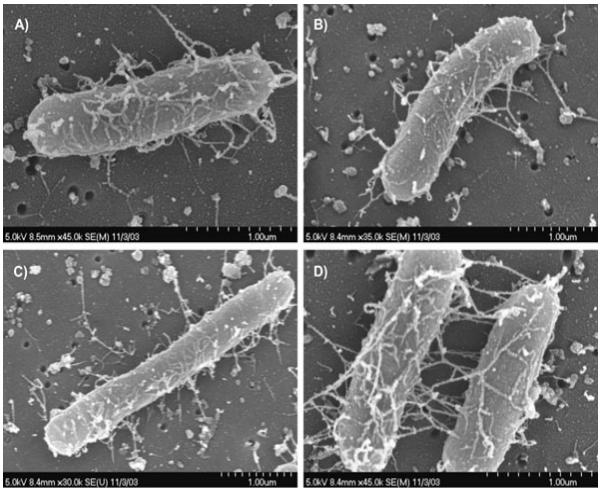

Figure 1-6. SEM images of *E. coli* cells bound with immuno-SWNT species. There was the possibility of occasionally finding two cells bound together by the nanotubes (Elkin, T. et al, 2005).

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Nevertheless, simultaneously to our investigation So *et al* (So, H.M., et al, 2008), reported the use of an array of carbon nanotube field effect transistors (CNTFET) functionalised with RNA-aptamer to detect *E.coli* in less than 20 minutes. Even if the detection time is low, authors suggest the increase in the number of arrays or the use of a network with a large sensing area in order to obtain similar results to those obtained using conventional methods. Therefore, it is clear that many attempts are still required to improve the performance parameters of these devices to obtain reliable and useful biosensors for the market.

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1.2. Objectives

The general objective of this thesis is the development of electrochemical biosensors for microorganisms in which CNTs are used as the transducer elements. The antigen-antibody interaction is used as the molecular recognition mechanism where the antibodies act as the sensing layers able to detect both pathogenic bacteria and fungi. The molecular recognition mechanism and the ability of the CNTs to transduce the presence of microorganisms make labels unnecessary.

The scope of the thesis covers the area from the synthesis of the CNTs to the exposure of different microorganisms. Therefore, the following specific objectives have been aimed at in this research:

Functionalise the devices with specific molecular recognition able to selectively detect *Salmonella* Infantis, *Candida albicans* and *Aspergillus flavus* in a sample and avoid the non-specific binding of undesired molecules by means of blocking agents.

Detect bacteria, yeasts and moulds in both known and unknown samples and determine their presence by means of the electrical and microscopy characterisation.

Explore the capability of CNTFET-biosensors to reach selectivity and sensitivity comparable to current detection methods of pathogenic microorganisms.

Improve the performance parameters of the available methods for the detection of microorganisms; thus, providing an alternative method that can be used in the reliable determination of pathogens in real samples.

The main added value of this thesis is to explore the ability of the CNTs to transduce the presence of pathogenic microorganisms into an electrical signal that can be measured. In this way, this thesis is the first attempt to provide an alternative method for the sensitive and selective detection of pathogenic microorganisms in a short time without any kind of labels; thereby, improving the performance parameters of the traditional existent methods.

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1.3. References

Alivisatos, P. Nature Biotechnology. **2004**. 22, 47-52.

Asleson, C.M., Bensen, E.S., Gale, C. A., Melms, A.S., Kurischko, C., Berman, J. Journal of Molecular and Cellular Biology. **2001**. 21, 1272–1284.

Carrascosa, L.G., Moreno, M., Álvarez, M., Lechuga, L.M. Trends in Analytical Chemistry. **2006**. 25, 3, 196-206.

Chen, H., Heng, C.K., Puiu, P.D., Zhou, X.D., Lee, A.C., Lim, T.M., Tan, S.N. Analytica Chimica Acta. **2005.** 554, 52–59.

de Boer E. and Beumer, R.R. International Journal of Food Microbiology. **1999.** 50, 119–130.

Dungchai, W., Siangproh, W., Chaicumpa., W., Tongtawe, P., Chailapakul, P. Talanta. **2008**. 77, 727–732.

Elechiguerra, J.L., Burt, J.L., Morones, J.R., Camacho, A., Gao, X., Lara, H.H., Yacaman, M.J. Journal of Nanobiotechnology. **2005**. 3,6.

Elkin, T., Jiang, X., Taylor, S., Lin, Y., Gu, L., Yang, H., Brown, J., Collins, S., Sun, Y. ChemBioChem. **2005**, 6, 640 –643.

Eraso, E., Moragues, M.D., Villar-Vidal, M., Sahand, I. H., González, N., Pontón, J., Quindós, G. Journal of Clinical Microbiology. **2006.** 44, 3340-3345.

Eustis, S. and El-Sayed, M. A. Chemical Society Reviews. **2006**. 35, 209–217.

G. Gautshchi, Piezoelectric Sensorics, Springer-Verlag, Berlin, **2002**.

Gordon, S.H., Jones, R.W., McClelland, J.F., Wicklow, D.T., Greene, R.V. Journal of Agricultural and Food Chemistry. **1999**. 47, 5267-5272.

Huang, T.S., Tzeng, Y., Liu, Y.K., Chen, Y.C., Walker, K.R., Guntupalli, R., Liu, C. Diamond and Related Materials. **2004**. 13, 1098-1102.

Ilic, B., Czaplewski, D., Zalatutdinov, M., Craighead, H.G., Neuzil, P., Campagnolo, C., Batt, C. Journal of Vaccum Science and Technology B. **2001**. 19, 2825-2828.

Kalele, S.A., Kundu, A.A., Gosavi, S.W., Deobagkar, D.N., Deobagkar, D.D., Kulkarni, S.K. Small. **2006.** 2, 335-338.

Kumar, S., Balakrishna, K., Batra, H. Biomedical and Environmental Sciences. **2008**. 21, 137-143.

Lazcka, O., Del Campo, F.J., Muñoz, F.X. Biosensors and Bioelectronics. **2007**. 22, 1205–1217.

Leaw, S.N., Chang, H.C., Barton, R., Bouchara, J.P., Chang, T.C. Journal of Clinical Microbiology. **2007**. 45, 2220 – 2229.

Lequin, R. M. Clinical Chemistry. **2005**. 51, 2415–2418.

Lin, Y., Jiang, X., Elkin, T., Fernando, K.A., Gu, L., Taylor, S., Yang, H., Jones, E., Wang, W., Sun, Y.P. Journal of Nanoscience and Nanotechnology. **2006.** 6, 868–871.

Liu, W.T. Journal of Bioscience and Bioenginnering. **2006**. 102, 1-7.

Liu, Y., Brandon, R., Cate, M., Peng. X., Stony, R., Johnson, M. Analytical Chemistry. **2007.** 79, 8796-8802.

Marcus, R. Current Opinion in Pediatrics. **2008**. 20, 79–84.

Mohanraj, V.J. and Chen, Y. Tropical Journal of Pharmaceutical Research. **2006**. 5, 561-573.

Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G., Erlich, H. Cold Spring Harbor Symposia on Quantitative Biology. **1986.** 51, 263–273.

Nugaeva, N., Gfeller, K.Y., Backmann, N., Lang, H.P., Duggelin, M., Hegner, M. Biosensors and Bioelectronics. **2005**. 21, 849–856.

Obuchowska, A. Analytical and Bioanalytical Chemistry. **2008**. 390, 1361-1371.

Pontón, J. and del Palacio, A. Revista Iberoamericana de Micología. **2007**. 24, 181-186.

Rotem, E., McKinstry, M., Hwang, J., Oppenheim, A.B., Fekete, R. A., Giulian, G., Merril, C., Nagashima, K., Adhya, S. Proceedings of the National Academic of Science. **2006**. 103, 4841-4845.

So, H.M., Park, D.W., Jeon, E.K., Kim, Y.H., Kim, S.K., Lee, C.K., Choi, S.Y., Kim, S.C., Chang, H., Lee, J.O. Small. **2008.** 4, 197–201.

Steven, H. and Martin, L. Applied Microbiology and Biotechnology. **2007.** 76, 513-519.

Su, X.L. and Li, Y. Analytical Chemistry. **2004**. 76, 4806-4810.

Yan, X., Ji, F.H., Thundat, T. Current Analytical Chemistry. **2006.** *2,* 297-307.

Yang, H., Li, H.P., Jiang, X.P. Microfluidics and Nanofluidics. **2008**. 5, 571-583.

http://www.genwaybio.com/gw_file.php?fid=6056. Last accessed in 18th, March, 2009.

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Chapter 2

Scientific bases

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The main aim of this chapter is to explain the fundamental concepts of the building blocks that take part in the biosensors developed in this thesis; from the molecular recognition element to the transducer. First, section 2.1 introduces the transducer element: the carbon nanotubes (CNT). In this section, the properties of CNT, their synthesis and their integration in field-effect transistors (FET) are explained. Section 2.2. defines the antibodies, the recognition element of our biosensors. Special attention is paid to their structure and to their physical adsorption on hydrophobic surfaces. Finally, section 2.3. describes the kind of pathogens that have been detected with our devices: bacteria, yeasts and fungi.

2.1. Transducer: Carbon Nanotubes (CNTs)

2.1.1. Definition, structure and types of CNTs

CNTs, discovered by Iijima, S. (Iijima, S. 1991), can be described as a graphene sheet rolled up into a nanoscale-tube (Dresselhaus, M.S. et al, 1996). A single sheet generates single-walled carbon nanotubes (SWCNTs) while multiple sheets produce multi-walled carbon nanotubes (MWCNTs) (see figure 2-1). SWCNTs have a minimum diameter of about 0.4 nm to 2 nm and and lengths up to 1.5 cm have been reported (Huang, S. et al, 2004). Such dimensions give rise to aspect ratios (length/diameter) of over ten million (Raffaellea, R.P. et al, 2005). SWCNTs can be either metallic or semiconducting (Fischer, J.E. 2006).

Noriaki Hamada and colleagues, at the NEC Laboratory in Tsukuba have calculated dispersion relations for small-diameter nanotubes showing that about one-third of small-diameter nanotubes are metallic, while the rest are semiconducting, depending on their diameter and chiral angle. By contrast, MWCNTs have diameters from 10 to 200 nm and lengths up to hundreds of microns with an adjacent shell separation of 0.34 nm (Khare, R., and Bose, S. 2005). They usually display a metallic character.

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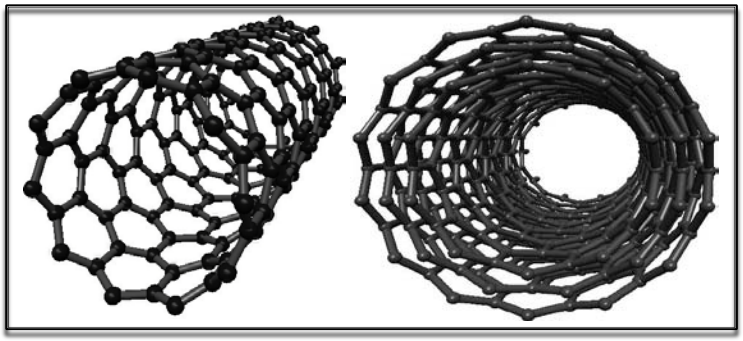

Figure 2-1. Schematic representation of the CNTs. A single-walled carbon nanotube (left) and a multi-walled carbon nanotube (right)

The atomic structure of a SWCNT is conventionally described by a pair of integers (*n*,*m)* denoting the relative position $C_h=na_1+ma_2$ of the pair of atoms on a graphene strip which, when rolled onto each other, form a tube (a_1 and a_2 are unit vectors of the hexagonal honeycomb lattice). The chiral vector C_h uniquely defines a particular (*n*,*m*) tube, as well as its chiral angle (Θ), which is the angle between C_h and a_1 (Charlier, J.C. and Roche, X.B. 2007). It can vary between 0 and 30º, which allow obtaining three possible configurations of CNTs (figure 2-2). Armchair nanotubes formed when *n* = *m* and the chiral angle is 30°. Zigzag nanotubes formed when either *n* or *m* is zero and the chiral angle is 0°. All other nanotubes, with chiral angles intermediate between 0° and 30°, are known as chiral nanotubes.

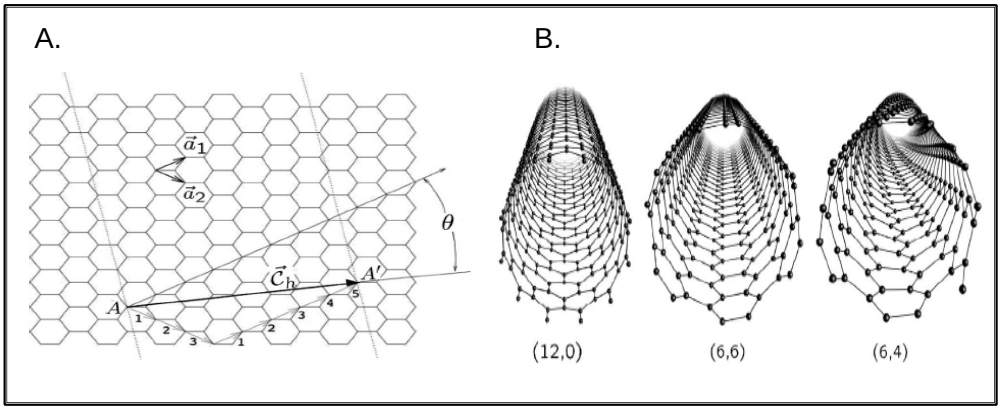

Figure 2-2. A). Graphene honeycomb network with lattice vectors a_1 and a_2. The chiral vector $C_h=5a_1+3a_2$ represents a possible wrapping of the two-dimensional graphene sheet

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into a tubular form. The direction perpendicular to C_h is the tube axis. The chiral angle Θ is defined by the C_h vector and the a_1 zigzag direction of the graphene lattice. B). Three different configurations of CNTs by rolling up a graphene sheet (12,0) zigzag, (6,6) armchair and (6,4) chiral nanotubes (Adapted from Charlier, J.C. and Roche, X.B. 2007).

A few published reports on measurements of the SWCNTs show that the armchair tubes are metallic (Cowley, J. M. et al, 1997.; Rao, A. M. et al, 1997), the zigzag tubes are semiconducting and chiral tubes are semiconducting or metallic, depending on the diameter of the tube and on the wrapping angle (Wildöer, J.W.G. et al, 1998). Both, diameter of the tube and the wrapping angle at the same time depend on the indices *n* and *m*. In general, if $n - m = 3q$, where *q* is an integer, the nanotube is metallic, whereas for $n - m \neq 3q$ it is semiconducting (Heller, I. et al, 2006). However because of the growth process of the CNTs is stochastic with respect to wrapping indices, one third of the tubes will be metallic (Fischer, J.E. 2006).

2.1.2. Synthesis of carbon nanotubes

Three methods are usually employed to synthesise CNTs. They involve arch-discharge, laser ablation and chemical vapour deposition (CVD). In this thesis we have utilized CVD as technique to produce the CNTs which are the transducer element in our biosensors. CVD method employs a carbon source (carbon monoxide, acetylene, methane, etc) which is decomposed in an oven by heating at temperatures in the range of 500 to 1100º. The carbon released by the decomposition of the gas, is deposited onto the surface of the catalyst particles (usually Ni, Fe and Co) which act as seeds to nucleate the growth of CNT (Trojanowicz, M. 2006). Depending on the operating conditions (temperature, catalyst, flow rate of gases, size of the particles) SWCNTs or MWCNTs can be obtained. Usually, SWCNTs are synthesized at higher temperatures (800-1100 ºC) than MWCNTs (Bourgoin, J.P. et al, 2006). CVD allows continuous fabrication, and may be the most favourable method for scale up and production (Poole, C.P. and

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Owens, F.J. 2003). Nevertheless, the control of the size of the catalyst particles in order to produce tubes with high purity and low diameter is still a challenge.

2.1.3. Electrical properties

The CNTs properties are also related to the electronic structure of the graphene sheet. It contains sp^2-hybridized carbon atoms. Three out of the four outer-shell electrons of these carbons participate in bonding with neighbour carbons while the fourth electron is in a *p*-orbital perpendicular to the hexagonal lattice. In a flat graphene sheet, these p-orbital electrons are distributed in the valence (π) and conduction (π*) bands, providing a semi-metallic character owing to their theoretically zero bandgap (Kim, S. N. et al, 2007).

The band with high energy is named ***conduction band*** while the band with low energy is the ***valence band***. According to the energy band model, it is possible to distinguish between metallic conductors, semiconductors and isolators by considering whether conductivity and valence band are separated by a gap (***the band gap)*** or whether they overlap. To move an electron from the valence band to the conduction band it is required energy, defined as E_g. In the case of isolating materials this E_g is higher than ~5 eV (Gründler, P. 2006) while in semiconducting nanotubes E_g is lower but has a dependence on the diameter. Consequently, smaller diameter nanotubes have larger band gaps (in a range between 0.3 to 0.8 eV for SWCNTs with diameters of 1 to 3 nm) (Heller, I. et al, 2006). In the case of metallic tubes there is no gap between the valence band and the conduction band.

Additionally to the classification of CNTs in metallic or semiconducting, another important parameter is the position of the Fermy energy *(E_F)* with respect to the charge neutrality point (CNP). For an undoped *C*NT the E_F coincides with the CNP ($E_F = 0$). Electron (*n)* or hole (*p)* doping produce a shift of the E_F up or downwards. If the doping induced Fermi level shifts are larger than the energy separation between the one-dimensional subbands, a semiconducting CNT is turned into a metallic one (Krüger, M. et al, 2001).

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Some of the electrical properties of the CNTs which made them extraordinary nanomaterials with many potential applications include:

- CNTs are quasi-ballistic carrier transport (Javey, A. et al., 2003.; Saito, R. et al, 1998) showing a great potential as building blocks of nano and micro-electronic devices.
- CNTs have remarkable conductivity and high specific surface area (up to 1600 m^2/g (Baughman, R.H. et al, 1999).
- Due to symmetric structure of the conduction and valence bands, CNTs can be used to make complementary circuits (Pourfath, M. et al, 2009).
- The tunability of the band-gap with the tube diameter make CNTs suitable candidates for opto-electronic devices, especially for infra-red (IR) applications due to the relatively narrow band gap (Freitag, M. et al., 2003).
- The presence of electrons and holes in semiconducting CNTs favours the development of transistors with high density of on-state current (Javey, A. et al, 2004).
- Individual SWCNTs can be used as nanoelectrodes for electrochemistry, yielding enhanced mass transport and high current densities equivalent to sub-10 nm hemispherical electrodes (Heller, I. et al, 2005).
- Networks of CNTs have potential applications as transparent and conductive coatings, with properties comparable to indium tin oxide (ITO), which is the current industry material standard (Hu, L. et al, 2004).
- Hu (Hu, L. et al, 2004) and Lay (Lay, M. D. et al, 2004) have shown that a carbon nanotube network containing both metallic and semiconducting nanotubes can operate as the conducting channel in a field effect transistor (FET) configuration and that their conductance can be tuned by electrostatic interaction with a solid-state gate or an electrolyte gate.
- Devices based on single tube have mobilities as high as 100,000 cm^2/Vs, (Duerkop, T. et al, 2004), current carrying capacities of 10^9 A/cm^2 (Yao, Z. et al, 2000) and ON/OFF current ratios as large as 10^5 (Javey, A. et al, 2002).

CNTs can be used in biosensing applications due to they have every atom on the surface and exposed to the environment. As a consequence, even small changes in the charge environment can affect strongly their electrical properties. Therefore, they have the potential for very high detection sensitivity through the depletion or accumulation of charge carriers, caused by the binding of charged biomolecules at the surface (Allen, B.L. 2007).

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2.2. Recognition element: Antibodies

Several research works have been reported regarding to the use of carbon nanotubes for the detection of biomolecules (Lin, Y. et al, 2004.; O`Connor, M., et al, 2004.; Pantarotto, D. et al, 2003.; Okuno, J. et al, 2007). The detection mechanism is mainly based on the antigen-antibody interaction. Antibodies also called immunoglobulins, are glycoproteins with a molecular weight ranges from 140 KDa to 970 KDa, secreted by specialized B lymphocytes known as plasma cells. They are synthesized by an animal in response to the presence of a foreign substance, the antigen. Antibodies display an extraordinary specificity and binding affinity for a given antigen being useful as analytical reagents in diagnostics, in environmental and food tests (immunoassays and immunosensors), and in separations (immunoaffinity chromatography) (Giacomelli, C.E. 2006). In this thesis immunoglobulin G (IgG) antibodies have been immobilized over the surface of the CNTs. They act as the recognition elements of our devices being able to detect different pathogenic microorganisms.

The structure of an immunoglobulin G presents three protein domains. Two of the domains are identical and form the arms of the Y-shape. They are also known as “Fab” domains in which each arm contains a site that can bind to an antigen, making IgG bivalent molecules. The third domain forms the base of the Y and it is known as “Fc” (fragment that cristallizes). The Fab segments are linked to the Fc by the hinge region, which varies in length and flexibility in the different antibody classes and isotypes (Vermeer, A.W.P. and Norde, W. 2000). This segment allows lateral and rotational movement of the two antigen binding domains. The two heavy-chain polypeptides in the Y structure are identical with about 55 KDa, and the carboxy-terminal regions of the two chain fold together to make the Fc domain. The two light chains are also identical with a molecular weight of about 25 KDa. All, polypeptide chains are held together by disulfide bridges and non-covalent bonds (Voet, P. et al, 2008) (see figure 2-3). According to the number of Y-like units and the type of heavy chain polypeptide, antibodies have been divided into five classes; IgG, IgM, IgA, IgE and IgD (Liljas, A. et al, 2009).

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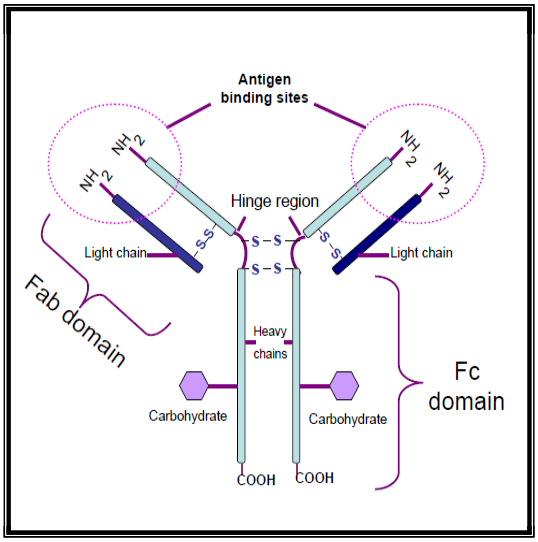

Figure 2-3. Schematic structure of the immunoglobulin G (IgG).

According to the specificity of an antibody against a given antigen they can be classified in two groups. The first one involves polyclonal antibodies. They are produced by immunization of an animal (often rabbit or goat) and then, they are extracted from the animal antiserum and purified to the desired level. The production process is relatively inexpensive, universal and large quantities of an antibody can be isolated from a single extraction. Polyclonal antibodies bind to multiple epitopes of a given antigen.

The second group includes monoclonal antibodies. They are produced by means of a more complex process involving the fusion of sensitized B lymphocytes with a myeloma cell (malignant cancer cell) preparation. The complex lymphocytes B-myeloma cell is implanted in the animal and then they are harvested and purified. A powerful feature of monoclonal antibodies is that the immortal nature of the hybridoma allows for its continual reculturing, thus providing a constant source of monoclonal antibodies. Monoclonal antibodies bind to only one epitope of a given antigen, being more specific than polyclonal antibodies (Voet, P. et al, 2008).

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2.2.1. Immobilization methods

The immobilization of immunoglobulin G (IgG) on CNTs is an important step in the development of immunosensors. The attachment involves covalent and non-covalent process. The first one provides stability but reduces the desirable electronic properties of SWNTs. By contrast, non-covalent immobilization preserves the primary structures of the SWNTs along with their electrical properties (Allen, B. L. et al, 2007). Therefore, in this thesis we have used non-covalent process to immobilize the IgG antibodies over the CNTs.

2.2.1.1. Adsorption of the IgG

Theoretically, the non-specific adsorption of proteins involving IgG, onto the nanotube surface is attributed to supramolecular interactions. Chen *et al* (Chen, J. et al, 2003), proven that after one hour of incubation proteins such as IgG, BSA and avidine were adsorbed onto the CNTs. This spontaneous adsorption has been attributed to hydrophobic interactions (Allen, B.L. et al, 2007) and it has also been associated with the affinity of carbon nanotubes by amino groups, as demonstrated by Bradley *et al* (Bradley, K. et al, 2004). Moreover, intermolecular interactions involving aromatic amino acids (i.e., histidine and tryptophan) in the polypeptide chains of the proteins can also contribute to the observed affinity of the peptides to carbon nanotubes (Wang, S. et al*,* 2003).

In the adsorption process the degree of hydrophobicity of the external surface of the protein play a key role in their immobilization. As a rule the more hydrophobic the protein is, the stronger is its affinity for a surface (figure 2-4). In addition, the more hydrophobic the surface is, the stronger adsorption is promoted (Vermeer, A.W.P. et al, 2000).

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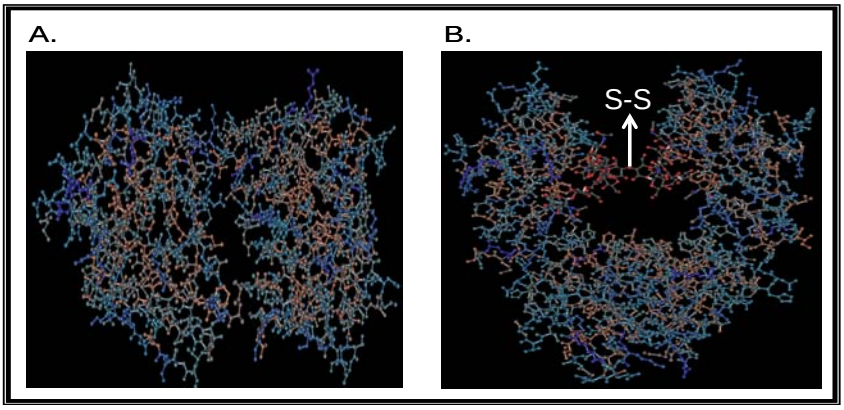

Figure 2-4. Molecular structure of a murine monoclonal antibody. A). Fab frgment B). Fc fragment in which can be clearly appreciated the disulphide bonds. The hydrophobic aminoacids in both fragments are shown in the dark blue color. The sequences were obtained from the National Center of Biotechnology Information (NCBI).

2.2.1.2. IgG-binding through bacterial proteins

Due to the fact that adsorption is mainly a non-covalent process, the protein is orientated onto the CNTs surface in such a way that acquires the lowest energy. This fact does not always assure that the Fab distal ends are orientated towards the most favourable interaction with the antigens. Therefore, to achieve a stable and orientated attachment of immunoglobulin molecules on a solid surface without disrupting their biological function (i.e. selectivity and specificity) it is required a method that allows to anchor only the Fc region of the immunoglobulin leaving the Fab domain available to attach the antigen.

An approach to find out a well-defined antibody surface is the use of bacterial proteins like protein A or protein G. They are found in the cell wall of some species of *Staphylococcus* and *Streptococcus* respectively. It has been suggested that these bacteria can evade the immune response by coating with host antibodies, thereby increasing their pathogenicity (Sloan, D.J. and Hellinga, H.W. 1999). Both, protein A and protein G display high specificity for the Fc domain of the IgG (Oh, B.K. et al, 2004). Protein A has been immobilized on a surface of CNTs (Chen, R.J. et al, 2003.; Byon, H.R. and Choi, H.C., 2006). Nevertheless, a drawback is that it

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lacks of Fc reactivity with IgG3, a subclass constituting approximately 8 % of total IgG (Bjorck, L. and Kronvall, G. 1984).

By contrast, protein G**,** has been demonstrated to be more generally IgG binding. It has different and separate sites to bind IgG. B1 is one of the domains of protein G with 56-residue that folds into a four-stranded β-sheet and one α-helix (figure 2-5) It has two separate IgG-binding sites on its surface, each interacting respectively with specific, independent sites on the Fab or Fc fragments of the antibody. A B1-Fc complex has shown that the Fc-binding site is mediated primarily by side-chain contacts between the two proteins. This interaction is predominantly polar rather than hydrophobic in character formed by a double “knobs-into-holes” interaction in which a knob from the B1 protrudes into a hole in the Fc region, and vice versa (figure 2-6). (Sloan, D.J. and Hellinga, H.W. 1999).

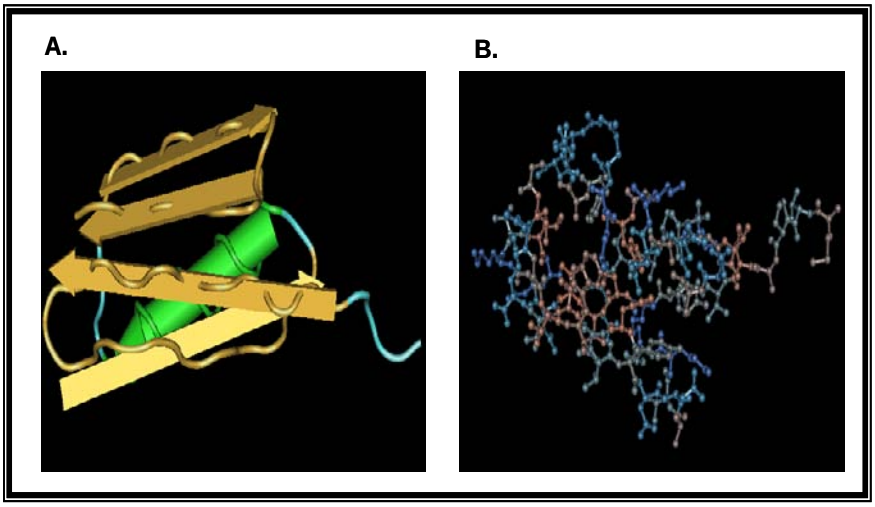

Figure 2-5. Molecular structure of the protein G. A). Secondary structure of the protein G showing four β-sheets and an α-helix. B). Hydrophobic aminoacids present in the protein (dark blue color). The sequence was obtained from the National Center of Biotechnology Information (NCBI).

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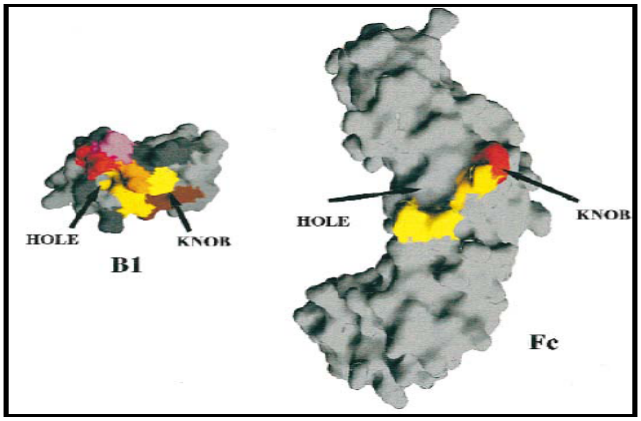

Figure 2-6. B1-Fc complex interaction, highlighting the position of the knobs and holes on the both proteins (Sloan, D.J. and Hellinga, H.W. 1999).

Nevertheless, protein G displays affinity Fab-binding sites of the IgG and it is also reactive with human albumin protein. As a result, genetic engineering has been used to produce molecules that retain their affinity only for the constant fraction "Fc" of the IgG, assuring a well orientation process of the antibodies (Lee, J.M. et al, 2007). In our research we used recombinant protein G that allows increasing the density of binding sites available for the antigen and therefore the sensitivity of the biosensors devices (figure 2-7).

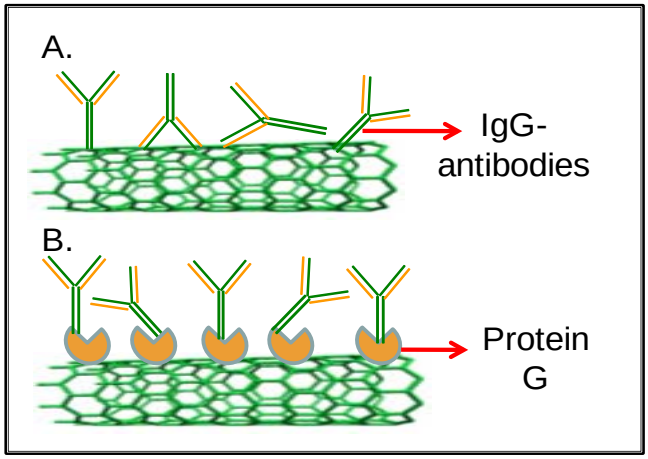

Figure 2-7. Schematic representation of the antibody immobilization process. A) Random adsorption of the IgG on a top of CNT. B) IgG binding through the protein G.

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2.2.1.3. Blocking agents

SWCNTs have all their carbon atoms at the surface; therefore they trend to interact with other compounds including proteins. In order to produce selective biosensors based on SWCNTs it is necessary to avoid the non-specific binding (NSB) of possible interferences present in the real samples. It can be achieved on one hand by using specific molecular receptors (in our case IgG antibodies) that only recognize the pathogen of interest avoiding cross-reactivity. On the other hand, it can be enhanced covering the gaps of the CNTs walls left unprotected by the adsorbed protein with specific blocking agents. Many molecules have been reported in the literature that are used for protecting various surfaces from proteins in mechanisms such as steric repulsion, hydration, and solvent structuring, employing π-π stacking or hydrophobic interactions (Zhao, Y. and Stoddart, J.F. 2009).

The most commonly used to protect carbon nanotubes are surfactants like Tween 20 (Chen, R.J. et al, 2004.; Star, A. et al, 2003.; Shim, M. et al, 2002), polymers like polyethylenglycol (PEG), polyethylene oxide (PEO) (Tuncel, A. 2000), polyethylene glycol methacrylate (Beyer, M. et al, 2006.; Chen, E.S. et al, 1998.; Martínez, M.T. et al, 2009) and gelatins like fish gelatin or fish skin gelatin (Sanchez, Z.C. et al, 2009).

Both, Tween 20 and PEG display protein-resistant properties. Tween 20 contains a long alkyl chain of twelve carbons (dodecyl chain), with twenty ethylene glycol groups and three terminal hydroxy (-OH) (Chi, Y.S. et al, 2007). Lin *et al* (Lin, Y. et al, 2004) showed that a Tween 20 coated semiconducting nanotube device did not show any changes in conductance upon exposure to various types and concentrations of protein solutions. They suggested that this surfactant formed a nearly uniform layer on the nanotube surface by favorable hydrophobic interactions.

In the case of PEG the mechanism of NSB is less understood. It seems that a brush-induced steric repulsion preventing contact between proteins and the underlying surface, and the hydration shells around the PEG moieties, can be the

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two dominating mechanisms (Marie, R. 2006). Martínez *et al* (Martínez, M.T. et al, 2009) showed that a multifunctional polymer consisting of polyethylene glycol and methacrylate groups as well as N-succinimidyl esters interacted hydrophobically with the CNT through an aliphatic backbone. At the same time this compound allows the covalent bond between CNTs and DNA.

The use of gelatin as blocking agent has been reported by Piao M.H.M. *et al* (Piao, M.H.M. et al, 2008) whose blocked their polyclonal antibody modified PVC-COOH electrodes with a PBS solution containing Tween 20 and getalin at 0.8 % (PBSTG) to avoid nonspecific binding bisphenol A (BPA). The frequency response was tested in both the absence and the presence of BPA. They did not observe changes in the background solution without BPA, indicating that there was not non-specific adsorption. Sánchez *et al* (Sanchez, Z.C. et al, 2009) used the same solution to protect CNTFETs against the non-specific binding of the same xenobiotic compound. They found that their CNTFETs were effectively protected against the NSB of the BPA and even of the small interferences like fluoranthene, pentacloronitrobenzene and malathionhe on the sidewalls of the CNTs. However the mechanism of interaction between this blocking agent and the CNTs is still unknown.

ISBN:978-84-692-9761-2/DL:T-203-2010

2.3. Devices based on Carbon Nanotube Field effect transistors (CNTFETs)

The development of CNTFETs was independently reported by the Dekker group (Tans, S.J. et al, 1998) and the Avouris group at IBM (Martel, R. et al, 1998) in 1998. They are electronic devices that use CNTs as conducting channels and display high sensitivity towards local chemical environments (Kauffmanab, D.R. and Star, A. 2008).

The structure of a FET consists of two metal electrodes called ***source*** and ***drain*** connected to the conducting channel. Moreover, a third electrode called ***gate*** is often used (figure 2-8). The gate can be used to electrostatically induce charges onto the tube. In semiconducting CNTs, a negative bias on the gate induces positive charges (holes) and a positive bias induces negative charges (electrons) (McEuen, P. 2003).

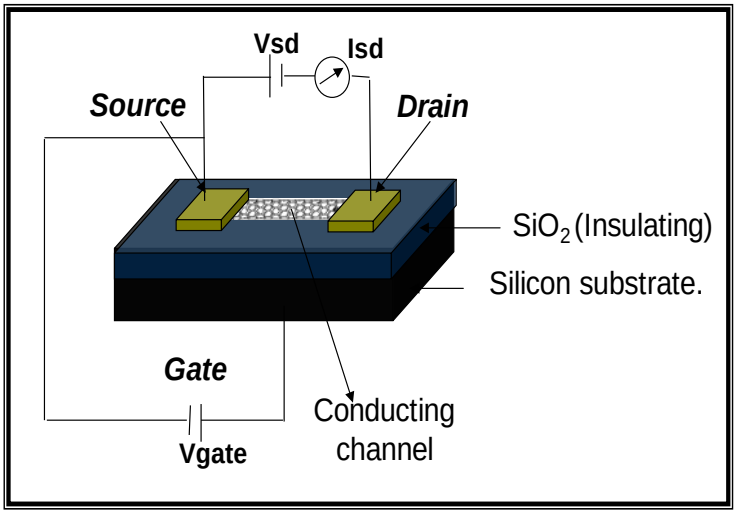

Figure 2-8. Schematic representation of a field effect transistor based on CNTs.

Based on the conducting channel two types of CNTFETs can be obtained. The first one is based on a single wall carbon nanotube (figure 2-9A). Dai and co-workers (Kong, J. et al, 2000) were the first to report the use of SWCNT to detect NO_2 and NH_3, where the resistance of a single semiconducting SWCNT was observed to

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increase or decrease by 2–3 orders of magnitude upon exposure to NO_2 or NH_3, respectively. This change was attributed to shifts in the valence band with respect to the Fermi level. FETs based on a single semiconducting tube are highly effective for specialized use with a relation ON/OFF of several orders of magnitude, being very sensitive for biosensing purpose. However, they are also impractical for mass production due to great variability in SWCNT (chirality and diameter) resulting in irregular device function (Vichchulada, P. et al, 2007). Moreover, the Schottky barrier may vary substantially from device to device.

The second type is based on a network of CNTs (figure 2-9B) in which the device operation depends on the density of the nanotubes (Hecht, D.S. et al, 2006.; Kocabas, C. et al, 2005.; Javey, A. et al, 2003.; Izard, N. et al, 2008). Although they are less sensitive than FETs based on a single nanotube, offer better reproducibility and manufacturability (Grüner, G. 2006). In these devices each CNT has its own threshold voltage and on–off ratio due to natural variations in CNT synthesis. Thus, the electronic characteristics of the devices depend on the sum of individual CNT thresholds and on–off ratios (Lin, A. et al, 2009). For a dense network, conductive nanotubes can act to screen the gate voltage, thus decreasing the on/off ratio of the device (Hu, L. et al, 2004).

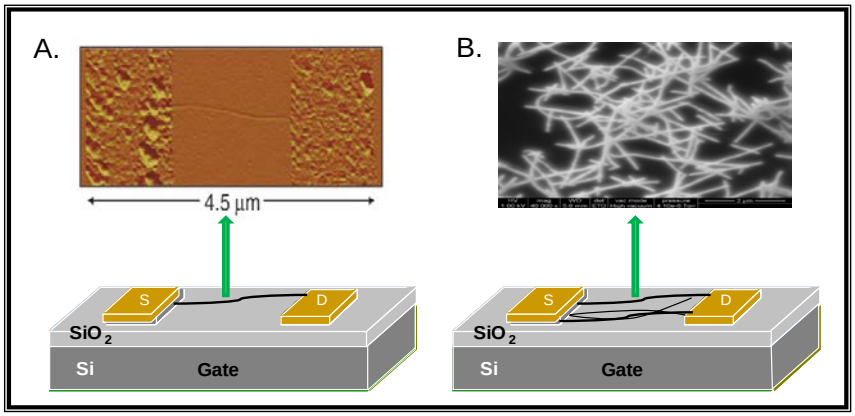

Figure 2-9. Field effect transistor. A) Based on a CNT. The inset shows an AFM image of a typical device. B) Based on a network of CNTs. The inset shows an ESEM image of a network of CNTs synthesized by CVD.

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FETs based on semiconducting SWCNTs typically exhibit *p*-type behaviour under ambient conditions due to charge transfer process between the nanotubes and adsorbed oxygen molecules from the air. However, chemical doping, either by charge transfer from alkali metals or by adsorption of electron-donating organic molecules can lead to *n*-type behaviour (Javey, A. et al, 2002) (figure 2-10).

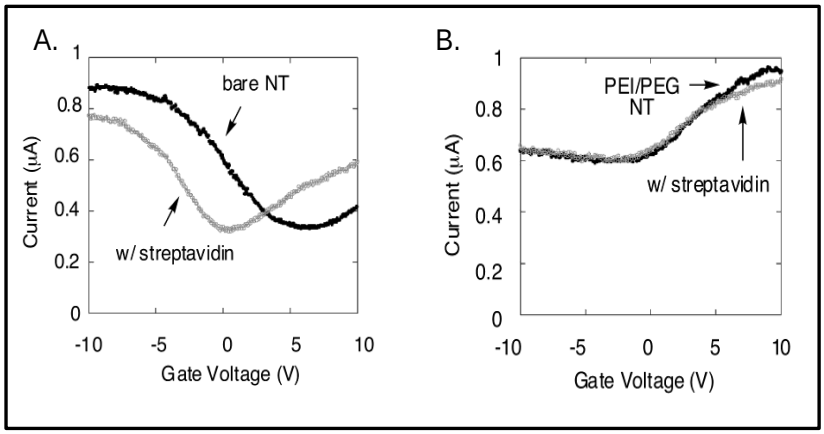

Figure 2-10. A) *p*-type behaviour of a CNTFET device before and after being exposed to streptavidin (B) *n*-type behaviour of a CNTFET coated with a mixture of PEI (polymer electron-donating) and PEG in the absence and presence of streptavidin (Adapted from Star, A. et al, 2003).

Although CNTs are very stable chemical structures, their conduction properties can be affected by chemical species adsorbed on the tube as well as the metal electrodes (McEuen, P. 2003). Between the CNT and the metal contact there is a potential barrier, called “Schottky barrier” (SB) which inhibits the transmission of holes from the metal electrode into the CNT. If the CNT and metal have different work functions, there is equilibrium at the Fermi level leading to a donation of electronic density from the metal into the partially depleted CNT valence band. Recently, it has been shown that high-work-function Pd contacts allow obtaining zero or slightly negative Schottky barriers (SBs) in semiconducting tubes with diameters of $d > 2$ nm (Javey, A. et al, 2004).

Towards very positive gate voltages, the SB at the CNT – metal electrode inhibits the transport of holes from the metal electrode into the CNT valence band, therefore the measured current is small and the device is said to be in the ‘‘OFF-state’’. Towards more negative gate voltages the SB decrease and increase hole transport into the CNT, the device is said to turn on, the current increase and the device is said to be in the ‘‘ON-state’’. Sweeping the gate voltage at a constant V_{sd} generates a current (or conductance increase) versus gate voltage V_g curve, I $-V_g$ called a transistor transfer characteristic (Kauffmanab, D.R. and Star, A. 2008).

In a dense network involving both metallic and semiconducting nanotubes, the conductance of the off state, will be dominated by the conductance of the metallic tubes. Assuming the same conductance for metallic and negatively biased semiconducting nanotubes, a ratio of the metallic to semiconducting tubes of 1 to 2 would suggest a modulation (on/off conductance ratio) of 3. However, in low density networks, percolation issues can also tend to increase the on/off ratio. For a sample with very few pathways from source to drain, there is a high probability that there will exist no continuous all-metallic tube paths, therefore yielding a slight off current, and an on/off ratio several orders of magnitude higher than the on/off ratio for a thick film (Hu, L. et al, 2004).

Figure 2-11 shows the gate dependence of conductance for a metallic and a semiconducting SWNT. Dotted curve shows that there is not gate dependence of conductance, indicating the presence of exclusively metallic tubes, while in a solid line shows a strong gate dependence of conductance of at least 4 orders of magnitude. This is representative of transport through purely semiconducting tubes. Balasubramanian *et al* (Balasubramanian, K. et al, 2004) report that a strong hysteresis is observed due to the electrical characterization is performed under ambient conditions. They explain that this phenomenon can be associated to oxygen-related charge traps either in the SiO_2 or to the presence of SiO_2 surface-bound water molecules.

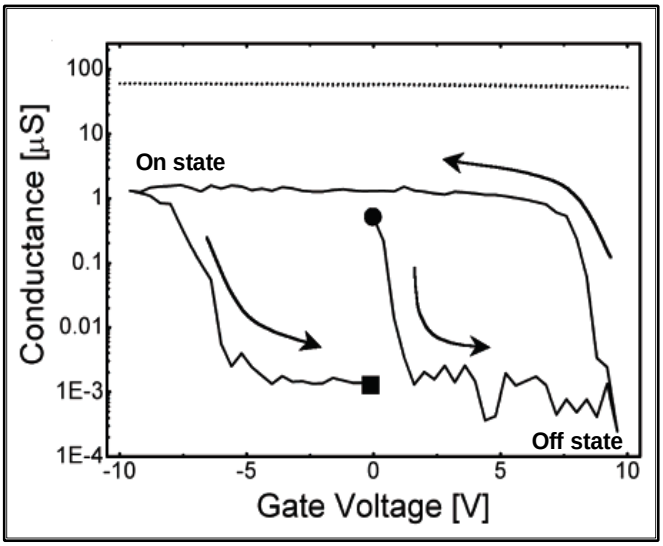

Figure 2-11. Gate dependence of conductance for a metallic SWNT (dotted line) and for a semiconducting SWNT (solid line), measured at room temperature. The gate voltage scan start at the point marked with the solid circle and stopped at the point marked with the solid square. A drain source voltage (Vsd) of 1 mV is used for measuring the conductance of the metallic SWNT and 100 mV for the semiconducting SWNT (Adapted from Balasubramanian, K. et al, 2004).

CNTFETs can be operated in two gate configurations. The first one, involve CNTFETs back-gated, which was applied in the present research work and was represented in figure 2-8 and 2-9. In this configuration the capacitor is a SiO_2 layer located between the *n* or *p*-doped Si substrate and the CNTs. The carbon nanotubes are connected to the source and drain electrodes. The third electrode "gate" is the so-called back-gate electrode since it is placed at the back of the device producing the final structure of a transistor. When a voltage is applied through the back-gate electrode, it makes possible to modulate the field strength across the insulating SiO_2, therefore the density of mobile charges in semiconducting SWCNTs are modified. As a consequence the conductivity of the device undergoes changes that can be measured and recorded. It is important to highlight that if the gate is placed in close contact with the CNTs the transconductance of the device can increase (Krüger, M. et al, 2001).

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This kind of configuration permits two important functions. On one hand, due to the CNTs are in close contact with the sample, the electrical signal generated can be easily related to the presence of the analyte. The generated field can be measured as a change in the resistance in the source-to-drain current. On the other hand, in this configuration the gate electrode is directly integrated into the device being unnecessary an external electrode (Cid, C., 2009).

The second type of configuration include CNTFETs electrolyte gated. Krüger *et al* (Krüger, M. et al, 2001) and Rosenblatt *et al* (Rosenblatt, S. et al, 2002) have shown that SWCNT-based field-effect transistors (FETs) can be electrolytically gated with high sensitivity. In this configuration a reference electrode (usually Pt) is placed in contact with a solution containing the analyte which at the same time is in contact with the SWCNTs (figure 2-12). The electrostatic potential difference between the solution and the CNTs is controlled through a gate voltage and the potential at the metal-liquid interface. Because the reaction is often carried out in buffer solution, biomolecules can reach the metal surface of the electrode altering the redox conditions at the metal-liquid interface. To overcome this problem Ag/AgCl reference electrode with a porous glass frit can be used ensuring that the redox conditions at the metal-liquid interface are well controlled and therefore, achieving artifact-free measurements (Minot, E.D. et al, 2007).

It has been found that the electrolyte-gated configuration increase the sensitivity of the CNTFETs devices, nevertheless the main disadvantage is related to the miniaturizing the reference electrode and/or conditions of the electrolyte when using Pt electrodes (Cid, C., 2009).

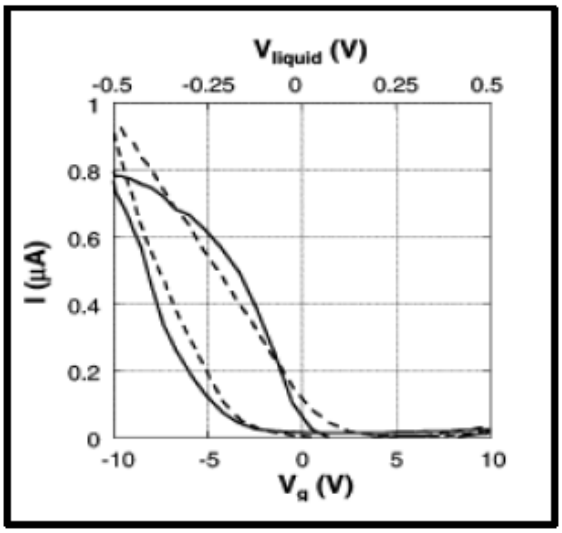

Figure 2-12. Transfer characteristics of a CNTFET in air (solid line), using the bottom gate, and in water (dotted line), using the liquid gate (Bradley, K. et al, 2004).

2.3.1. Biosensing applications of the CNTFETs

Different strategies can be applied to detect an analyte. In the case of FET based on CNTs the current flows through their tubular structure, therefore, any change in the surrounding environment modify the device characteristics being easily quantified (Grüner, G. 2006). When a molecule is adsorbed on the device, two main phenomena can take place; the first one involves a charge transfer process in which molecules provide or withdraw electrons to the tube. The second one is a scattering process, in which the adsorbed molecules act as scattering source (Grüner, G. 2006). Electrical measurements can help to distinguish between these two effects. If there is a charge transfer process, the threshold voltage becomes more positive (electron acceptor) or more negative (electron donor). In contrast, if a scattering mechanism occurs the overall conductance drops (Allen, B.L. et al, 2007) (figure 2-13).

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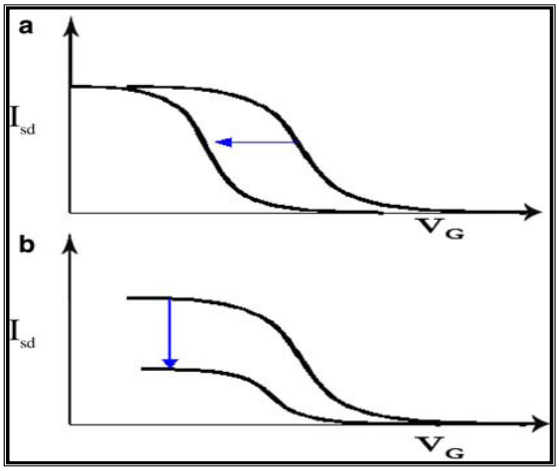

Figure 2-13. Change in the transistor device characteristics. a) Electron transfer from a protein to the tube b) Potential scattering (Gruner, G. 2006).

The detection of biological molecules using CNTs is a rapidly growing field. SWCNTs possess diameter comparable to large biological molecules, giving them the size compatibility necessary to ‘‘wire into’’ the system of interest. The surface area of individual SWCNTs is about 1600 m^2g^{-1}, the highest value for any known material allowing absorption process (Cinke, M. et al, 2002). They also have very high surface-to-weight ratio (~300 m^2g^{-1}) making them accessible to both electrochemistry and immobilization of biomolecules (Grüner, G. 2006). Furthermore, the low charge carrier density of SWCNTs is directly comparable to the surface charge density of proteins, which intuitively makes SWCNTs well suited for electronic detection that relies on electrostatic interactions with analyte biomolecules (Heller, I. et al, 2008).

CNTFETs have been used to detect glucose (Besteman, K. et al, 2003), xenocompounds (Sanchez, Z.C. et al, 2009), proteins like IgG (Cid, C. et al, 2008), DNA (Gui, E.L., 2007.; Star, A., 2006.; Martínez, M. T. et al, 2009), viruses (Dastagir, T., 2007) among others. Nevertheless, untill 2006 nobody had been detected bacteria or higher cells using CNTFETs. Only the interaction between bacteria and CNTs had been previously studied.

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For instance, Huang *et al* (Huang, T. S. et al, 2004) proven that anti-*Salmonella* and *anti-S. aureus* antibodies immobilized onto the CNTs allowed to detect concentrations up to 10^8 cfu/mL of these two bacteria. Gu *et al* (Gu, L. et al, 2005), reported the use of SWCNTs functionalized with galactose to detect *E.coli.* The complex Gal-SWCNT was mixed with $5x10^8$ cfu/mL of the bacteria during one hour. After that, the complex Gal-SWCNT-*E.coli* was observed in the SEM and the results showed a strong interaction between the complex and the bacteria. Elkin *et al* (Elkin, T. et al, 2005) used immuno-carbon nanotubes functionalized with BSA and anti-*E.coli O157:H7* antibody. The complex SWCNT-BSA-antibody was finally exposed to ~10^9 cfu/mL of the bacteria. The antigen-antibody interaction was proven through the confocal microscopy.

Lin *et al* (Lin, Y. et al, 2006), used the complex MWCNT synthesized by CVD using as catalyst xilene a precursor of the ferrocene. The resulting MWCNTs, containing residues of ferromagnetic catalyst, were coated with BSA and anti-*E.coli* antibody and were exposed to *E.coli O157:H7* at room temperature for 1 hour. By means of immunomagnetic separation the authors were able to detect ~ 40 cfu/100 µL of the bacteria in pure culture. Simultaneously to the publication of our first paper, So *et al* (So, H. et al, 2008) have also published an electrochemical sensor based on CNTFETs to detect the bacteria *E.coli*. They functionalized an array of CNTFETs with aptamers as the recognition element and were able to detect up to 93 cfu/mL of *E. coli* in water in less than 20 minutes.

In this research work, we tried to develop selective and sensitive biosensors able to detect different types of pathogenic microorganisms including bacteria, moulds and yeast with high relevance in both the food and medical sectors. Moreover, different blocking agents were used in order to protect the sidewalls of the CNTs from the non-specific binding of possible interference present in real samples. In this way, our devices were applied for biosensing purpose in simple and complex matriz. A brief explanation follows about the structure and antigenic determinants of bacteria, yeasts and moulds are given.

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2.3.1.1. Bacteria

They are unicellular organisms that can be both pathogenic and non-pathogenic with diameters having a range of 0.2 µm y 5 µm. Bacteria can be classified according to the composition of their cell wall in Gram positive, Gram negative and alcohol resistant. Gram positive bacteria, possess a cell wall with high content of peptidoglucane (about 90 %) and low amounts of polymers like teichoic acids (figure 2-14). Certain teichoic acids are intimately associated with lipids; therefore they are called lipotheichoic acids (Madigan, M.T. et al, 2000). Both teichoic and lipoteichoic acids are responsible for the antigenic properties of these organisms. By contrast, in Gram negative bacteria the amount of peptidoglucane is lower than Gram positive (about 10 %). In addition, they present an external membrane composed of lipopolysaccharides (LPS) (figure 1-14). These LPS contain lateral polysaccharides known as somatic antigen (O) which vary depending on the type of bacteria. The lipid portion of the LPS, referred to as lipid A is associated with the toxic properties of these organisms.

As can be seen, gram positive and gram negative bacteria are antigenically different; therefore, antibodies should not display cross-reactivity in the presence of a mixture of both types of microorganisms. Furthermore, bacteria can present surface structures such as, flagellum or capsules, which also determine their antigenicity. Flagellums are composed by the flagellar protein called flagellin*;* by contrast capsules are formed by polysaccharides containing glycoproteins and different polysaccharides. This structure represents and plays an important role in the attachment of the pathogenic microorganism to their host. Therefore, flagellar antigen (H) or capsular antigen (K) can be recognized by the antibodies respectively.

UNIVERSITAT ROVIRA I VIRGILI
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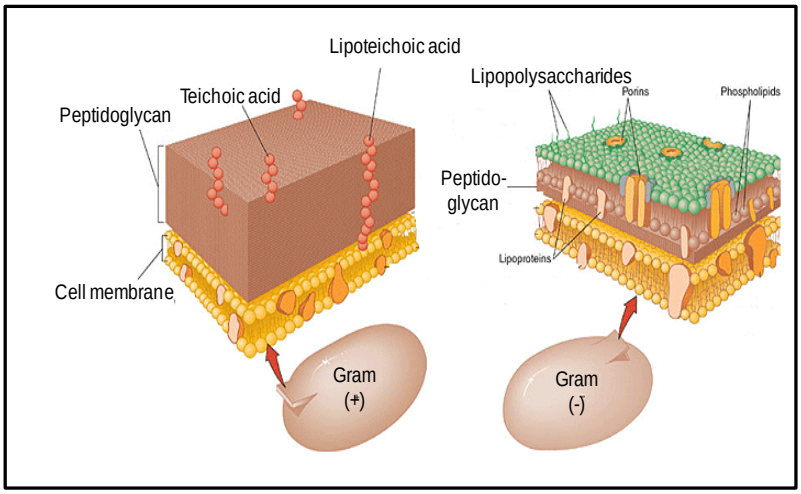

Figure 2-14. Schematic representation of the cell wall of gram positive and gram negative bacteria (Adapted from T. Betsy, J. Keogh, 2005).

2.3.1.2. Yeasts

Yeasts are unicellular fungi, usually spherical, oval, or cylindrical, and cell division generally takes place by budding. In this process a new cell forms as a small outgrowth of the old cell, the bud gradually enlarges and then separates. They can be distinguished microscopically from bacteria by their size (typically measuring 3–4 µm in diameter) and by the presence of nucleus. Although most yeast reproduce only as single cells, under some conditions some yeast can form filaments. This is the case of *Candida albicans,* which display a filamentous phase increasing their pathogenicity (Madigan, M.T. et al, 2000).

The cell wall of the yeast is composed basically by polysaccharide, proteins and lipids. The most important polysaccharides include; chitin (polymer of n-acetil-glucosamine), glucanes (polymers of glucose) and mannans (polymers of manose that can be linked to other type of monosaccharide). Structurally, mannans consist of an internal base, an external chain and oligomannosides with labile base. The

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external region of the chain determines the antigenic specificity. Figure 2-15 shows a schematic representation of a yeast cell.

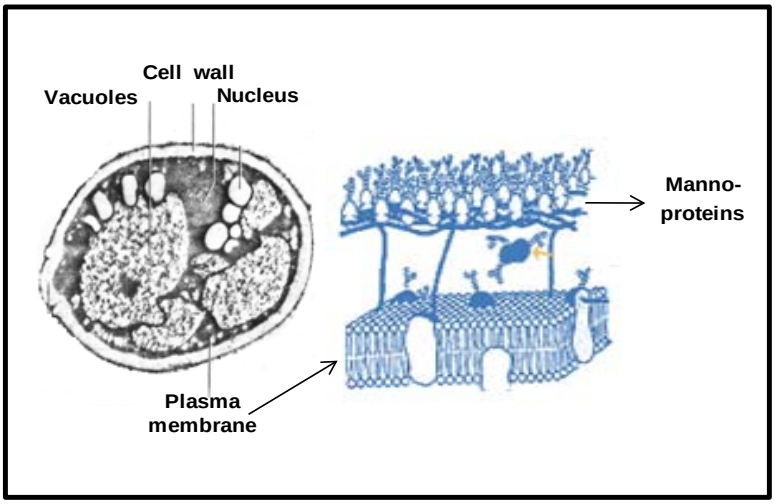

Figure 2-15. Schematic representation of the morphological structure of yeast.

2.3.1.3. Moulds

They are filamentous fungi, widespread in nature and are frequently found on stale bread, cheese or fruit. A single filament is called hypha. It usually grows together across a surface and forms a compact structure called a mycelium. From the fungal mycelium, other hyphal branches may reach the air producing aerial branches spores called conidia (figure 2-16). They are asexual spores and are responsible of the final color for the fungi which may be black, blue-green, red, yellow or brown. As well as in yeasts, the cell wall of the moulds is generally composed of 80-90 % polysaccharide, with proteins, lipids, polyphosphates and inorganic ions. Mannans and galactomannas are often responsible for the immunological response against moulds and yeast (McGinnis, M.R. and Borgers, M. 1989).

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These fungi can cause disease through three main mechanisms. The first involves allergic reactions after being exposed to specific fungal species. *Aspergillus* spp, is commonly associated with hypersensitivity causing asthma. A second fungal disease mechanism is the production of mycotoxins. The best known examples are the aflatoxins produced by *A. flavus,* a fungus frequent in grains. The mycotoxin can induce tumors in animals. The third fungal disease-produced mechanims is through an overgrow of a fungus on or in the body, producing mycosis which can vary from superficial to serius life-threatening diseases (Madigan, M.T. et al, 2000).

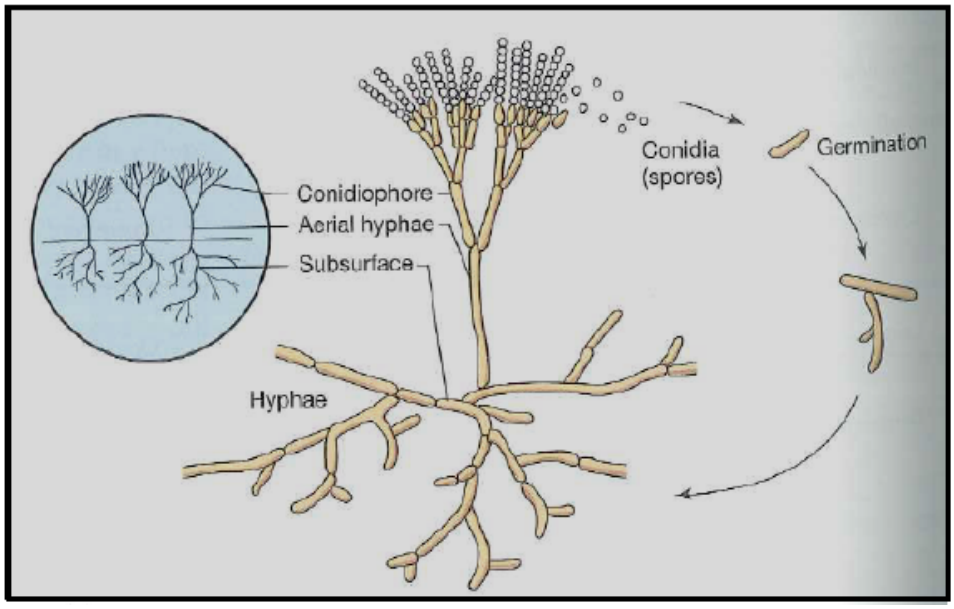

Figure 2-16. Diagram of the different structures a mould displays along its life cycle (Madigan, M.T. et al, 2000).

ISBN:978-84-692-9761-2/DL:T-203-2010

2.4. References

Allen, B.L., Kichambare, P.D., Star, A. Advanced Materials. **2007**, 19, 1439–1451.

Balasubramanian, K., Sordan, R., Burghard, M., Kern, K. Nano Letters. **2004**. 4, 827-830.

Baughman, R.H., Cui, C., Zakhidov, A.A., Iqbal, Z., Barisci, J.N., Spinks, G.M., Wallace, G.G., Mazzoldi, A., De Rossi, D., Rinzler, A.G., Jaschinski, O., Roth, S., Kertesz, M. Science. **1999**. 284, 1340–1344.

Besteman, K., Lee, J. O., Wiertz, F.G.M., Heering, H.A. , Dekker, C. Nano Letters. **2003**. 3,727-730.

T. Betsy, J. Keogh, Microbiology Demystified, McGraw-Hill Publishing, New York, **2005.**

Beyer, M., Felgenhauer, T., Bischoff, F. R., Breitling, F., Stadler, V. Biomaterials. **2006**. 27, 3505-3514.

Bjorck, L. and Kronvall, G. The Journal of Immunology. **1984**. 133, 969-974.

J.P. Bourgoin, A. Loiseau, J.F. Nierengarten, Nanoscience, Springer, Berlin. **2006**.

Bradley, K., Briman, M., Star, A., Grüner, G. Nano Letters. **2004**. 4, 253-256.

Byon, H.R., Choi, H.C. Journal of American Chemical Society. **2006**. 128, 2188-2189.

Cinke, M., Li, J., Chen, B., Cassell, A., Delzeit, L., Han, J., Meyyappan, M. Chemical Physics Letters. **2002**, 365, 69–74.

Charlier, J.C., Roche, X.B. Reviews of Modern Physics. **2007**. 79, 677-732.

Chen, R.J., Bangsaruntip, S., Drouvalakis, K.A., Shi, N.W., Shim, M., Li, Y., Kim, W., Utz, P.J., Dai, H. Proceedings of the National Academy of Sciences. **2003**. 100, 4984-4989.

Chen, E. S.; Chen, E. C. M. Bioelectrochemistry and Bioenergetics. **1998**. 46, 15-19.

Chen, R. J., Choi, H. C., Bangsaruntip, S., Yenilmez, E., Tang, X., Wang, Q., Chang, Y. L., Dai, H. Journal of American Chemical Society. **2004.** 126, 1563-1568.

Chi, Y.S., Byon, H.R., Choi, H.C., Choi, I. S. ChemBioChem. **2007**. 8, 1380-1387.

Cid, C., Riu, J., Maroto, A., Rius, F.X. Analyst. **2008**. 133, 1005–1008.

Cid C. **2009**. Doctoral Thesis. Sensors based on carbon nanotube field-effect transistors and molecular recognition approaches. Department of Analytical and Organic Chemistry. Universitat Rovira I Virgilli.

Cowley, J.M., Nikolaev, P., Thess, A., Smalley, R.E. Chemical Physics Letters. **1997**. 265, 379–384.

Dastagir, T., Forzani, E.S., Zhang, R., Amlani, I., Nagahara, L.A., Tsui, R., Tao, N. Analyst. **2007**.132, 738-740.

M.S. Dresselhaus, G. Dresselhaus, P.C. Eklund, Science of Fullerenes and Carbon Nanotubes, Academic Press, New York, **1996**.

Duerkop, T., Getty, S., Cobas, E., Fuhrer, M. Nano Letters. **2004**. 4, 35-39.

Elkin, T., Jiang, X., Taylor, S., Lin, Y., Gu, L., Yang, H., Brown, J., Collins, S., Sun, Y. ChemBioChem. **2005**. 6, 640-643.

ISBN:978-84-692-9761-2/DL:T-203-2010

J.E. Fischer, Nanotubes and Nanofibers, Taylor & Francis Group, LLC, New York, **2006.**

Freitag, M., Martin, Y., Misewich, J.A., Martel, R., Avouris Ph. Nano Letters. **2003**. 3, 1067–1071.

Giacomelli, C.E. Encyclopedia of Surface and Colloid Science. **2006**. DOI 10.1081/E-ESCS-120000092.

P. Gründler, Chemical Sensors, Springer-Verlag, Berlin, **2006**.

Grüner, G. Analytical and Bioanalytical Chemistry. **2006**. 384, 322-335.

Gui, E.L., Li, L.J., Zhang, K., Xu, Y., Dong, X., Ho, X., Lee, P.S., Kasim, J., Shen, Z.X., Rogers, J.A., Mhaisalkar, S.G. Journal of the American Chemistry Society. **2007**.129, 14427-14432.

Gu, L., Elkin, T., Jiang, X., Li, H., Lin, Y., Qu, L., Tzeng, T.R.J., Joseph, R., Sun, Y.P. Chemical Communications. **2005**. 874-876.

Hecht, D.S., Ramirez, R.J.A., Briman, M., Artukovic, E., Chichak, K.S., Stoddart, J.F., Grüner, G. Nano Letters. **2006**. 6, 2031–2036.

Heller, I., Kong, J., Heering, H.A., Williams, K.A., Lemay, S.G., Dekker, C. Nano Letters. **2005**. 5, 137-142.

Heller, I., Kong, J., Williams, K.A., Dekker, C., Lemay, S.G. Journal of the American Chemical Society. **2006**. 128, 7353-7359.

Heller,I., Janssens, A.M., Mannik, J., Minot, E.D., Lemay, S.G., Dekker, C. Nano Letters. **2008**. 8, 591-595.

Hu, L., Hecht, D.S., Gruner, G. Nano Letters. **2004**. 4, 2513-2517.

Huang, S., Woodson, M., Smalley, R., Liu, J. Nano Letters. **2004**. 4, 1025–1028.

Iijima, S. Nature.**1991**. 354, 56.

Izard, N., Kazaoui, S., Hata, K., Okazaki, T., Saito, T., Iijima, S., Minami, N. Applied Physics Letters. **2008**. 92, 243112.

Javey, A., Kim, H., Brink, M., Wang, Q., Ural, A., Guo, J., McIntyre, P., McEuen, P., Lundstrom, M., Dai, H. Journal of Nature Materials. **2002**. 1, 241-246.

Javey, A., Guo, J., Wang,Q., Lundstrom, M., Dai, H. Nature. **2003**. 424, 7.

Javey, A., Guo, J., Farmer, D.B., Wang, Q., Wang, D.W., Gordon, R.G., Lundstrom, M., Dai, H. Nano Letters. **2004**. 4, 447-450.

Kauffmanab, D.R. and Star, A. Chemical Society Reviews. **2008**. 37, 1197–1206.

Khare, R. and Bose, S. Journal of Minerals & Materials Characterization & Engineering. **2005**. 4, 31-46.

Kim, S.N., Rusling, J.F., Papadimitrakopoulos, F. Advanced Materials. **2007**. 19, 3214-3228.

Kong, J., Franklin, N.R., Zhou, C.W., Chapline, M.G., Peng, S., Cho, K.J., Dai, H.J. Science. **2000**. 287, 622–625.

Kocabas, C., Hur, S.H., Gaur, A., Meitl, M.A., Shim, M., Rogers, J.A. Small. **2005**. 1,1110-1116.

Krüger, M., Buitelaar, M.R., Nussbaumer, T., Schönenberger, C., Forro, L. Applied Physcis Letters. **2001**. 78, 1291-1293.

Lay, M.D., Novak, J.P., Snow, E.S. Nano Letters. **2004**. 4, 603-606.

Lee, J.M., Park, H.K., Jung, Y., Kim, J.K., Jung, S.O., Chung, B.H. Analytical chemistry. **2007**. 79, 2680-2687.

A. Liljas, L. Liljas, J. Pirkur, G, Lindblom, P. Nissen, M. Kjeldgaard, Textbook of Structural Biology, World Scientific Publishing Co. Pte. Ltd, Singapore, **2009**.

Lin, A., Patil, N., Ryu, K., Badmaev, A., De Arco, L., Zhou,C., Mitra, S., Wong, H.S. IEEE Transactions on Nanotechnology. **2009**. 8,1.

Lin, Y., Allard, L.F., Sun, Y.P. Journal of Physical Chemistry B. **2008**. 108, 3760-3764.

Lin, Y., Taylor, S., Li, H., Fernando, K.A., Qu, L., Wang, W., Gu, L., Zhou, B., Sun, Y.P. Journal of Materials Chemistry. **2004**. 14, 527-541.

Lin, Y., Jiang, X., Elkin, T., Fernando, K.A., Gu, L., Taylor, S., Yang, H., Jones, E., Wang, W., Sun, Y.P. Journal of Nanoscience and Nanotechnology. **2006**. 6, 868-871.

M.T. Madigan, J.M. Martinko, J. Parker, Brock Biology of Microorganisms. Ninth ed., Prentice Hall, Inc, New Jersey, **2000**.

Martínez, M.T., Tseng, Y.C., Ormategui, N., Loinaz, I., Eritja, R., Bokor, J. Nano Letters. **2009**. 9, 530-536.

Marie, R., Beech, J.P., Vörös, J., Tegenfeldt, J.O., Höök, F. Langmuir. **2006.** 22, 10103-10108.

Martel, R., Schmidt, T., Shea, H.R., Hertel, T., Avouris, P. Applied Physics Letters. **1998**. 73, 2447.

McEuen, P. Single wall carbon nanotubes. Physics World. **2000**.31-36

M.R. McGinnis, M. Borgers, Current Topics in Medical Mycology, Springer-Verlag, New York. **1989**.

Minot, E.D., Janssens, A.M., Heller, I., Heering, H.A., Dekker, C., Lemay, S.G. Applied Physical Letters. **2007**. 91, 093507.

O`Connor, M., Kim, S.N., Killard, A.J., Foster, R.J., Smyth, M.R., Papadimitrakopoulos, F., Rusling, J.F. Analyst. **2004**. 129, 1176-1180.

Oh, B.K., Lee, W., Kim, Y.K., Lee, W.H., Choi, J.W. Journal of Biotechnology. **2004**. 111, 1-8.

Okuno, J., Maehashi, K., Kerman, K., Takamura, Y., Matsumoto, K., Tamiya, E. Biosensors and Bioelectronics. **2007**. 22, 2377-2381.

Pantarotto, D., Partidos, C.D., Graff, R., Hoebeke, J., Briand, J.P., Prato, M., Bianco, A. Journal of the American Chemical Society. **2003**. 125, 6160-6164.

Piao, M.H.M., Noh, M.A. Rahman, H., Won, M.S., Shim, Y.B. Electroanalysis. **2008**. 20, 30–37.

C.P. Poole, F.J. Owens, Introduction to Nanotechnology, Wiley-Interscience, Hoboken, New Jersey , **2003**.

Pourfath, M., Kosina, H., Selberherr, S. Downloaded on June 4, **2009** from IEEE Xplore.

Raffaellea, R.P., Landia, B.J., Harrisb, J.D., Baileyb, S.G., Hepp, A.F. Materials Science and Engineering B. **2005**. 116, 233–243.

Rao, A. M., Richter, E., Bandow, S., Chase, B., Eklund, P.C., Williams, K.A., Fang, S., Subbaswamy, K.R., Menon, M., Thess, A., Smalley, R.E., Dresselhaus, G., Dresselhaus, M. S. Science. **1997**. 275, 187–190.

ISBN:978-84-692-9761-2/DL:T-203-2010

Rosenblatt, S., Yaish, Y., Park, J., Gore, J., Sazonova, V., McEuen, P. L. Nano Letters. **2002**. 2, 869-872.

R. Saito, G., Dresselhaus, M.S. Dresselhaus, Physical Properties of Carbon Nanotubes, Imperial College Press, London, **1998**.

Sánchez-Acevedo, Z.C., Riu, J., Rius, F. X. Biosensors and Bioelectronics. **2009**. 24, 2842–2846.

Shim, M., Kam, N.W.S., Chen, R.J., Li, Y., Dai, H. Nano Letters. **2002**. 2, 285-288.

Sloan, D.J. and Hellinga, H.W. Protein Science. **1999**. 8, 1643–1648.

So, H.M., Park, D. W., Jeon, E. K., Kim, Y. H., Kim, S.K., Lee, C.K., Choi, S. Y, Kim, S.C., Chang, H., Lee, J.O. Small. **2008**. 4, 197-201.

Star, A., Gabriel, J.C.P., Bradley, K., Grüner, G. Nano Letters. **2003**. 3, 459-463.

Star, A., Tu, E., Niemann, J., Gabriel, J.C.P., Joiner, C.S., Valcke, C. Proceedings of the National Academy of Sciences. **2006**. 103, 921-926.

Tans, S.J., Devoret, M.H., Dai, H., Thess, A., Smalley, R.E., Geerligs, L.J., Dekker, C. Nature. **1997.** 386, 474.

Trojanowicz, M. Trends in Analytical Chemistry. **2006**. 25, 480-489.

Tuncel, A. Colloid and Polymer Science. **2000**, 278, 1126-1138.

Vermeer, A.W.P., Norde, W., van Amerongen, A. Journal of Biophysics. **2000**. 79, 2150-2154.

Vermeer, A.W.P. and Norde, W. Journal of Biophysics. **2000**. 78, 394-404.

ISBN:978-84-692-9761-2/DL:T-203-2010

Vichchulada, P., Zhang, Q., Lay, M.D. Analyst. **2007**. 132, 719–723.

P. Voet, J. Voet, C. Pratt, Principles of Biochemistry, third ed., John Wiley & Sons, INC, **2008**.

Wang, S., Humphreys, E. S., Chung, S.Y., Delduco, D. F., Lustig, S. R., Wang, H., Parker, K. N., Rizzo, N. W., Subramoney, S., Chiang, Y.M., Jagota, A. Nature Materials. **2003**. 2, 196.

Wildöer, J.W.G., Venema, L.C., Rinzler, A.G., Smalley, R.E., Dekker, C. Nature. **1998**. 391, 59.

Yao, Z., Kane, C.L., Dekker, C. Physics Reviews Letters. **2000**. 84, 2941-2944.

Zhao, Y.L. and Stoddart, J.F. Accounts of Chemical Research. **2009**. 42, 1161-1171.

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Chapter 3

Experimental development of the biofunctionalized devices

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3.1. Introduction

This chapter presents a detailed description of the materials, reagents, equipment and procedures used for the development of the CNTFET sensors. The objective is to give a general idea concerning the manufacture of the biosensors, from the synthesis of the carbon nanotubes to the functionalization and detection process as well as the preparation of the pathogenic microorganisms detected are explained along with the characterization techniques of the devices. Finally, the aim of this chapter is to supply a complete protocol that can be reproduced and therefore be used in practice for future research.

3.2. Apparatus, materials, and reagents

3.2.1. Cells preparation

3.2.1.1. Apparatus

- Autoclave (Selecta)
- Mini orbital shaker (Stuart)
- Biosafety chamber, Telstar Technologies, S.L. Model Bio II A (Izasa)
- Memmert incubator (Afora)

3.2.1.2. Strains

3.2.1.2.1. Bacteria

- *Salmonella* Infantis S34/11(Faculty of Medicine and Health Sciences at the Universitat Rovira i Virgili)
- *Shigella sonnei* CECT 413(Faculty of Medicine and Health Sciences at the Universitat Rovira i Virgili)
- *Streptococcus pyogenes* CECT 985T(Faculty of Medicine and Health Sciences at the Universitat Rovira i Virgili)

3.2.1.2.2. Yeast

- *C. albicans* CECT 1001 (Spanish Type Culture Collection, Valencia,Spain)
- *Saccharomyces cerevisiae* CECT 1443 (Spanish Type Culture Collection, Valencia,Spain)
- *C. albidus* CECT 11978 (Spanish Type Culture Collection, Valencia,Spain)

3.2.1.2.3. Moulds

- *Aspergillus flavus* CECT 2684 (Spanish Type Culture Collection, Valencia,Spain)
- *Penicillium chrysogenum* CECT 2307 (Spanish Type Culture Collection, Valencia,Spain)

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- *Fusarium oxysporum* CECT 2154 (Spanish Type Culture Collection, Valencia,Spain)

3.2.1.3. Media

- Trypticase soy agar (TSA), Breckton-Dickinson (Comercial Belles S.A.)
- Trypticase soy broth (TSB), Beckton-Dickinson (Comercial Belles S.A.)
- Sabouraud Dextrosa Agar (SDA), Oxoid
- Oxytetracycline, gentamicine yeast agar (OGY), Oxoid
- Brain heart infusion broth (BHI), Scharlau Chemie Microbiology (Comercial Belles S.A.)
- Buffered peptone water, Scharlau Chemie Microbiology (Comercial Belles S.A.)

3.2.2. Synthesis of the CNTs

3.2.2.1. Apparatus

- Horizontal split tube furnace HST 12/600 (Carbolite)
- Quartz tubular reactor (4 cm x 120 cm) (Afora)
- Ultrasonic bath 100 W (Selecta)
- Spin coater WS-400B-6NPP/LITE (Laurell Technologies Corporation).
- Mass controllers for methane and hydrogen including PC software (Bronkhorst, Ruurlo)

3.2.2.2. Materials

Substrates of Si/SiO_2. 500 nm of SiO_2 grown thermally over type n-type low resistivity Si. Dimensions: 0.5 x 0.5 cm (D+T Microelectrónica, National Microelectronics Centre).

3.2.2.3. Reagents

- Methane C-45 (Carburos metálicos)
- Hydrogen C-50 (Carburos metálicos)
- Argon C-50 (Carburos metálicos)

ISBN:978-84-692-9761-2/DL:T-203-2010

- Nitrogen C-50 (Carburos metálicos)
- Iron nitrate (III) nonahydrated 99.99 % (Sigma)
- Acetone 99.5 % (Sigma)
- Isopropanol 99.5 % (Sigma)
- Deionized and charcoal-treated water (18.2 MΩ·cm specific resistance) obtained with Milli-Q PLUS reagent-grade water system (Millipore).

3.2.3. Development of the CNTFETs

3.2.3.1. Materials

Silver ink Electrodag ® 1415 M (Acheson Industries, Scheemdam, Netherlands)

3.2.4. Functionalization of the CNTFETs

3.2.4.1. Proteins

- IgG anti-*Salmonella* OBT0958 (i.e. a rabbit polyclonal antibody for *Salmonella* “O” & “H” antigens (4–5 mg/mL)), Oxford Biotechnology Ltd. (Oxford, UK)
- IgG anti-*Candida*, 1750-5070 (i.e. a monoclonal mouse anti-*C. albicans* (2.8 mg/mL)), Oxford Biotechnology Ltd (Oxford, U.K.).
- IgG anti-*Aspergillus* 0071-1300 (i.e. a polyclonal rabbit anti-*Aspergillus* (1 mg/mL)), Oxford Biotechnology Ltd. (Oxford, U.K.)
- Protein G from *Streptococcus* sp. recombinant, expressed in *Escherichia coli* (1 mg/mL) (Sigma).

3.2.4.2. Reagents

- Dulbecco phosphate buffered saline solution (PBS), 150 mM, pH: 7.1 - 7.5 (Sigma)
- Tween 20 (Sigma)
- Gelatine from cold water fish skin (Sigma)

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- Deionized and charcoal-treated water (18.2 MΩ·cm specific resistance) obtained with Milli-Q PLUS reagent-grade water system (Millipore)

3.2.5. Characterization of the CNTFETs

3.2.5.1. Electrical characterization

- Precision semiconductor parameter analyser Agilent 4157A (Agilent Technologies)
- MP 1008 Manual probe station (Wentworth Laboratories)
- Faraday box (SIRM)
- Anti-vibration table (SIRM)

3.2.5.2. Microscopy characterization

- Scanning electron microscope (SEM), JSM 6400 (Jeol, Tokyo, Japan)
- Environmental-Scanning electron microscope (ESEM), Quanta 600, (FEI, Hillsboro, OR, USA)
- Atomic force microscope (AFM), PicoPlus (Molecular Imaging, Tempe, AZ, USA)

3.2.5.3. Ellipsometry characterization

- Woollam VASE spectroscopic ellipsometer (J.A. Woollam Co. Lincoln, USA)
- Software WVASE32 (J.A. Woollam Co. Lincoln, USA)

3.3. Procedures

3.3.1. Preparation of the pathogenic microorganisms

3.3.1.1. Bacteria

- Take a sample of the bacteria from the initial culture stored at -20 ºC and reactivate it by inoculating in 10 mL of sterile TSB at 37 ºC for 12 h.

UNIVERSITAT ROVIRA I VIRGILI
BIOSENSORS BASED ON CARBON NANOTUBE FIELD EFFECT TRANSISTORS (CNTFETs) FOR DETECTING PATHOGENIC MICROORGANISMS
Raquel Amanda Villamizar Gallardo
ISBN:978-84-692-9761-2/DL:T-203-2010

- Then, pelletize the sample in a centrifuge at 6000 rpm for 15 minutes and discard the supernatant.
- Resuspend the pellet in 10 mL of sterile 0.85 % saline solution and enumerate the bacteria contained in the saline solution by plating serial dilutions on TSA plates and incubating at 37 ºC for 24–48 h (figure 3-1).
- Prepare additional dilutions in sterile 0.85 % saline solution to produce the desired final sample concentration of each strain.

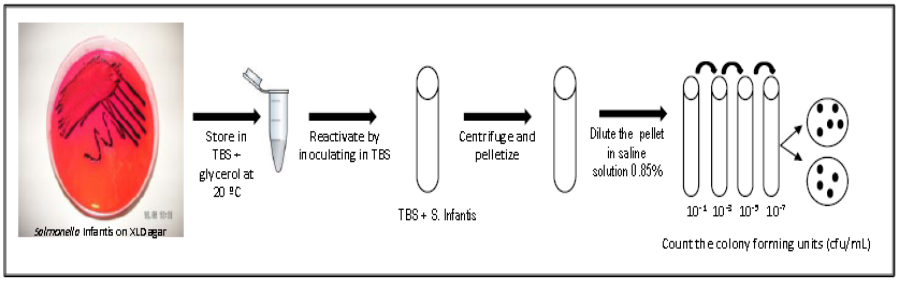

Figure 3-1. Cell culture preparation of *Salmonella* Infantis.

3.3.1.2. Yeast

- Rehydrate the stock strains with sterile water and subculture at least three times on SDA and incubated at 35 ºC for 48 h to test their viability.
- Take isolated colonies and resuspend them on sterile 0.85 % saline solution.
- Enumerate the yeast contained in the saline solution by plating serial dilutions on SDA plates and incubating at 35 ºC for 24–48 h (Chang, H.C. et al, 2000) (figure 3-2).
- Prepare additional dilutions in sterile 0.85 % saline solution to produce the desired final sample concentrations of each strain.
-

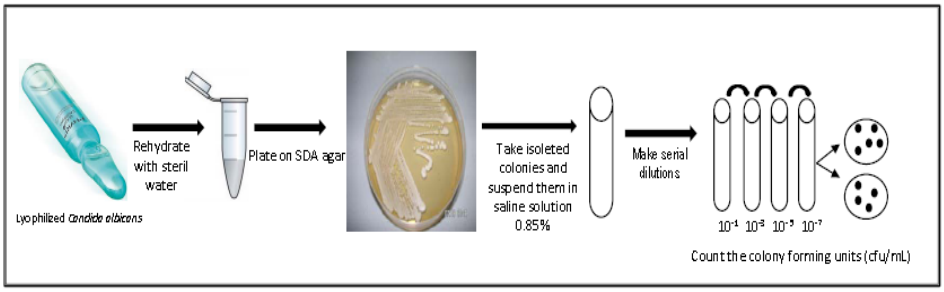

Figure 3-2. Cell culture preparation of *Candida albicans*.

3.3.1.3. Moulds

- Rehydrate the stock strains with sterile water and plate them in SDA at 30 ºC for 5-7 days (Hao, W. et al, 2008) to check their viability.
- Wash the spores from the surface of the medium with PBS solution.
- Take flaks of 100 mL of BHI, inoculate them with 1 mL of the spore suspension obtained before and incubate at 30 ºC, 120 rpm for 7 days (Tsai G.J. and Yu S.C., 1999) (figure 3-3).
- Harvest the cultures and separate the mycelium by filtrating with Whatman No. 5 filter paper (Hetherington, S.V. 1994).
- Collect the mycelium, wash it with sterile distilled water and dry in the oven at 105 ºC until stable weight (De Vos, M.M. and Nelis, H.J. 2003).
- Dilute the previous samples in PBS (w/v) to produce the desired final sample concentration of each strain.

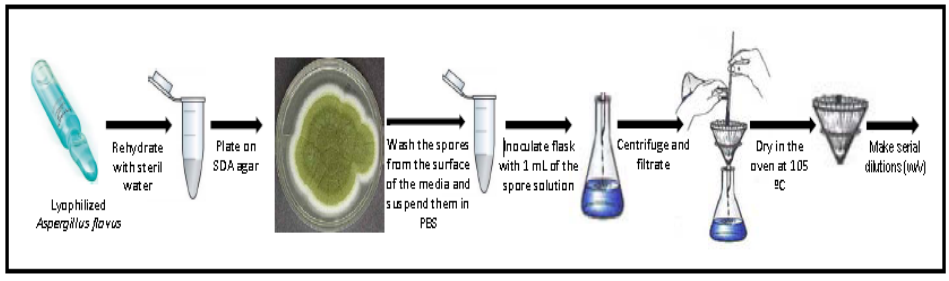

Figure 3-3. Cell culture preparation of *Aspergillus flavus*.

UNIVERSITAT ROVIRA I VIRGILI
BIOSENSORS BASED ON CARBON NANOTUBE FIELD EFFECT TRANSISTORS (CNTFETs) FOR DETECTING PATHOGENIC MICROORGANISMS
Raquel Amanda Villamizar Gallardo
ISBN:978-84-692-9761-2/DL:T-203-2010

3.3.2. Synthesis of the SWCNTs

- Prepare the catalyst solution by dissolving iron nitrate in isopropanol up to a concentration of 100 µg/mL.
- Sonicate the solution in an ultrasonic bath, for 10-15 minutes.
- Clean the Si/SiO_2 substrate by making successive immersions in acetone, isopropanol, and deionized water in the ultrasonic bath. Each immersion should last about 5 minutes.
- Dry with nitrogen.
- Spin-coat three times 20 µL of the catalyst solution over the Si/SiO_2 at 3000 rpm and leave it spinning until the solvent is completely evaporated (about 30 seconds).
- Place the Si/SiO_2 substrates into the quartz reactor and switch on the oven at 900 ºC.
- Introduce an argon current of 1000 sccm until the maximum temperature is reached. After that, close the argon flow and simultaneously introduce 600 sccm of methane and 200 sccm of hydrogen for 20 minutes (figure 3-4).
- Finally, close the methane and hydrogen flows while argon flow is introduced again into the quartz reactor until the system cools down to room temperature (Cid C. 2009).

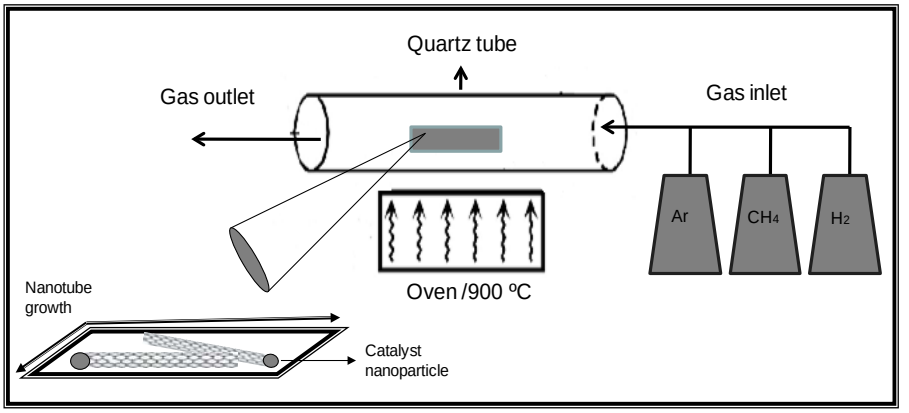

Figure 3-4. Schematic representation of the chemical vapour deposition (CVD) process used for the synthesis of SWCNT at 900 ºC. The zoom shows the growth mechanism of the nanostructures.

UNIVERSITAT ROVIRA I VIRGILI
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ISBN:978-84-692-9761-2/DL:T-203-2010

3.3.3. Development of the CNTFETs

As it has been explained in chapter 2, the structure of a FET consists of two metal electrodes named *source* and *drain* connected to the semiconducting channel forming a transistor. Perpendicular to this structure, we find a capacitor made of the semiconducting nantubes, the SiO_2 dielectric layer and the semiconducting *n*-type doped Si layer. Different methods have been used to develop the sensor. Photolithography is one of the common methods used to grafting the electrodes. Although this method generates well reproducible devices it is also expensive. Therefore, in the present research we use a homemade screen printing process. This process allows obtaining cheap devices in a very short time.

The process involve the use of a conductive ink, Electrodag® 1415 M, made of silver and 4-methylpentan-2-one. By using a home-made designed silicone mask, the silver ink is deposited manually over the Si/SiO_2 substrate containing the as-grown network of SWCNTs. The mask allows printing the metallic electrodes at the desired spots (figure 3-5). Subsequently in order to fix the electrodes to the surface, the chips are cured in an oven at 150 ºC for 10 min (Cid C. 2009).

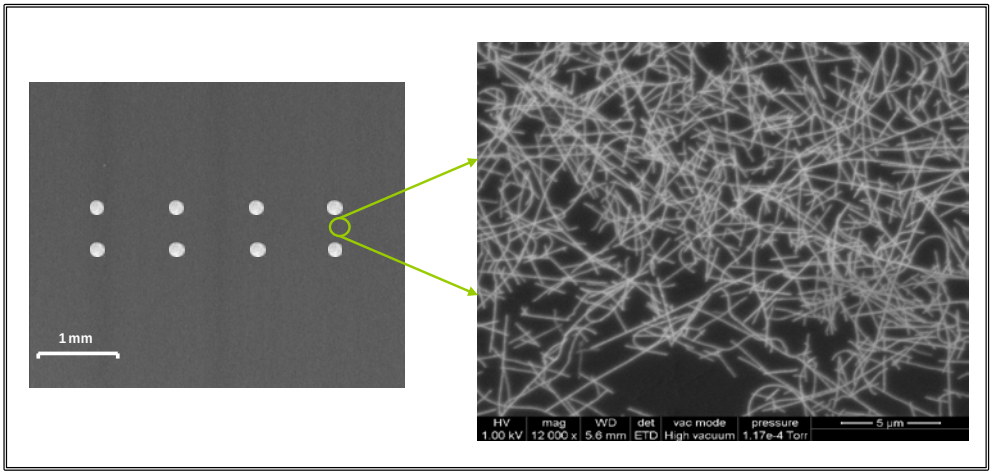

Figure 3-5. SEM image of the home-made screen-printed electrodes. The size of the electrodes is about 200 μm^2 and the distance between the source and drain is approximately 500 µm. The zoom shows an E-SEM image of the typical network of SWCNTs obtained after a CVD process.

ISBN:978-84-692-9761-2/DL:T-203-2010

3.3.4. Functionalization process

In the development of our sensor devices, two methods have been used to immobilize the molecular receptors (antibodies) onto the transducers (SWCNTs).

3.3.4.1. Direct adsorption of IgG onto the CNTs

The direct adsorption of immunoglobulin on a surface is a non-covalent process governed mainly by hydrophobic interactions between antibodies and the solid surface (Chen, R.J. et al, 2003). Since the adsorption is a random process, it can leave free gaps in the surface. Therefore, in order to avoid the non-specific binding of other proteins or possible interferences on the SWCNTs a solution of Tween 20 is used as blocking agent (Chi, Y.S. et al, 2007). The functionalization process is carried out as follows:

- Dilute the commercial antibody up to the desired concentration (i.e. 10 µg/mL) in phosphate buffered saline solution (PBS) (15 mM, pH=7.4).
- Immerse the CNTFET obtained before in the prepared antibody solution for 1 hour at 37 ºC to have the antibodies adsorbed onto the SWCNTs (Lin, Y. et al, 2006).
- Rinse with PBS (15 mM, pH=7.4), deionized water and dry with nitrogen (Ilic, B., et al, 2000).
- Immerse the CNTFET previously coated with the antibodies in a solution containing Tween 20 at 0.5 % for 2 hours (figure 3-6). Subsequently, rinse with deionized water and dry with nitrogen (So, H.M. et al, 2005).
- After this process, the devices are ready to use to detect the microorganism[1]

3.3.4.2. IgG binding through the protein G

In order to obtain well-orientated antibodies linked to the SWCNT surface, bacterial proteins like protein G can be used. They are found in the cell wall of most species of *Staphylococcus* and *Streptococcus* and display high specificity for the Fc domain

[1] CNTFETs with the IgG directly adsorbed were used to detect the bacteria *Salmonella* Infantis and the yeast *Candida albicans*.

ISBN:978-84-692-9761-2/DL:T-203-2010

of the IgG, thus leaving the Fab region available for detecting the target (Oh, B.K. et al, 2004). This functionalization process involves the following steps:

- Dilute the commercial protein G up to the desired concentration (e.g. 5 µg/mL) in phosphate buffered saline solution (PBS) (15 mM, pH=7.4).
- Immerse the CNTFET in the prepared protein G solution for 30 minutes at 37 ºC to have the protein adsorbed over the SWCNT (Lin, Y. et al, 2006).
- Rinse with PBS (15 mM, pH=7.4), deionized water and dry with nitrogen (Ilic, B. et al, 2000).
- Immerse the CNTFET previously coated with protein G in a PBSTG solution containing 1.5 % of Tween 20 and 2 % of gelatin for 3 hours in order to avoid the non-specific adsorption of the antibodies onto the transducer (Piao, M.H. et al, 2008) and to protect the SWCNTs from the interferences of the sample matrix (figure 3-6).
- Rinse again with deionized water and dry with nitrogen (Ilic, B. et al, 2000) and immerse the CNTFET previously coated with protein G and PBSTG in an antibody solution (8 µg/mL) for 1 hour at 37 ºC to have the proteins attached on a top of the protein G.
- Finally, the devices are ready to detect the microorganism[2].

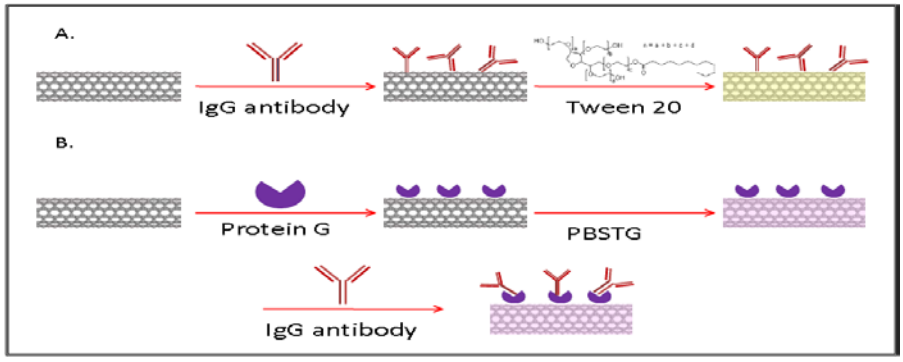

Figure 3-6. Schematic representation of the functionalization process. A) Direct adsorption of the IgG antibodies onto the SWCNTs B). Immobilization of the IgG antibodies by using protein G.

[2] CNTFETs with the IgG adsorbed onto protein G was used to detect the mould *Aspergillus flavus.*

3.3.5. Characterization of the biofunctionalized CNTFET

3.3.5.1. Spectroscopic Ellipsometry characterization

Spectroscopic ellipsometry is a very suitable technique to characterize biofunctionalized surfaces (Bae, Y.M. et al, 2005). This method is used to investigate aspects such as the thickness of protein layers absorbed on the surface of our devices. The process involves the follow steps:

- Characterize a pure wafer of Si/SiO_2 and the wafer containing the as-grown network of CNTs before starting the functionalization process by using an spectroscopic ellipsometer.
- Functionalize the devices as has been explained above (3.3.4)[3]
- Measure the ellipsometric angles Ψ and Δ after each functionalization step, using the wavelength range between 270 to 1700 nm (0.73–4.59 eV),[4] at three different angles of incidence: 55º, 65º and 75º and at three different spots.
- Analize the data by using the commercial software (WVASE32) and develop the model to determine the thickness of the protein layers deposited onto the SWCNTs (figure 3-7).

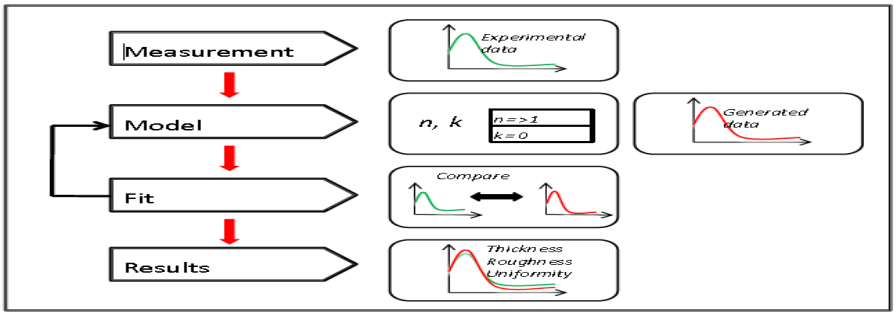

Figure 3-7. Ellipsometry data analysis after each functionalization step[5].

[3] In this case the concentration of the Tween 20 and gelatin were reduced to 0.05 % and 0.8 % respectively. This ratio has been previously reported as an effective blocking agent for small molecules (Piao, M.H. et al, 2008).

[4] Although the measurements were made using a wide wavelength range, for the data analysis only the results obtained in the visible range were taken into account.

[5] The optical constants "*n*" (index of refraction) and "*K*" (extinction coefficient) describe how light propagates through the carbon nanotubes before and after being functionalised.

ISBN:978-84-692-9761-2/DL:T-203-2010

3.3.5.2. Electrical characterization

The electrical characterization of CNTFETs devices is the main tool to observe the biosensing process from the synthesis of the carbon nanotubes to the detection of the analyte. In our devices two electrodes *source* and *drain* are connected to the transducer channel (a network of SWCNTs grown on a Si/SiO_2 surface by CVD). The silicon substrate is used as a back gate (third electrode of the FET configuration) while the SiO_2 acts as a dielectric layer able to isolate electrically the SWCNTs from the silicon layer. A semiconductor parameter analyzer at room temperature is used to perform this characterization process. Two thin tungsten tips of the probe station are contacted to the ***source*** and ***drain*** electrodes. The tips are connected to the semiconductor parameter analyzer (figure 3-8).

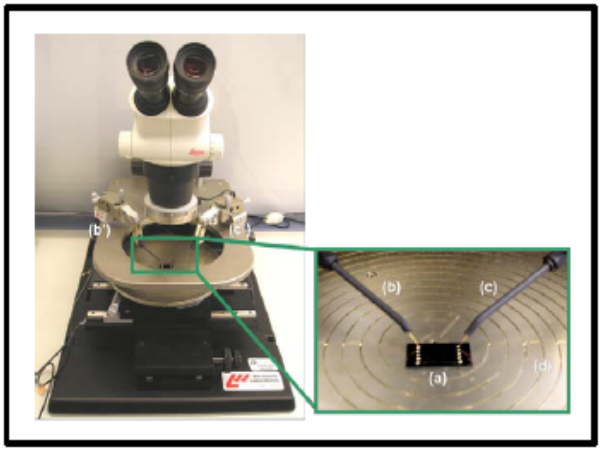

Figure 3-8. Probe station used for the electrical characterization of the CNTFETs devices. a) CNTFET sensor. b and c) Tungsten tips contacting the *source* and *drain* electrodes respectively.

The electrical characterization is performed as follows:

- Apply a source-to-drain sweep voltage (Vsd) (+250 mV to -250 mV) keeping constant the gate voltage (Vg) at 0 V. This step allows obtaining;

a) the resistance of the channel (R = V / I), b) the optimum value of Vsd [6], c) the source-to-gate current ("gate leakage") at Vg = 0 V [7] (Cid C. 2009).

- Register the electrical current while sweeping the Vg [8]. From this curve, we can know whether the CNT network is semi-conducting or metallic. This curve, i.e. also known as the "device characteristics" or "gate dependence", is also registered after each functionalisation step and after exposing the functionalised devices to the pathogenic microorganisms.

3.3.5.3. Microscopic characterization

The CNTFET are microscopically characterized with Atomic Force Microscopy (AFM), Scanning Electron Microscopy (SEM) and Environmental Scanning Electron Microscopy (ESEM). AFM is a well known technique that provides direct images of the surface topography (Kuhlmeier, D. et al, 2003) without special sample preparation. This technique allows to mesure the height of the CNTs. In this way, it is possible: 1) to verify that the synthesized CNTs are indeed single-walled carbon nanotubes; and 2) to follow the functionalisation steps by measuring the height after each functionalisation. AFM is also a contactless technique being possible to reuse the samples after their analysis. The disadvantage of AFM is that it is quite slow. Moreover, it is impossible to obtain an overall image of the whole sample. This technique was used in this research to obtain surface images from 1µm x 1µm to 20 µm x 20µm.

SEM and ESEM are techniques that allow obtaining images of a sample surface with a high range of magnification by scanning it with a high-energy beam. These electrons interact with the sample producing signals that contain information about

[6] In our devices the optimum Vsd is the one that provides electrical current values of a few µA and is usually found between 100 mV and 250 mV.

[7] Devices with a gate leakage higher than 1 nA cannot be used. The gate leakage is sometimes due to physical damage in the isolating layer (SiO_2) but also it can be due to a high density of the as-grown SWCNTs. To overcome this problem, the network of the SWCNTs containing the source and drain electrodes is isolated by cutting the surface around the electrodes.

[8] The best value of Vg is the one that produces the highest source-drain current values but also, the one that keeps the capacity to maximize the modulation of the signal.

its topography and composition. The most common method used to obtain the images is by using the secondary electron imaging mode, which can produce very high-resolution images of a sample surface, revealing details about 1 to 5 nm in size. Furthermore they provide large depth field. SEM often requires sample pre-treatment being useful only for electrically conductive samples. Moreover, the sample must be dehydrated due to it only operate in high or low vacuum. By contrast in E-SEM electrically or non-electrically conductive samples can be analysed. In addition, E-SEM allows imaging in high vacuum, low vacuum and environmental conditions. Both techniques, SEM and ESEM do not allow calculating the height of the CNTs and the potential applied often burn the CNTs. Therefore, the samples cannot be reused.

These techniques are used in this research to known the density of the CNT networks and to verify the presence of antibodies and microorganisms on the CNT networks. The voltage used vary from 15 kV for the as-grown carbon nanotubes to 1 or 2 kV for the carbon nanotubes after being coated with the antibodies and after interaction with the microorganisms. The working distance used is in a range of 8 mm to 10 mm. We did not metalize the sample because a same device was observed several times after each functionalization step.

3.4. References

Bae, Y.M., Oh, B.K., Lee, W., Lee, W.H., Choi, J.W. Biosensors and Bioelectronics. **2005**. 21,103-110.

Cid, C. **2009**. Doctoral Thesis. Sensors based on carbon nanotube field-effect transistors and molecular recognition approaches. Department of Analytical and Organic Chemistry. Universitat Rovira i Virgili.

Chang, H.C., Chang, J.J., Huang, A.H., Chang, T.C. Journal of Clinical Microbiology. **2000**. 38, 971–976.

Chen, R.J., Bangsaruntip, S., Drouvalakis, K.A., Kam, N.W., Shim, M., Li, Y., Kim, W., Utz, P.J., Dai, H. Proceedings of the National Academy of Sciences. **2003**. 100, 4984–4989.

Chi, Y.S., Byon, H.R., Choi, H.C., Choi, I. S. ChemBioChem. **2007**. 8, 1380 – 1387.

De Vos, M.M. and Nelis, H.J. Journal of Microbiological Methods. **2003**. 55, 557–564.

Hao, W., Pan, Y.X., Ding, Y.Q., Xiao, S., Yin, K., Wang, Y.D., Qiu, L.W., Zhang, Q.L., Woo, P.C.Y., Lau, S.K.P., Yuen, K.Y., Che, X.Y. Clinical and Vaccine Immunology. **2008**. 15, 194–202.

Hetherington, S.V., Henwick, S., Parham, D.M., Patrick, C.C. Clinical and Diagnostic Laboratory Immunology. **1994**. 1, 63-67.

Kuhlmeier, D., Rodda, E., Kolarik, L.O., Furlong, D.N., Bilitewski, U. Biosensors and Bioelectronics. **2003**.18, 925-936.

Ilic, B., Czaplewski, D., Craighead, G. Applied Physics Letters. **2000**. 77, 450–452.

Lin, Y., Jiang, X., Elkin, T., Fernando, K.A., Gu, L., Taylor, S., Yang, H., Jones, E., Wang, W., Sun, Y.P. Journal of Nanoscience and Nanotechnology. **2006**. 6, 868–871.

ISBN:978-84-692-9761-2/DL:T-203-2010

Oh, B.K., Lee, W., Kim, Y.K., Lee, W.H., Choi, J.W. Journal of Biotechnology. **2004**. 111, 1-8.

Piao, M.H., Noh, M.H., Rahman, M.A., Won, M.S., Shim, Y.B. Electroanalysis. **2008**. 20, 30–37.

So, H.M., Won, K., Kim, Y.H., Kim, B.K., Ryu, B.H., Na, P.S., Kim, H., Lee, J.O. Journal of the American Chemical Society. **2005**. 127, 11906–11907.

Tsai, G.J. and Yu, S.C. International Journal of Food Microbiology. **1999**. 50, 181–189.

UNIVERSITAT ROVIRA I VIRGILI
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Chapter 4

CNTFET for pathogenic bacteria determination

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4.1. Introduction

The growing incidence of pathogenic bacteria in foods is a considerable public health problem around the world. For that reason, regulatory agencies such as the Food and Drug Administration (FDA) have established control quality programs in order to avoid pathogens in foods. Hazard analysis critical control point (HACCP) is a preventive management program from the FDA applied in the food industry to take corrective actions during the food manufacturing process thus reducing possible microbial contamination in the final product(http://www.fda.gov/Food/FoodSafety/HazardAnalysis CriticalControlPointsHACCP/default.htm).

A great deal of pathogenic bacteria has been related to the generation of foodborne diseases. Some of the most significant pathogens include *Salmonella* spp., *Escherichia coli*, *Staphylococcus aureus*, *Campylobacter jejuni* and *Bacillus cereus* among others (Taux, R.V. 1997). Salmonellosis is an infectious disease produced by *Salmonella* that is an important pathogen in both developed and developing countries.

Many analytical tests for pathogenic bacteria including *Salmonella* are available in the market. Current methods for detecting contamination of foodstuffs with *Salmonella* consist of conventional culture techniques, biochemical test, immune-based assays, and nucleic-acid based methods (Taitt, C.R. et al, 2004). The culturing and plating method is the oldest bacterial detection technique and remains the standard detection method. Selective media can be used to detect specific bacteria species. They usually contain inhibitors (compounds that stop or delay the growth of non-targeted strains) or particular substrates that only the targeted bacteria is able to degrade producing specific colours characteristic of each specie. The final detection and enumeration is often performed by ocular inspection (Lazcka, O. et al, 2007). However, conventional methods require long analysis time and in some cases pre-enrichment are required to recover and detect the pathogen (Koyuncu, S. and Haggblom, P. 2009). Moreover, additional biochemical test are often required to identify the species.

Biochemical testing uses the enzymatic machinery of microorganisms to degrade specific compounds present in a media. The test often requires between 4 to 24 h of incubation time and fresh cultures to obtain reliable results. Wilson, G. (Wilson, G. 2004) developed a biochemical screening protocol for *Salmonella* and *Shigella* that uses rapid enzymatic reactions. It had a sensitivity of 100 % and a specificity of 94 %. This researcher reported the presumptive presence of *Salmonella* 3 h after colony isolation. However, further testing on other types of media is recommended by the author.

Immuno-assays rely on the specific binding of an antibody to an antigen (de Boer, E. and Beumer, R.R. 1999) Commercial immunoassays for microorganisms like the enzyme linked-immunosorbent assay (ELISA) combine the specificity of antibodies and the sensitivity of antibodies or antigens coupled to an enzyme achieving sensitivities and specificities of 100 % (Janyapoon, K. et al, 2000). However, the need of specific labels in the case of the typical sandwich assay makes the method labour intensive.

The sensitivity in conventional ELISA test can be defined as the proportion of true positives correctly identified [agreed positives/(agreed positives+false negatives) x 100 %] while specificity can defined as the proportion of true negatives correctly identified [agreed negatives/(false positives+agreed negatives) x 100 %] (Abass, E.M. et al, 2006).

Nucleic-acid methods are based on the extraction and amplification of target DNA/RNA with specifics probe using the well known polymerase chain reaction (PCR) (Scheu, P.M. et al, 1998). To improve the sensitivity and reduce the time analysis of pathogens. Eyigör A et al, (Eyigör, A. et al, 2007) designed a PCR-ELISA method to detect *Salmonella* DNA from selective primary enrichment culture of chicken intestine. The detection limit of PCR-ELISA for pure culture of *Salmonella.*Enteritidis 64 K was 50 cfu/mL and for intestinal samples artificially contaminated with *Salmonella* Enteritidis 64 K was found to be 70 cfu/mL. Sensitivity and specificity of the assay was determined as 100 %

The immunocapture and the use of PCR is other approach to enhance the pathogen detection process (Jeníková, G. et al, 2000.; Leon-Velarde, C.G. et al, 2009.; Liébana, S et al, 2009). Liébana *et al* (Liébana, S et al, 2009), reported the use of magnetic beads coated with antibodies against *Salmonella* coupled to an amplification of the genetic material by PCR and the use of electrochemical magneto-genosensing to capture, concentrate and detect *Salmonella* in skim milk samples. They obtained a limit of detection of 1 cfu/mL in 3.5 h without any pretreatment. Furthermore, the authors stated that if the skim milk is pre-enriched for 6 h, the method is able to selectively detect as low as 0.04 cfu/mL (1 cfu / 25 g of milk) of *Salmonella*.

In the last decades researches have been trying to develop devices able to made analysis in situ and in real time. The importance of this kind of elements comes from their high specificity and sensitivity, which allow the detection of a broad spectrum of analytes in complex sample matrices (food, blood, serum, urine, etc). Biosensors are devices that are composed by a biological (protein, nucleic acid) or biomimetic recognition element (peptide, aptamer) in close contact with a transducer. Depending on the detection system the biosensors can be classified in optical, mass, electrochemical and thermal sensors. In addition, biosensors can also be classified as direct, in which the analyte of interest is detected by the sensor, or indirect, in which a preliminary biochemical reaction takes place and the products of that reaction are then detected by the sensor (Ivnitski, D. et al, 1999).

Electrochemical biosensors are devices based on the observation of an electrical measurement (current, impedance, potential) due to chemical interactions occurring at the sensor sample matrix interface (Lazcka, O. et al, 2007). Rishpon *et al* (Rishpon, J. et al, 1992) described a rapid and sensitive method based on an enzyme-tagged immuno-electrochemical assay to detect pathogenic bacteria. In these devices antibodies are immobilized on disposable carbon filter disc electrodes and are used to capture antigens in test solutions. The system was able to detect as low as 10 cell/mL in less than 10 minutes of *Staphylococcus aureus* and *Escherichia coli*. Nevertheless, this method requires like the conventional ELISA

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the use of an enzyme-labeled antibody and specific substrates to obtain the potentiometric signal.

Shabani *et al* (Shabani, A. et al, 2008**)** have recently proposed a novel method based on a bacteriophage-modified microarray for the specific and direct detection of *E. coli.* The system use bacteriophage T4 as recognition receptors immobilized covalently onto functionalized screen-printed carbon electrode (SPE) microarrays. Impedance measurements were recorded to detect the binding of *E. coli* to the T4 phages. The researchers stated that using this method it is possible to determine 20 cfu/mL. In addition, because phage lyse only living bacteria, the method can differentiate living from dead bacteria. Delibato *et al* (Delibato, E. et al, 2009) reported the use of an immunosensor array, based on a 96-well electrochemical plate coupled with immunomagnetic beads (ELIME array), for the detection of *Salmonella* in meat samples. The immunosensor array was able to detect a low number of *Salmonella* cells (1-10 cfu/25 g) after only 6 h of incubation in a pre-enrichment broth. Barreiros, M (Barreiros, M. et al, 2009) developed and electrochemical impedance spectroscopy (EIS) to detect pathogenic *E. coli O157:H7* bacteria via a label free immunoassay-based detection method. Sensors were able to detect very low concentration of *E. coli* with limits of detection as low as 10-100 cfu/mL.

Recently, in our research group, Zelada-Guillén *et al* (Zelada-Guillén, G. et al, 2009) reported a potentiometric aptasensor based on carbon nanotube for the detection of only 1 cfu/mL of *Salmonella* Typhi in real time. Therefore, electrochemical biosensors are particularly interesting because they are usually inexpensive, are well adapted to miniaturization, and can provide disposable-type chips for field applications.

This chapter presents the first attempt to develop an electrochemical biosensor based on carbon nanotube field effect transistors (CNTFET) to detect pathogenic bacteria and it is thoroughly explained in the published article presented below. Briefly, the biosensor consists of a network of CNTs, previously synthesized by CVD, that are used as the transducer element. Antibodies are the molecular

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receptor able to detect selectively the bacteria *Salmonella* Infantis. These antibodies are adsorbed onto the sidewalls of the CNTs by means of hydrophobic interactions allowing the detection of the bacteria and at the same time preserving the electrical properties of the transducer. Non-specific interactions from the sample components with the CNTs are avoided by using a blocking agent. The developed sensor was characterized by using both electrical and microscopic techniques.The direct detection system together with the excellent electrical properties of the CNTs produces a label-free and sensitive biosensor.

Furthermore, this chapter supplies an additional experimental session that compiles other tests performed that were not included in the published paper. Finally the complementary conclusions and the references are reported.

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4.2. Article:

"Fast detection of Salmonella Infantis with carbon nanotube field effect transistors". Biosensors and Bioelectronics. 2008. 24, 279-283.

Raquel A. Villamizar[τ‡], Alicia Maroto*[‡], F. Xavier Rius[‡], Isabel Inza [ŧ] and Maria J. Figueras [ŧ]

[‡] Department of Analytical and Organic Chemistry. Universitat Rovira i Virgili. Marcel.lí. Domingo, s/n. 43007. Tarragona, Spain.

[ŧ] Department of Basic Medical Sciences. Faculty of Medicine and Health Sciences. Universitat Rovira i Virgili. Reus. Spain.

[τ] On leave from the Department of Microbiology. University of Pamplona. Colombia.

UNIVERSITAT ROVIRA I VIRGILI
BIOSENSORS BASED ON CARBON NANOTUBE FIELD EFFECT TRANSISTORS (CNTFETs) FOR DETECTING PATHOGENIC MICROORGANISMS
Raquel Amanda Villamizar Gallardo
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ABSTRACT

In this paper we report a fast, sensitive and label-free biosensor for the selective determination of *Salmonella* Infantis. It is based on a field-effect transistor (FET) in which a network of single-walled carbon nantotubes (SWCNTs) acts as the conductor channel. Anti-*Salmonella* antibodies were adsorbed onto the SWCNTs and subsequently the SWCNTs were protected with Tween 20 to prevent the non specific binding of other bacteria or proteins. Our FET devices were exposed to increasing concentrations of *S*. Infantis and were able to detect at least 100 cfu/mL in 1 hour. To evaluate the selectivity of our FET devices, *Streptococcus pyogenes* and *Shigella sonnei* were tested as potential competing bacteria for *Salmonella*. At a concentration of 500 cfu/mL, neither *Streptococcus* nor *Shigella* interfered with the detection of *Salmonella*. Therefore, these devices could be used as useful label-free platforms to detect *S*. Infantis and, by using the suitable antibody, other bacteria or viruses.

KEYWORDS: Biosensor, Field-Effect Transistor, Carbon nanotubes, Bacteria, Salmonella

INTRODUCTION

Salmonella enterica subsp*. enterica* serotype Infantis (*S*. Infantis) is a rod-shaped bacterium that is found in the bacterial flora of living reptiles and amphibians. It is also very common in poultry, red meat, raw egg shells, unpasteurized milk and their dairy products. *S*. Infantis can cause gastroenteritis and can be transmitted through the ingestion of contaminated food or water (Boyle et al., 2007; Plym et al., 2006; Shahada et al., 2006). It is one of the most significant pathogenic microorganisms in food-borne infections in humans due to its multidrug resistance. For this reason, there is a widespread need to protect public health by developing fast and reliable techniques to detect it at low concentrations in food and water.

Conventional culturing methods are reliable but very time consuming (i.e. typically requiring 3-7 days) and other methods such as the polymerase chain reaction (PCR) or enzyme-linked immunosorbant assays (ELISA) require between 8-48h. Some biosensors have been developed for the label-free detection of pathogens,

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although they still have limits of detection (LODs) of about 10^3 cfu/mL (Taylor et al., 2006).

Single-walled carbon nanotubes (SWCNTs) have outstanding electrical and mechanical properties. Tans *et al* (Tans et al., 1998) reported the first Field Effect Transistor (FET) based on a single SWCNT and Kong *et al* (Kong et al., 2000) used it to develop the first Carbon Nanotube Field Effect Transistor (CNTFET) chemical sensor. The functionalization of SWCNTs gives rise to the development of selective CNTFET biosensors based on the principles of molecular recognition. Star *et al* used a CNTFET based on a single SWCNT to detect streptavidin by means of the biotin-streptavidin interaction (Star et al., 2003). Byon *et al* detected protein-protein interactions at 1pM concentration with a highly sensitive FET based on networks of SWCNTs (Byon et al., 2006). CNTFETs functionalized with aptamers have been applied to detect either thrombin (So et al., 2005) or immunoglobulin E (Maehashi et al., 2006).

So far CNTFETs have not been applied to detect bacteria or higher organisms. Only the interaction between CNTs and bacteria has already been studied. Huang *et al* (Huang et al., 2004) adsorbed anti-*Salmonella* antibodies on CNTs and then exposed it to 108 cfu/mL of *Salmonella typhimurium* for 1 hour. The bacteria linked to the CNTs were observed with scanning electron microscopy (SEM). Elkin *et al* (Elkin et al., 2005) prepared immuno-carbon nanotubes by functionalising SWCNTs with albumin serum bovine (BSA) and anti-*E.coli* antibodies. The interaction between the immuno-SWNTs and *E. coli* cells was evidenced both by SEM and the use of a green fluorescent protein secondary antibody as a marker.

Lin *et al* (Lin et al., 2006) used anti-*E.coli* antibodies to functionalize multi-walled carbon nanotubes (MWCNTs) with encapsulated ferromagnectic elements. After the immunomagnetic separation of the MWCNTs, they were able to selectively detect 400 cfu/mL of *E.coli*.

The antigen-antibody interaction implemented as the sensing layer together with the capability of CNTs to transduce the charge transfer by using FETs can be further exploited to detect cells. In this study, we report the development of fast and

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sensitive CNTFET to selectively detect at least 100 cfu/mL of *S.* Infantis in one hour. The recognition layer of the CNTFET consists of the anti-*Salmonella* antibodies adsorbed onto the CNTs. Tween 20 was used to avoid the non-specific binding (NSB) of other bacteria or proteins. The molecular recognition mechanism used and the ability of the CNTs to transduce the presence of *S*. Infantis makes labels unnecessary.

EXPERIMENTAL SECTION

Materials

Anti-*Salmonella* (i.e. a rabbit polyclonal antibody for *Salmonella* "O" & "H" antigens (4-5mg/mL), was purchased from Oxford Biotechnology Ltd. (Oxford, U.K). It was dissolved in phosphate buffer saline (PBS) to a final concentration of 10 ppm (pH=7.2) and stored at -20 ºC until use. Tween 20 and PBS were obtained from Sigma-Aldrich. Trypticase soy agar (TSA) and trypticase soy broth (TSB) were provided by Beckton-Dickinson and prepared according to their specifications. Silver ink was provided by Acheson industries.

Bacterial preparation

Stocks of strains of *S*. Infantis S34/11, *Shigella sonnei* (*S. sonnei*) CECT 413 and *Streptococcus pyogenes* (*S. pyogenes*) CECT 985T were obtained from the Faculty of Medicine and Health Sciences at the Universitat Rovira i Virgili. They were stored at -20 ºC with glycerol. *S.* Infantis was reactivated by inoculating the bacteria in 10 mL of sterile TSB at 37 ºC for 12 hours. Then, the sample was pelletized in a centrifuge at 6000 rpm for 15 minutes and the supernatant was discarded. The pellet was resuspended in 10 mL of sterile 0.85 % saline solution. The bacteria contained in the saline solution were enumerated by plating 10^7-fold serial dilutions on TSA plates and incubating at 37 ºC for 24-48 hours. Further dilutions in sterile 0.85 % saline solution were made to produce the final samples at 100, 300 and 500 cfu/mL of *S*. Infantis. The same procedure was followed to prepare 500 cfu/mL of *S. sonnei* and *S. pyogenes*.

ISBN:978-84-692-9761-2/DL:T-203-2010

Apparatus

Atomic force microscopy (AFM), Pico Plus (Molecular Imaging) in tapping mode was used to obtain the images of the networks of CNTs. Scanning electron microscopy (SEM), JSM 6400 (Jeol) was used to obtain the images of the functionalization process of the CNTs. SEM images were obtained by applying a voltage of 5 KV at a working distance of 8 mm. Electrical measurements were made using a 4157A Agilent semiconductor parameter analyzer and a Wentworth Laboratories MP1008 probe station.

Development of the CNTFETs

The CNTs networks were synthesized on top of a 500 nm layer of silicon dioxide thermally grown on highly n-type doped silicon chips (total area 0,5 x 0,5 cm^2) by chemical vapour deposition (CVD). A solution of 100 ppm iron nitrate in isopropanol was used as the catalyst. The CVD was performed at 900 ºC for 20 minutes with 600 sccm of methane and 200 sccm of hydrogen. AFM (Figure 1A) shows that a dense network of SWCNTs with an average height of 1.5 nm was obtained after the CVD. The source and drain electrodes of the CNTFETs were screen-printed with silver ink.

The gap between both electrodes was 0.5 mm and the size of the electrodes was 200 µm x 200 µm. The gate electrode was an aluminium layer on the back side of Si. The CNTFETs were electrically characterized by recording the current vs. the gate voltage. To obtain the instrumental variability, we carried out all the electrical measurements three times and we plotted the mean value and the range of the measurements.

Functionalization of the CNTFETs

The CNTFETs were functionalised as follows. First, the CNTFETs were incubated for 1 hour at 37 ºC in a 10 ppm solution of anti-*Salmonella* antibodies in PBS solution 15 mM (Lin et al., 2006; Bradley et al., 2004). Then, the CNTFETs were rinsed with PBS solution and distilled water, dried with nitrogen (Ilic et al., 2000) and immersed for 2 hours in a solution of 0.5 % Tween 20 dissolved in PBS 15 mM

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(So et al., 2005). The CNTFETs were again thoroughly rinsed with distilled water, dried with nitrogen and ready to be used to detect *S*. Infantis. Figure 1A shows a scheme of the CNTFET and Figure 1B shows the interaction of *S.* Infantis with the antibodies adsorbed onto the CNTs.

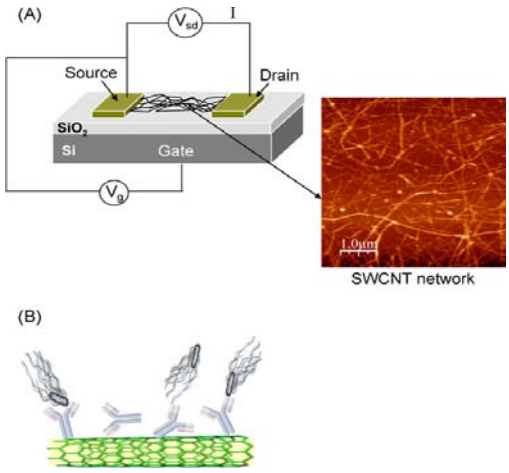

Figure 1. A) Experimental set up for detecting S*.* Infantis with a network of CNTFETs functionalized with anti-*Salmonella* antibodies. B) Antigen-antibody interaction of *S.* Infantis with a SWCNT functionalized with anti-*Salmonella* antibodies and protected with Tween 20.

Detection of *S*. Infantis

The functionalized CNTFETs were exposed to increasing concentrations of *S.* Infantis (i.e. first to 100, then to 300 and finally to 500 cfu/mL). For each concentration, the CNTFETs were immersed for 1 hour at 37 ºC, rinsed thoroughly with distilled water, dried with nitrogen and electrically characterized. For each concentration we scanned the area between the electrodes (500 μm^2) with SEM to count the number of bacteria attached to the CNTs.

Selectivity of the CNTFETs

Selectivity was checked in the presence of two bacteria: *S. pyogenes* and *S. sonnei*. A functionalized CNTFET was first immersed in a bacteria solution containing 500 cfu/mL of *S. pyogenes* for 1 hour at 37 ºC, thoroughly rinsed with

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distilled water, dried with nitrogen and electrically characterized. Subsequently it was exposed to 500 cfu/mL *S.* Infantis under the same conditions mentioned above. This procedure was also followed for 500 cfu/mL of *S. sonnei.* All these experiments were confirmed microscopically with SEM.

RESULTS AND DISCUSSION

The CNTFETs were electrically characterized after each functionalization step in dry conditions by measuring three times the dependence of the source-drain current, I, on the back gate voltage, Vg, in the range +10 V to -10 V (Bradley et al., 2004). The bias voltage, Vsd, was fixed at 250 mV. Figure 2 shows how each functionalization step affects the electrical current of a typical CNTFET. Each electrical current plotted corresponds to the mean value and range of the replicates. The range of the measured electrical current was in all cases less than 10 % of the electrical current measured. A *p*-type behaviour was observed for the as grown networks due to the electron withdrawal of adsorbed oxygen molecules from the air (Star et al., 2003).

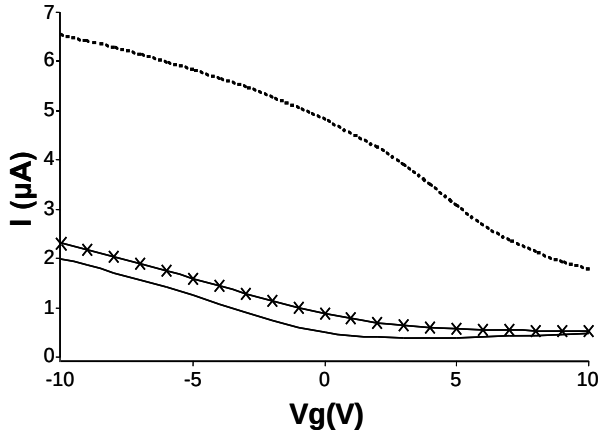

Figure 2. Gate voltage dependence of the source-drain current of a typical CNTFET after each functionalization step. Pristine CNTs (----); anti-*Salmonella* antibodies adsorbed on the CNTs (and CNTs covered with anti-*Salmonella* antibodies and Tween 20) (—)

Anti-*Salmonella* antibodies were adsorbed on the carbon nanotubes. This phenomenon has been attributed to several origins, among them, the size

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compatibility between the two entities (Shim et al., 2002) and the role of hydrophobic interactions (Chen et al., 2003). We observed through the SEM that one hour was enough to immobilize anti-*Salmonella* antibodies onto the side walls of CNTs. We took SEM images of a CNTFET just after the antibodies had been immobilised and the CNTFET had been submerged in PBS solution for 24 hours.

We observed a uniform layer of antibodies, suggesting that they were strongly adsorbed on the CNTs since the density of antibodies adsorbed did not decrease 24 hours later. The electrical current of the device decreased after the adsorption of anti-*Salmonella* due to the charge transfer process in which the antibodies provide electrons to the CNTs (Grüner et al., 2006). These electrons are provided by the amine groups of aminoacids with base-containing residues (i.e. arginine, histidine and lysine) that are located at the external envelope of the protein. Each adsorbed amine donates 0.04 electrons to the nanotubes giving rise to a decrease of the electrical current and to a shift in the threshold voltage of the semiconducting CNTs (Bradley et al., 2004).

The CNTFET electrical current decreased slightly after the adsorption of Tween 20, as previously observed by So *et al* (So et al., 2005). Tween 20 is a surfactant with linear aliphatic chains that adsorbs on the walls of CNTs by hydrophobic interactions and protects them against the non-specific binding (NSB) of proteins (Byon et al., 2006; So et al., 2005; Chen et al., 2003) or bacteria. In our case, Tween 20 covers the gaps of the CNTs walls left unprotected by the adsorbed anti-*Salmonella* protein. These functionalized devices were subsequently exposed to solutions of *S.* Infantis for 1 hour at 37 ºC. Figure 3 shows a SEM image of a FET device exposed to *Salmonella*. Notice that the immobilization of *anti- Salmonella* antibodies can be observed as bright dots on the SWCNTs. The bacteria are linked to the CNTs through antigen-antibody interaction.

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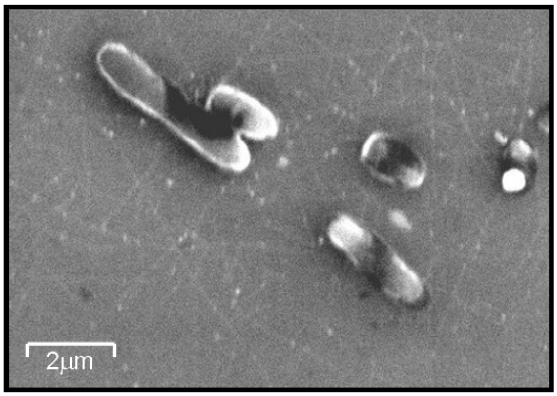

Figure 3. SEM image of a typical functionalized device after exposure to 500 cfu/mL of bacteria for 1 hour at 37 ºC. *S.* Infantis is linked to the CNTs network through antigen-antibody interactions. The small dots along the carbon nanotubes, some of them much brighter, are the antibodies adsorbed onto the CNTs.

The functionalized CNTFETs were exposed to increasing concentrations of *S.* Infantis. For each concentration, the CNTFETs were immersed for 1 hour at 37 ºC, rinsed thoroughly with distilled water, dried with nitrogen and electrically characterized by measuring three times the I-V characteristics and by plotting the mean value and range of the measurement values. Figure 4 shows the I-V characteristics (for a bias=0.25 V) of a functionalized CNTFET before exposure to *S.* Infantis and after exposure to 100, 300 and 500 cfu/mL. The inset in Figure 4 shows that the adsorption of *S.* Infantis significantly decreases the electrical current at negative gate voltages (i.e. from a 45 % decrease for 100 cfu/mL to an 80 % decrease for 500 cfu/mL). The error bars (that correspond to the range of the electrical current measured for the three replicates) show that the variability of the electrical current was lower than 10 %. The decrease of the electrical current is probably due to the antibodies interacting with the somatic O antigen (O-antigen) and the flagellar H antigen (H-antigen) present in the outer membrane and the flagellums of *S.* Infantis, respectively.

The O-antigen consists of a large polymer of repeating oligosaccharide units giving the major antigen specificity of the organism, because the monosaccharide units vary among species and even among strains. The H-antigen is composed of a single globular protein, flagellin (Johnson et al., 1967; Wang et al., 2003). In a

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similar way as with the adsorption of the antibody, either the charge transfer mainly from the amino groups of the bacterial proteins or the distortion of the CNTs could be the reason for the conductance decrease (Star et al., 2003; Grüner et al., 2006) The inset in figure 4 shows that the higher the concentration of *Salmonella* is, the more the electrical current decreases. Since the bacteria were prepared in sterile 0.85 % NaCl solution, we checked the possible effect of the interferences due to the matrix components, i.e. if the decrease of the electrical current could also be due to the presence of NaCl. As a control experiment, we exposed the devices to a 0.85 % NaCl solution for 1 hour at 37 ºC and no significant change of the electrical current was observed. The measured response of the devices exposed to *Salmonella* was stable for at least twenty-four hours, i.e. the electrical current measured showed no drift and a variability lower than 10 %.

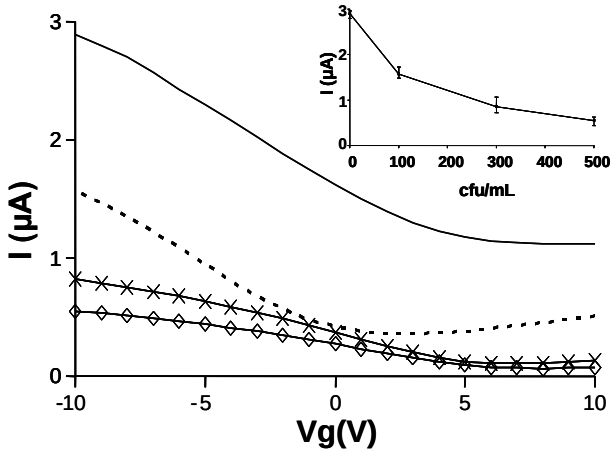

Figure 4. Gate voltage dependence of the source-drain current of a typical CNTFET before exposure to *S.* Infantis (–) and after exposure to 100 cfu/mL (----); 300 cfu/mL (-X-) and 500 cfu/mL (-◊-) of S. Infantis. Each electrical current plotted corresponds to the mean value of three replicates. The inset shows the behaviour of the source-drain current vs. increasing bacteria concentration (cfu/mL) at Vg=- 10 V. The error bars correspond to the range of electrical current measured for the three replicates.

SEM confirmed that the number of bacteria attached to the CNTs was proportional to the concentration of *Salmonella* in solution. A functionalized CNTFET was exposed to different bacteria concentrations for one hour each, thoroughly rinsed

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with distilled water and dried with nitrogen. For each concentration we scanned the area between the electrodes (500 μm^2) with SEM to count the number of bacteria attached to the CNTs. We counted 25, 36 and 63 bacteria after exposing the CNTFET to 100, 300 and 500 cfu/mL, respectively.

As a control experiment, we submerged CNTFET devices in Tween 20 without having adsorbed the anti-*Salmonella* antibodies on the CNTs. These devices were subsequently exposed to 500 cfu/mL S. Infantis for 1 hour at 37 ºC. Figure 5 shows that the electrical current changed slightly after the device was exposed to 500 cfu/mL of *S.* Infantis. The electrical current plotted corresponds to the mean value of the three measurements. The error bars of the inset (that correspond to the range of the current values) show that the slight change of the electrical current is due to the variability of the electrical current. In this way we proved that Tween 20 was effectively protecting the CNTs against the NSB of bacteria and that the decrease in the electrical current was only due to antigen-antibody interaction.

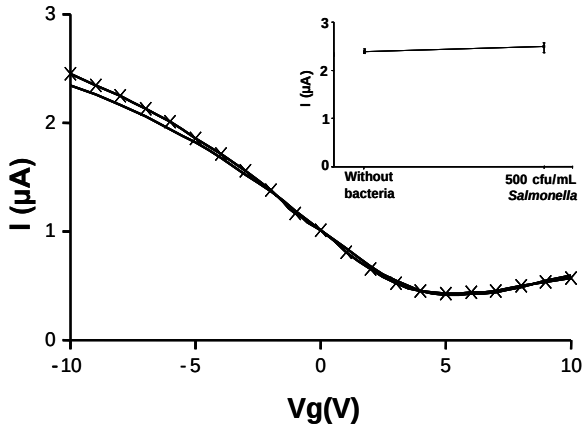

Figure 5. Electrical behaviour of a Tween 20 modified-CNTFET before exposure to *S*. Infantis(–) and after exposure to 500 cfu/mL (-X-) of *S*. Infantis. Each electrical current plotted corresponds to the mean value of three replicates. The inset shows the behaviour of the source-drain current at Vg=-10 V. The error bars correspond to the range of the electrical current measured for the three replicates.

ISBN:978-84-692-9761-2/DL:T-203-2010

The effectiveness of Tween 20 against the NSB was also checked with SEM. A CNTFET (functionalized with Tween 20 but not with anti-*Salmonella*) was exposed to 100, 300 and 500 cfu/mL *S*. Infantis for one hour each, thoroughly rinsed with distilled water and dried with nitrogen. For each concentration we scanned the area between the electrodes (500 μm^2) with SEM. Only one bacterium was bound when the device was exposed to 100 cfu/mL of *S*. Infantis whereas two bacteria were observed when it was exposed to 300 and 500 cfu/mL of *S.* Infantis.

Selectivity of our sensors devices was checked in the presence of two bacteria: *S. pyogenes* and *S*. *sonnei*. These pathogens are normal part of animal flora and can be found together with *Salmonella* in contaminated food or water. *S. pyogenes* should not have any cross-reactions with anti-*Salmonella* antibody because it is a Gram positive bacterium that is totally different from *S.* Infantis. On the other hand, *S. sonnei* is a Gram negative bacterium that is morphologically and antigenically similar to *S.* Infantis. However, no cross-reaction was observed with *S. sonnei*. A functionalized CNTFET was first immersed in a bacteria solution containing 500 cfu/mL of *S. pyogenes* for 1 hour at 37 ºC, thoroughly rinsed with distilled water, dried with nitrogen and electrically characterized. Subsequently it was exposed to 500 cfu/mL *S.* Infantis under the same conditions mentioned above. This procedure was also followed with another functionalized CNTFET for 500 cfu/mL of *S. sonnei.* Figures 6A and 6B show that the electrical current changes slightly after the CNTFETs are exposed either to *S. pyogenes* or *S. sonnei* whereas it decreases between 50-60 % after exposure to *S.* Infantis. The slight changes of the electrical current after exposing the devices to *S. pyogenes* and *S. sonnei* are due to the variability of the electrical current.

ISBN:978-84-692-9761-2/DL:T-203-2010

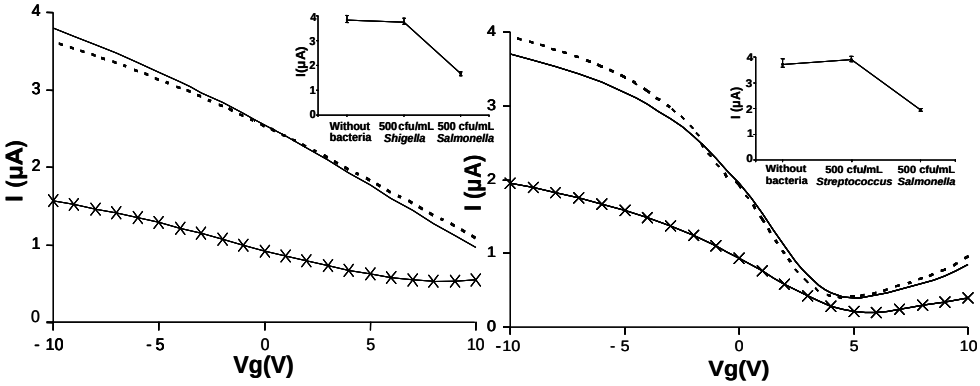

Figure 6. A) Gate voltage dependence of the source-drain current of a functionalized CNTFET before exposure to the bacteria (—) and after exposure to 500 cfu/mL of *S. sonnei* (----); and 500 cfu/mL of *S.* Infantis (-x-). B) Gate voltage dependence of the source-drain current of a functionalized CNTFET before exposure to the bacteria (—) and after exposure to 500 cfu/mL of *S. pyogenes* (----); and 500 cfu/mL of *S.* Infantis (-x-). Each electrical current plotted corresponds to the mean value of three replicates. The inset shows the behaviour of the source-drain current at Vg=-10 V. The error bars correspond to the range of the electrical current measured for the three replicates.

Selectivity was also checked with SEM. A functionalized CNTFET was exposed to 100, 300 and 500 cfu/mL of *S. sonnei* for one hour each, thoroughly rinsed with water and dried with nitrogen. For each concentration, we scanned the area between the electrodes (˜500 μm) with SEM. We observed only two bacteria bound to the CNTFET after it had been exposed to 100 cfu/mL. No further bacteria were attached after the CNTFET was exposed to 300 and 500 cfu/mL of *S. sonnei*. The same procedure was followed for *S. pyogenes*. In this case, no bacteria were attached to the CNTs after a functionalized CNTFET device was exposed to 100, 300 and 500 cfu/mL *S. pyogenes*. We can conclude that at a concentration of 500 cfu/mL, neither *S. sonnei* nor *S. pyogenes* interfered with the detection of *S.* Infantis.

Therefore, Tween 20 avoids the NSB of other bacteria and the antibody has no significant cross reaction with Gram negative bacteria that are similar to *S.* Infantis. Our sensor devices are then able to detect selectively at least 100 cfu/mL of *S.* Infantis. This limit of detection is similar to the one obtained for *Salmonella* with

other type of recently developed biosensors based on quartz crystal microbalance (Su et al., 2005), piezoelectric transducers (Olsen et al., 2006) and surface plasmon resonance (Oh et al., 2004).

CONCLUSIONS

We have developed a fast, sensitive and label-free biosensor for the selective detection of *S.* Infantis. It is based on the transduction power of CNTFETs combined with the recognition capacity of the antigen-antibody interaction. It can detect at least 100 cfu/mL of *S.* Infantis in just one hour. This is clearly better than the time responses of the current techniques used for detecting this pathogen. This biosensor, then, could be used as a useful label-free platform to detect other pathogenic bacteria, viruses or eukaryotic cells like moulds and yeast by using an adequate molecular receptor. The performance of these devices will be evaluated by applying them to other strains of *Salmonella* and also to food products contaminated with *Salmonella*.

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REFERENCES

Boyle, E.C., Bishop, J.L., Grassl, G.A., Finlay, B.B., 2007. Journal of Bacteriology. 189, 1489-1495.

Bradley, K., Briman, M., Star, A., Gruner, G., 2004. Nano Letters. 4, 253-256.

Byon, H. R., Choi, H. C., 2006. Journal of the American Chemical Soci ety. 128, 2188-2189.

ISBN:978-84-692-9761-2/DL:T-203-2010

Chen, R. J., Bangsaruntip, S., Drouvalakis, K. A., Kam, N. W., Shim, M., Li, Y., Kim, W., Utz, P. J., Dai, H., 2003. Proceedings of the National Academy of Sciences of the United States of America. 100, 4984-4989.

Elkin T., Jiang X., Taylor S., Lin Y., Gu L., Yang H., Brown J., Collins S., Sun Y., 2005. ChemBioChem. 6, 640 –643.

Grüner, G., 2006. Analytical and Bioanalytical Chemistry. 384, 322-335.

Huang, T. S., Tzeng, Y., Liu, Y. K., Chen, Y. C., Walker, K. R., Guntupalli, R., Liu, C., 2004. Diamond and Related Materials. 13, 1098-1102.

Ilic, B., Czaplewski, D., Craighead, G., 2000. Applied Physics Letters. 77, 450-452.

Johnson, E. M., 1967. Journal of Bacteriology. 94, 2018-2021.

Kong, J., Franklin, N. R., Zhou, C., Chapline, M. G., Peng, S., Cho, K., Dai, H., 2000. Science. 287, 622-625.

Lin, Y., Jiang, X., Elkin, T., Fernando, K.A., Gu, L., Taylor, S., Yang, H., Jones, E., Wang, W., Sun, Y.P., 2006. Journal of Nanoscience and Nanotechnology. 6, 868-871.

Maehashi, K., Katsura, T., Kerma, K., Takamura, Y., Matsumoto, K., Tamiya, E., 2006. Analitycal Chemistry. 79, 782-787.

Oh, B.K., Kim, Y.K., Park, Lee W.H., Choi J.W., 2004. Biosensors and Bioelectronics. 19, 1497-1504.

Olsen, E.V., Sorokulova, I.B., Petrenko V.A., Chen, I.H., Barbaree, J.M., Vodyanoy, V.J., 2006. Biosensors and Bioelectronics. 21, 1434-1442.

Plym, F. L., Wierup, M., 2006. Revue Scientifique et Technique. 25, 541-554.

Shahada, F., Chuma, T., Tobata, T., Okamoto, K., Sueyoshi, M., Takase, K., 2006. International Journal of Antimicrobial Agents. 28, 302–307.

Shim, M., Kam, N. W. S., Chen, R. J., Li, Y., Dai, H., 2002. Nano Letters. 2, 285-288.

So, H. M., Won, K., Kim, Y. H., Kim, B. K., Ryu, B. H., Na, P. S., Kim, H., Lee, J. O., 2005. Journal of the American Chemical Society. 127, 11906-11907.

Star, A., Gabriel, J. C., Bradley, K., Gruner, G., 2003. Nano Letters. 3, 459-463.

Su, X.L., Li, Y., 2005. Biosensors and Bioelectronics. 21, 840-848.

Tans, S. J., Verschueren, A. R., Dekker, C., 1998. Nature. 393, 49-52.

Taylor, A. D., Ladd J., Yu Q., Chen S., Homola J., Jiang S., 2006. Biosensors and Bioelectronics. 22, 752–758.

Wang, L., Rothemund, D., Curd, H., Reeves, P.R., 2003. Journal of Bacteriology.

185, 2936–2943.

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4.3. Supplementary experimental section

4.3.1. Microbiological test

Microbiological cultures were performed in order to confirm the purity of *Salmonella* strain. Figure 4-1A shows an image of the typical growth of *Salmonella* in xylose lysine desoxycholate agar (XLD agar). It has red appearance due to the indicator phenol red with a pH of approximately 7.4. This media contains a small amount of sodium desoxycholate that provide the selectivity. Moreover, it contains xylose, a sugar that it is fermented by practically all enterics except for the shigellae, and this property enables the differentiation of *Shigella* species.

Salmonella, is able to ferment the sugar xylose producing acid. It causes a descense in the pH making that the phenol red indicator changes the color from red to yellow. *Shigella* cannot do this and therefore remain red. After xylose is fermented, *Salmonella* decarboxylate lysine, increasing the pH once again to alkaline and therefore, the final colonies appeare red colored. At this pH *Salmonella* species can produce hydrogen sulphide from the reduction of thiosulphate. This is indicated by ferric ammonium citrate producing black or black-centred colonies. Black centre allowed differentiating from *Shigella*.*Salmonella* was also observed by using SEM. It can be seen in figure 4-1B the rod-shape cells with a range from 0.7 to 1.5 µm in diameter and from 2 to 5 µm in length.

Figure 4-1. A) Typical grow of *Salmonella* Infantis in XLD agar. B) SEM image of the bacteria.

4.3.2. Covalent immobilization of the antibody

We initially applied a covalent process in order to immobilize the molecular receptor on a polymer deposited on top of the previously grown SWCNTs. The functionalization process was as follow: First, the as-grown SWCNTs were submerged in a 10 % solution of PEI in water for four hours at room temperature (rt) and rinsed thoroughly with water. Then, the devices were immersed during 2 hours at rt in a 2.5 % solution of glutaraldehyde (GA) in water. GA was then used as a crosslinker to covalently bind the anti-*Salmonella* antibodies to the PEI. One of the aldehyde groups of GA reacts with the amine groups of the PEI to form imine groups. After that, the devices were submerged in a 10 µL/mL solution of IgG in PBS (15 mM, pH 7.4) to link the antibodies to the CNTs through the free aldehyde group of the GA. Subsequently, the devices were immersed for 1 hour in glycine 0.1 M at pH=7 to block the remaining free aldehyde groups of the GA that did not react with the antibodies (Yakovleva, J. et al, 2002.; Su, X. et al, 1999). The CNTFETs were again thoroughly rinsed with distilled water, dried with nitrogen and ready to be used to detect *Salmonella* Infantis. Figure 4-2 shows a schematic representation of the whole functionalisation process and figure 4-3 shows an image taken from the SEM of a functionalised device after exposure to *S*. Infantis.

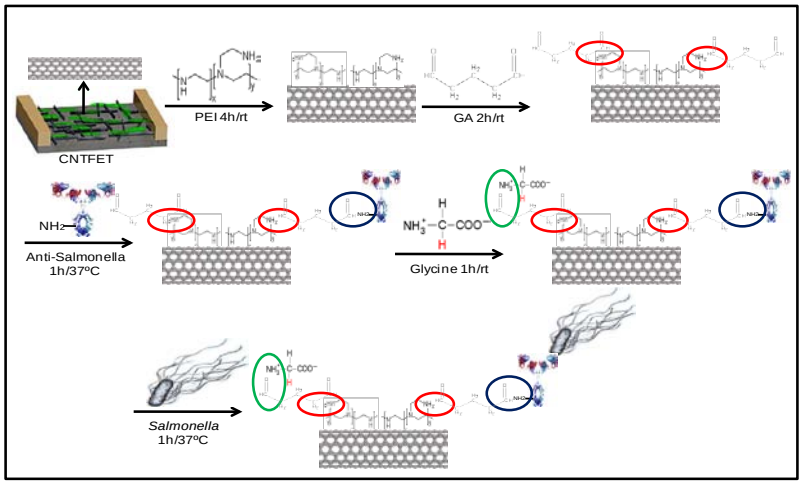

Figure 4-2. Experimental set up for detecting S. Infantis. The CNTs represent the network used as transducer elements. Anti-*Salmonella* antibodies are covalently linked to the PEI

through the crossliker glutaraldehyde. The bacteria are detected by means of the antigen-antibody interaction.

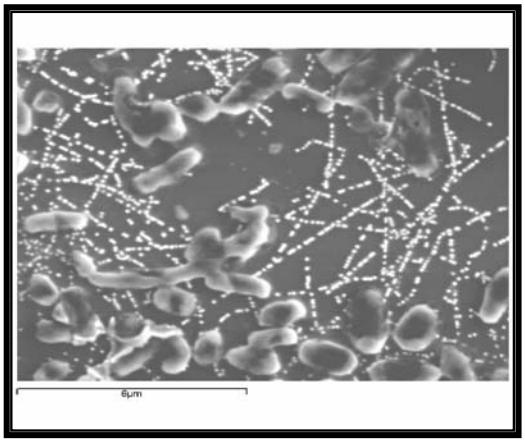

Figure 4-3. SEM image of a functionalized CNTFET after being exposed to 1000 cfu/mL of *S*. Infantis. The bright dot seems to be groups of anti-*Salmonella* antibodies aligned on top of the SWCNTs.

The CNTFETs devices were electrically characterized after each functionalisation step in dry conditions by measuring three times the dependence of the source-drain current, I, on the back gate voltage, Vg, in the range +10 V to -10 V (Bradley, K. et al., 2004). The bias voltage, Vsd, was fixed at 250 mV. Figure 4-4 shows how each functionalisation step affects the electrical current of a typical CNTFET. A *n*-type behaviour was observed for the as grown networks after being coated with PEI. It is probably due to the electron donating property of amine groups of the polymer that can lead to a change from *p*- to *n*-type behaviour (Javey, A. et al, 2002).

The electrical current decreases significantly after GA was linked to the PEI as well as when the antibody and glycine were bond to the system. Finally, when the device was exposed to the bacteria the electrical current became nearly zero. This is probably due to a deformation of the sidewalls of the CNTs taking into account that the bacteria are almost two thousand times larger than the transducer (CNTs). As a result, it can be concluded that the electrical properties of the devices were dramatically affected along the functionalization process hindering the final detection of the bacteria.

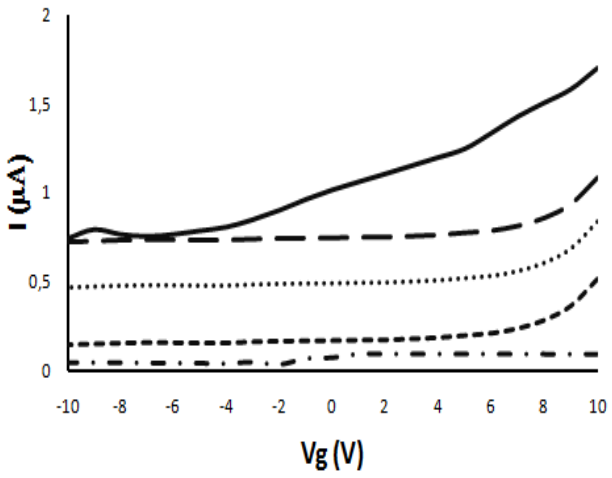

Figure 4-4. Gate voltage dependence of the source-drain current of a typical CNTFET after each functionalization step. As-grown CNTs coated with PEI (—); glutaraldehyde (– –), anti-*Salmonella* antibodies (·····), glycine (----); and after exposure to 100 cfu/mL of S. Infantis (– · –) .

4.3.2. Non-covalent immobilization of the antibody

It is well known that the electrical properties of the CNTs can be affected due to covalent functionalization process. Therefore, we decided to apply a non-covalent adsorption of the molecular receptors on the transducer.

Anti-*Salmonella* antibodies were absorbed onto the CNTs after 1 hour of incubation at 37 ºC. As it was previously commented in chapter 2, the non-specific adsorption of proteins involving IgG onto the nanotube surface is attributed to hydrophobic interactions (Allen, B. L. et al, 2007), the affinity of carbon nanotubes by amino groups (Bradley, K. et al, 2004) and intermolecular interactions involving aromatic amino acids (i.e., histidine and tryptophan) in the polypeptide chains of the proteins (Wang, S. et al, 2003).

Figure 4-5 shows AFM images of the as-grown CNTs before and after being coated with the antibodies. It can be clearly appreciated that the height of the as-grown CNTs is in a range of about 1.6 nm to 1.8 nm. After the antibodies are adsorbed the height of the nanotubes increase to about 8 nm to 10 nm. IgG structure is based

on two Fab and one Fc domain and a hinge, the region between the Fab and Fc. The height of the biomolecule is about 12 nm in the right position, it means with the Fab region pointing straight to the analyte. However, hinge region allow lateral and rotational movements of the molecule (Tronin, A. et al., 1995) thus causing different configuration of the antibody which could in principle cause different height values (i.e. from 8 nm to 10 nm).

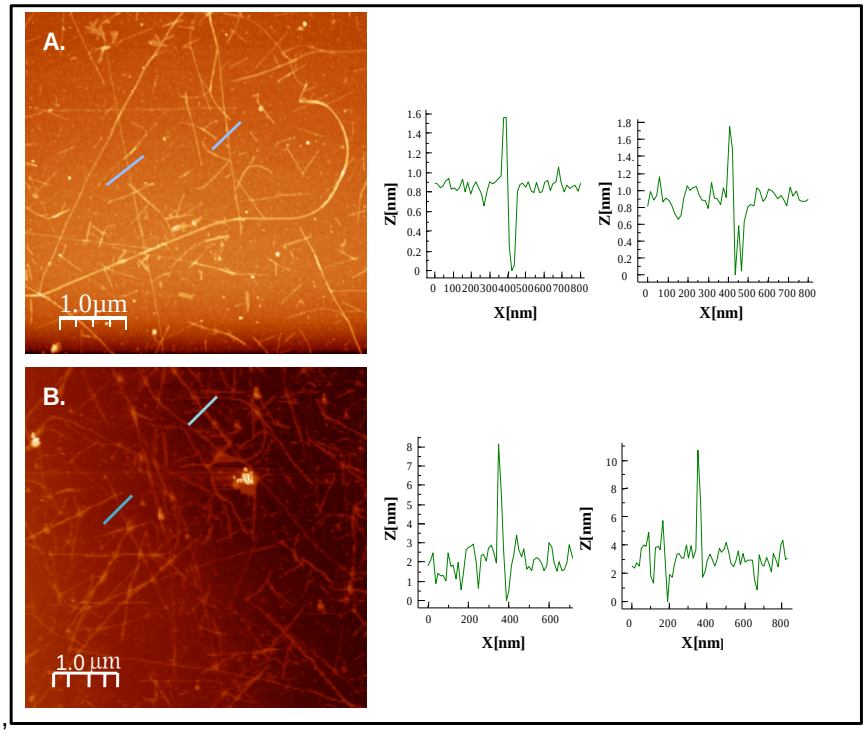

Figure 4-5. AFM images (left) and height profiles (right) recorded for (A) As-grown CNT network. (B) CNT network after the immobilization of IgG.

4.3.4. Response time of the sensor

In the article we report that the selected response time (1 hour) was based on bibliographical data about the kinetics of the reaction between the antibody and the bacteria. Nevertheless an experiment to determine this reaction time was performed.

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For this purpose, CNTFET devices were immersed in a solution containing the lowest assayed concentration of *S.* Infantis (100 cfu/mL) at 37 ºC. Every 15 minutes we rinsed the devices thoroughly with water, dried it and measured the electrical current (see Figure 4-6)After each measurement the devices were again submerged in the solution containing 100 cfu/mL *S*. Infantis for another 15 minutes and electrically characterized. The highest decrease for the current intensity was obtained between 30 and 60 minutes. The current decreased further from 60 to 75 minutes but not significantly. Therefore, the suitable reaction time and, therefore, the response time of our devices was 60 minutes.

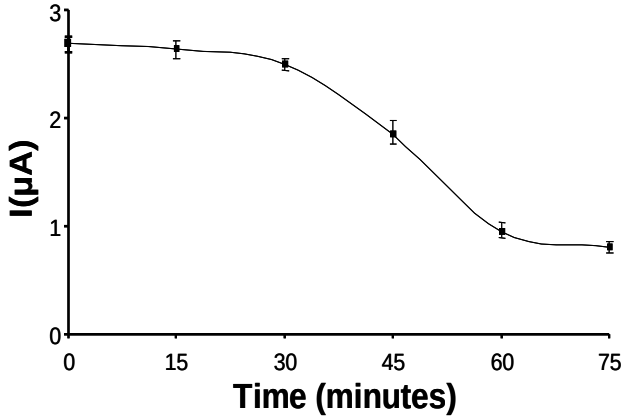

Figure 4-6. Source-drain current (at Vg=-10 V) obtained for a functionalized device after exposure to 100 cfu/mL of *S*. Infantis for 75 minutes. The source-drain current was measured every 15 minutes. Each electrical current plotted and the error bars correspond respectively to the mean value and range of three replicates measured in repeatability conditions.

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4.4. Complementary conclusions

Microscopy analysis showed that antibodies were irreversibly and uniformly adsorbed on top of the CNTs in only one hour. Although the orientation of the antibodies was not specifically addressed, most of them must be correctly oriented in the experimental conditions used, making it possible to detect, at least, 100 cfu/mL of S. Infantis.

The use of PEI to immobilise antibodies dramatically affected the electrical properties of the transducer. PEI changed the p-type behaviour to n-type behaviour and, more importantly, the four functionalization steps significantly decreased the conductance of the CNT networks. As a result, the low conductance of the functionalized CNTFET hindered their use as valuable devices to detect the presence of *Salmonella.*

By contrast, the direct adsorption of the antibodies on the CNT is a more feasible process since it preserved the electrical properties of the CNTs making it possible to determine from 100 to 500 cfu/mL of *Salmonella.*

Our devices are direct biosensors like those reported by Shabani *et al* (Shabani, A et al, 2008) label-free and easy to manufacture compared to the electrochemical device developed by Rishpon *et al* (Rishpon, J et al, 1992). However, the limit of detection and the response time is lower compared to those obtained by Barreiros *et al* (Barreiros, M et al, 2009) and Zelada-Guillén *et al* (Zelada-Guillén, G et al, 2009).

Therefore, further research is required in order to produce efficient biodevices with optimal performance parameters. It is probable that an enhanced orientation of the antibodies would increase the sensitivity of the devices. On ther hand, due to the proteic nature of the molecular receptor it is difficult to regenerate the sensors. To solve this problem, other type of receptors like aptamers or peptides could be assayed. In addition the improved biosensors must be tested with real samples.

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4.5. References

Abass, E.M., Mansour, D., Mutasim, M., Hussein, M., Harith, A. Journal of Medical Microbiology. **2006**. 55, 1193-1196.

Allen, B. L., Kichambare, P. D., Star, A. Advanced Materials. **2007**.19, 1439–1451.

Barreiros, M., Sporera, C., Sanvicensc, N., Pascuald, N., Errachida, A., Martineza, E., Marcod, M.-P., Teixeirae, V., Samiter, J. Procedia Chemistry. **2009.** 1, 1291–1294.

Bradley, K., Briman, M., Star, A., Gruner, G. Nano Letters. **2004**. 4, 253-256.

Delibato, E., Volpe, G., Romanazzo, D., De Medici, D., Toti, L., Moscone, D., Palleschi, G. Journal of Agricultural and Food Chemistry. **2009**. 57, 7200–7204.

de Boer, E. and Beumer, R.R. International Journal of Food Microbiology. **1999.** 50,119 –130.

Eyigör, A., Goncagül, G., Carli, K.T. Journal of Environmental Biology. **2007**. 1, 45-49.

Ivnitski, D., Hamid, I.A., Atanasov, P., Wilkins, E. Biosensors and Bioelectronics. **1999.** 14, 599–624.

Janyapoon, K., Korbsrisate, S., Thamapa, H., Thongmin, S., Kanjanahareutai, K., Wongpredee, N., Sarasombath, S. Clinical and Diagnostic Laboratory Immunology. **2000.** 7, 977–979.

Javey, A., Kim, H., Brink, M., Wang, Q., Ural, A., Guo, J., McIntyre, P., McEuen, P., Lundstrom, M., Dai, H. Journal of Nature Materials. **2002**. 1, 241-246.

ISBN:978-84-692-9761-2/DL:T-203-2010

Jeníková, G., Pazlarová, J., Demnerová, K. International Microbiology. **2000.** 3, 225–229.

Koyuncu, S. and Haggblom, P. BMC Veterinary Research. **2009**. 5,6.

Lazcka, O., Del Campob, F.J., Muñoz, F.X. Biosensors and Bioelectronics. **2007.** 22, 1205–1217.

Leon-Velarde, C.G., Zosherafatein, L., Odumeru, J.A. Journal of Microbiological Methods. **2009.** 79, 13-17.

Liébana, S., Lermo, A., Campoy, S., Barbé, J., Alegret, S., Pividori, M.I. Analytical Chemistry. **2009.** 81, 5812–5820.

J. Rishpon, Y. Gezundhajt, L. Soussan, L.R. Margalit, E. Hadas, Biosensor design and application, Oxford University Press, Oxford, **1992**.

Shabani, A., Zourob, M., Allain, B., Marquette, C.A., Lawrence, M.F., Mandeville, R. Analytical Chemistry. **2008**. 80, 9475–9482.

Scheu, P.M., Berghof, K., Stahl, U. Food Microbiology. **1998**. 15, 13–31.

Su, X., Chew, F.T., Li, S.F.Y. Analytical Biochemistry. **1999.** 273, 66 -72.

Taux, R.V. Emerging Infectious Diseases. **1997**. 4, 425-434.

Taitt, C.R., Shubin, Y.S., Angel, E., Ligler, F.S. Applied and Environmental Microbiology. **2004.** 70, 152–158.

Tronin, A., Dubrovsky, T., Nicolini, C. Langmuir. **1995.** 11, 385–389.

Wang, S., Humphreys, E. S., Chung, S.Y., Delduco, D. F., Lustig, S. R., Wang, H., Parker, K. N., Rizzo, N. W., Subramoney, S., Chiang, Y.M., Jagota, A. Nature Materials. **2003**. 2, 196.

Wilson, G. Journal of Clinical Microbiology. **2004**. 42, 4821–4823.

Yakovleva, J., Davidsson, R., Bengtsson, M., Laurell, T., Emneus, J. Analytical Chemistry. **2002**. 74, 2994-3004.

Zelada-Guilllén, G.A., Riu, J., Düzgün, A., Rius, F.X. Angewandte Chemie International Edition. **2009**. 48, 7334 –7337.

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Chapter 5

CNTFET for pathogenic yeast determination

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5.1. Introduction

Mycosis is one of the most common pathology that affects human beings. *Candida albicans* is an opportunistic fungi, ubiquitously distributed on the body microflora, able to produce both superficial and systemic infections (Trama, J.P. et al., 2005). Candidiasis is the so-called disease produced by *Candida.* In Spain the invasive candidiasis is the most important systemic mycosis and the main factor related with its production is the drug´s abuse (Pontón, J and del Palacio, A., 2007). Vaginal candidiasis causes 20–25 % of infectious vaginitis cases, ranked second among the bacterial vaginosis (Trama, J.P. et al., 2005).

Identification of *C*. *albicans* in clinical microbiology is usually made by using standard culture media (Cooke,V.M. et al, 2002.; Munin, E., 2007). Depending on the species, medium and detection system, the pathogen is detected within 24 to 72 hours. However, current culture methodologies have shown the occurrence of up to 35 % false results (Horvath, L.L. et al, 2005). As a consequence improved media based on chromogenic compounds have been developed (Chunchanur, S.K. et al, 2009). Although the culture is the standard method; it requires well trained technician and several days to obtain a reliable result.

The use of commercial kits like peptide nucleic acid fluorescent in situ hybridization (PNA-FISH) allows identifying *Candida* species isolated from blood cultures in 3 hours. However, an additional incubation for 24 hours is required to final determination of the yeast. Therefore, this confirmation method is time and reagent consuming.

PCR-based methods are also used to detect yeast (Wellinghausen, N. et al, 2009.; Maaroufi, F. et al, 2003.; McMullan, R. et al, 2008). Maaroufi *et al (*Maaroufi, F. et al, 2003) described a rapid and reproducible PCR assay for quantification of the *Candida albicans* ribosomal DNA (rDNA) in clinical blood samples. The assay for *C. albicans* exhibited a low limit of detection (5 cfu/mL of blood) and an excellent reproducibility (96 to 99 %).

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McMullan *et al.* proposed a method for early detection and identification of *Candida*. After manual extraction of DNA from serum, PCR was performed. This method shortens the procedure time up to 6 hours with similar accuracy outcome compared to conventional methods. However, processing requires multiple steps, and it may take longer to apply this method in clinical practice.

Immuno-based methods have also been reported for the detection of *Candida* (Moudni, E.B. et al, 1998.; Marcilla, A. et al, 1999.; Matsui, H et al, 2009). Matsui *et al*, developed an immunochromatographic method that enables the detection of vaginal *Candida* spp. The immunochromatographic strip was prepared with anti-*Candida* mannan polyclonal antibody. With this technique it is possible to detect as few as 10^4 cfu/mL of *Candida* spp. in about 30 minutes without using expensive equipment or skill. Overall, the sensitivity, specificity, positive predictive value, and negative predictive value of this method appeared to be 80.3, 99.3, 98.0, and 92.0 %, respectively.

Sensors have been recently introduced as alternative method to detect this pathogen (Chen, H. et al, 2005.; Mulero, R. et al, 2009). Mulero *et al*, describe a solid-state micro-scale pore in which a steady electrolyte current flow through the pore. Changes in the current were taken when individual yeasts travel through the pore. With this method was possible to detect about 200 cells/mL of *Candida albicans* in few seconds being possible to distinguish individual cells by their structural properties. In order to achieve a clinically applicable technique pre-processing of the sample would be necessary to reduce or eliminate potentially pore clogging particles from sample such as red blood cells, white blood cells, and platelets.

Nevertheless many efforts are still required to develop a method with improved performance parameters like rapidness, sensitivity and high selectivity. This chapter reports the first carbon nanotube field effect transistor (CNTFET) applied to the detection of a yeast. To improve the performance parameters of our devices, a monoclonal antibody highly specific for *C. albicans* was used as molecular receptor. CNTs were the transducer element able to detect the presence of at least 50 cfu/mL

of the yeast in only one hour. Possible interferences from the sample like proteins were avoided by using Tween 20 at 0.5 % as a protecting polymer that covers the CNTs. This biosensor is stable, easy to manipulate and provides an inexpensive option to in vitro screening of yeast.

Finally, this chapter provides additional experimental information to those included in the published paper, general conclusions and references.

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5.2. Article:

"Improved detection of Candida albicans with carbon nanotubes field effect transistors". Sensors and Actuators, B: Chemical. 2009.136, 451-457.

Raquel A. Villamizar[τ], Alicia Maroto* F. Xavier Rius

Department of Analytical and Organic Chemistry. Universitat Rovira i Virgili Marcel.lí. Domingo, s/n. 43007. Tarragona, Spain.

[τ] On leave from the Department of Microbiology. University of Pamplona. Km 1, vía Bucaramanga, N. de S. Colombia.

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Abstract

A biosensor for detecting *Candida albicans* has been developed to improve the performance characteristics of the currently available methods. It is based on a field-effect transistor (FET) in which a network of single-walled carbon nantotubes (SWCNTs) acts as the conductor channel. Monoclonal anti-*Candida* antibodies were adsorbed onto the SWCNT to provide specific binding sites for fungal antigens. Tween 20 at 0.5 % was used as a blocking agent to prevent the non-specific binding of other yeasts or proteins. Our FET devices were exposed to increasing concentrations of *C. albicans* and were able to detect at least 50 cfu/mL in only 1 hour. The sensor response remained stable for more than ten days. To evaluate the selectivity of our FET devices, *Cryptococcus albidus* and *Saccharomyces cerevisiae* were tested as potential competing yeasts for *Candida albicans*. The results showed that biosensors based on a carbon nanotube field effect transistor are useful tools to detect pathogenic yeasts like *Candida albicans* at low concentrations displaying high selectivity.

Keywords: Biosensor, Field-Effect Transistor, Carbon nanotubes, Yeast, *Candida albicans.*

Introduction

Candida albicans (*C. albicans*) is a yeast-like fungal organism that can make the morphologic transition from yeast to hyphal, thereby increasing its pathogenicity [1]. It can be found on human skin, in the digestive tract, mouth, throat, oesophagus, vagina and other mucus membranes causing a wide spectrum of diseases from simple cutaneous mycosis to complicated cases of candidiasis. Candidiasis is mainly an endogenous infection produced by the overgrowth of the human body's own fungi. In some cases, it can also be acquired from exogenous sources like catheters or prosthetic devices, person-to-person transmission or vertical transmission [2]. This yeast can affect especially patients with immunodeficient, neoplasya, diabetes, lymphomas among others.

ISBN:978-84-692-9761-2/DL:T-203-2010

Conventional diagnoses of *Candida* involve methods such as germ tube or colorimetric tests. Nevertheless, up to 5 % of the *C. albicans* isolates have been reported as germ tube negative [3]. Blood cultures for *Candida* species generally display low sensitivity and require additional tests to determine marker antigens. Some traditional culture media allows bacterial growth, thus reducing the recovery rate of target yeast from the samples [4]. Selective chromogenic agar medium has also been used to detect yeast with medical importance but they are time-consuming [5]. Serological tests for *Candida* involve different techniques such as counterimmunoelectrophoresis and radioimmunoassay. However they display variable sensitivity and lack of specificity, with up to 40 % false positive and negative rates [6]. Enzyme-linked immunosorbent assay (ELISA) is highly sensitive but requires the use of labels. Molecular biology techniques such as PCR and hybridization assays are reliable and highly sensitive but they are also reagent and time-consuming [7].

Mass sensors [8-11] and electrochemical sensors [12] have also been developed to detect both pathogenic and non-pathogenic yeasts and moulds. In spite of the emerging methods for detecting fungi, the detection range is still quite high (ca. 10^2 cfu/mL). This means that a simple, easy-to-perform and sensitive assay for detecting yeasts like *Candida* is still not available.

Single-walled carbon nanotubes (SWCNT) are one-dimensional nanostructures with all the carbon atoms on the surface, allowing optimal interaction with biomolecules in their chemical environment [13]. Dekker's group in 1998 reported the first carbon nanotube field effect transistor (CNTFET) [14]. CNTFETs with a selective coating have become a suitable system for biosensing applications because they combine the principles of molecular recognition through the recognition layer with the transduction capabilities of the carbon nanotubes. They have been used to detect DNA [15-17], proteins like thrombin and IgE and IgG by means of antibodies and aptamers as molecular receptors [18-20], viruses by means of peptide nucleic acids [21] and bacteria [22, 23].

ISBN:978-84-692-9761-2/DL:T-203-2010

So far, CNTFETs have not been used to detect eukaryotic cells. It is therefore worthwhile to broaden the application field to include these organisms. Here, we report the development of a sensitive CNTFET for the fast detection of C*. albicans*. As a recognition element, we adsorbed onto the SWCNTs monoclonal anti-*Candida* antibodies, which were able to provide specific binding sites for fungal membrane antigens. Tween 20 was used as a blocking agent to prevent the non-specific binding (NSB) of other yeasts [18, 24, 25]. In the presence of competing yeasts like *Cryptococcus albidus* the sensor showed high selectivity. Our devices were able to detect 50 cfu/mL with a time response of one hour, eliminating the need for a special labelling process. The operational performance parameters of our devices represent a clear advantage over traditional as well as some current methods used in the detection of pathogenic yeasts.

Experimental

Antibodies and biochemicals

Anti-*Candida* (i.e. a monoclonal mouse anti-*Candida albicans* (2.8 mg/mL) was purchased from Oxford Biotechnology Ltd. (Oxford, U.K.). It was dissolved in phosphate buffer saline (PBS) to a final concentration of 10 ppm (pH=7.2) and stored at -20 ºC until use. Tween 20 and PBS were obtained from Sigma-Aldrich. Sabouraud Dextrosa Agar (SDA) was provided by Oxoid and prepared according to their specifications. Silver ink Electrodag 1415M was provided by Acheson industries (Europe) Ltd (Scheemda, The Netherlands).

Yeast preparation

Lyophilized strains of *C. albicans* CECT 1001, *Saccharomyces cerevisiae* (*S. cerevisiae*) CECT 1443 and *Cryptococcus albidus* (*C. albidus*) CECT 11978 were obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain). They were rehydrated with sterile water, subcultured at least three times on SDA and incubated at 35 ºC for 48 hours to test their viability. Isolated colonies were resuspended on sterile 0.85 % saline solution. The yeast cells contained in the saline solution were enumerated by plating 10^8-fold serial dilutions on SDA plates and incubating at 35 ºC for 24-48 hours [26]. Further dilutions in sterile 0.85 %

ISBN:978-84-692-9761-2/DL:T-203-2010

saline solution were performed to produce the final samples at 50, 200 and 400 cfu/mL of *C*. *albicans*. The same procedure was followed to prepare 50, 200 and 400 cfu/mL of *S. cerevisiae* and *C. albidus.*

Apparatus

Atomic force microscopy (AFM), Pico Plus (Molecular Imaging) was used in tapping mode to characterize the SWCNTs before and after they were functionalised with the antibodies. Scanning electron microscopy (SEM), JSM 6400 (Jeol) was used to take images of the as-grown networks of SWCNTs and the functionalisation process. SEM images were obtained by applying a voltage of 1kV at a working distance of 8 mm. Electrical measurements were taken using a 4157A Agilent semiconductor parameter analyzer and a Wentworth Laboratories MP1008 probe station.

Development of the CNTFETs

The SWCNT networks were synthesized on top of a 500 nm layer of silicon dioxide thermally grown on highly doped n-type silicon chips (total area 0.5 cm x 0.5 cm) using chemical vapour deposition (CVD). A solution of 100ppm iron nitrate in isopropanol was used as the catalyst. The CVD was performed at 900 ºC for 20 minutes with 600 sccm of methane and 200 sccm of hydrogen. Figure 1 A-1 shows that a dense network of SWCNTs was obtained after the CVD. The average height of the SWCNTs (obtained from AFM) was about 1.5 nm. The source and drain electrodes of the CNTFETs were screen-printed with silver ink. The gap between both electrodes was 0.5 mm and the size of the electrodes was 200 µm x 200 µm. The gate electrode was an aluminium layer on the back side of Si. The CNTFETs were electrically characterised by recording the current vs. the gate voltage. To obtain the instrumental variability, we carried out all the electrical characterization measurements three times and we plotted the mean value and the range value of the measurements.

UNIVERSITAT ROVIRA I VIRGILI
BIOSENSORS BASED ON CARBON NANOTUBE FIELD EFFECT TRANSISTORS (CNTFETs) FOR DETECTING PATHOGENIC MICROORGANISMS
Raquel Amanda Villamizar Gallardo
ISBN:978-84-692-9761-2/DL:T-203-2010

Functionalisation of the CNTFETs

The functionalisation protocol was performed similarly to that previously described for the detection of *Salmonella* [22]. The CNTFETs were incubated for one hour at 37 ºC in a 5 ppm solution of anti-*Candida* antibodies in 15 mM PBS solution. The CNTFETs were then rinsed with PBS solution and distilled water, dried with nitrogen [27] and immersed for 2 hours in a solution of 0.5 % Tween 20 dissolved in 15 mM PBS [18]. The CNTFETs were again thoroughly rinsed with distilled water, dried with nitrogen and ready to be used to detect *C*. *albicans*. Figure 1 shows the experimental set up for detecting *C. albicans* using a functionalised CNTFET. Figure 1A-1 shows a CNTFET functionalised with the monoclonal anti-*Candida* antibodies. The average height of the antibodies (obtained from AFM) was about 9 nm. Figure 1B-1 shows that, as is well known, the yeasts do not retain their shape after being dried and having a high vacuum applied in the SEM.

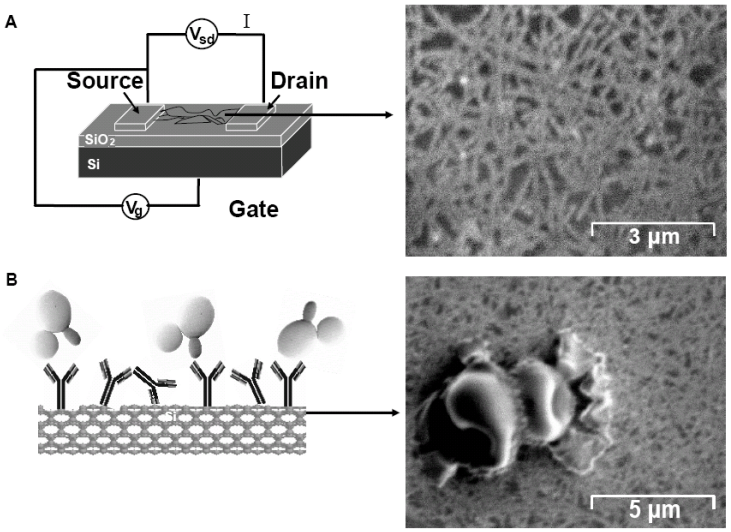

Figure 1. A) Schematic structure of the CNTFET device. A1) SEM image of a typical network of SWCNTs obtained by CVD and coated with anti-*Candida* antibodies. B) Antigen-antibody interaction of *C. albicans* with a SWCNT functionalised with anti-*Candida* antibodies and protected with Tween 20. B2) A SEM image of a functionalised CNTFET after exposure to *C. albicans.*

ISBN:978-84-692-9761-2/DL:T-203-2010

Detection of *C. albicans*

The functionalised CNTFETs were exposed to increasing concentrations of *C. albicans* (i.e. 50, 200 and 400 cfu/mL). For each concentration, the CNTFETs were immersed for one hour at 37 ºC, rinsed thoroughly with distilled water, dried with nitrogen and electrically characterised. For each concentration we scanned the area between the electrodes (~500 μm^2) with SEM to count the number of yeasts attached to the SWCNTs.

Determination of the response time

A functionalised CNTFET was immersed in a solution containing 50 cfu/mL of *C. albicans* at 37 ºC for one hour. Every 15 minutes the CNTFET was thoroughly rinsed with water, dried with nitrogen, electrically characterised, and again submerged in the solution containing 50 cfu/mL of *C. albicans* for another 15 minutes.

Selectivity of the CNTFETs

Selectivity was checked in the presence of two yeasts: *C. albidus* and *S. cerevisiae*. A functionalised CNTFET was first immersed in a yeast solution containing 400 cfu/mL of *C. albidus* for one hour at 37 ºC, thoroughly rinsed with distilled water, dried with nitrogen and electrically characterised. It was subsequently exposed to 400 cfu/mL of *C. albicans* under the same conditions mentioned above. The same procedure was followed for 400 cfu/mL of *S. cerevisiae.* All these experiments were confirmed microscopically with SEM.

Interference assay

Our sensor devices were also proven against possible interferences that can be found out in real samples of diseased patients. We took a functionalised CNTFET and it was exposed to different concentrations of BSA obtained from Sigma-Aldrich, (i.e. 1mg/L, 10 mg/L, 100 mg/L, 1000 mg/L and 10000 mg/L) for 1 hour each. The devices were washed with a solution of PBS and distilled water after exposure to each concentration, then were dried with nitrogen and electrically characterized.

ISBN:978-84-692-9761-2/DL:T-203-2010

Results and discussion

The electrical behaviour of the CNTFETs was monitored after each functionalisation step. We measured the dependence of the source-drain current, I, on the back gate voltage, V_g, in the +5 V to -5 V range at room temperature in dry conditions three times. The bias voltage, V_{sd}, was fixed at 250 mV. Figure 2 shows how each functionalisation step affects the electrical current of a typical CNTFET. A p-type behaviour was observed for the pristine networks due to the electron withdrawal of adsorbed oxygen molecules from the air which coincides with that found by Star *et al* [28].

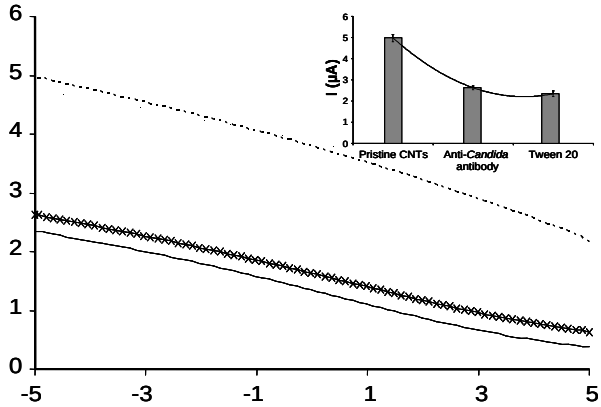

Figure 2. Gate voltage dependence of the source-drain current of a typical CNTFET after each functionalisation step. Pristine CNTs (----); anti- *Candida* antibodies adsorbed on the CNTs (–X–) and CNTs covered with anti-*Candida* antibodies and Tween 20 (—).The inset shows the behaviour of the source-drain current after each functionalisation step at Vg= -5 V.

Anti-*Candida* antibodies were adsorbed on the CNTs. Although the mechanism of non-specific adsorption of proteins onto SWCNTs is still unknown [29], it is thought that supramolecular interactions between the aromatic amino acids (i.e., histidine and tryptophan) present in the polypeptide chains of the proteins and the SWCNTs may help to clarify the affinity of the peptides towards this nanostructure [30]. This phenomenon also has been attributed to size compatibility between the two entities

[31]. We observed through the AFM that 1 h was enough to immobilize anti-*Candida* antibodies onto the sidewalls of SWCNTs.

We took AFM images of a CNTFET just after the antibodies had been immobilized and the CNTFET had been submerged in PBS solution for 24 h. We found small points non-uniformly distributed onto the sidewalls of the SWCNTs after one hour, which remained attached after 24 h, suggesting that they were strongly adsorbed.

The absorbed anti-*Candida* antibodies caused a change in the electrical properties of the device, which may be explained by a charge transfer process in which the aminoacids with base-containing residues (i.e. arginine, histidine and lysine) located on the external envelope of the protein provide electrons. According to Bradley K *et al*. [32] each adsorbed amine donates 0.04 electrons to the nanotubes giving rise to a decrease in the electrical current and to a shift in the threshold voltage of the semiconducting CNTs.

The CNTFET electrical current decreased slightly after the adsorption of Tween 20, as previously observed by So *et al.* [18]. Tween 20 is a surfactant with linear aliphatic chains that adsorbs on the walls of SWCNTs through hydrophobic interactions and protects them against the non-specific binding (NSB) of proteins [18, 24, 25] and bacteria [22, 23]. In this study, Tween 20 covered the gaps on the walls of the CNTs that were left unprotected by the adsorbed anti-*Candida* protein and also reduce the aggregation of the hydrophobic domains present in the polypeptide chain of the anti-*Candida* IgG. Since the yeast was prepared in sterile 0.85 % NaCl solution, we tested whether the electrical current might be affected by this matrix. We exposed the devices to a 0.85 % NaCl solution for 1 h at 37 ºC and no significant change in the electrical current was observed.

A functionalised CNTFET was later taken to determine the response time of our devices. We immersed it in a solution containing 50 cfu/mL of *C. albicans* at 37 ºC for 15 minutes and then thoroughly rinsed it with water, dried with nitrogen and measured the electrical current. After each measurement the devices were again submerged in the solution containing 50 cfu/mL for another 15 minutes and electrically characterised. Figure 3 shows that the highest decrease (i.e. about 34

ISBN:978-84-692-9761-2/DL:T-203-2010

%) in current intensity was obtained after 60 minutes of incubation. No further decrease was obtained later on. We chose a response time of one hour for our devices. After 10 days the signal was recorded again and it remained stable.

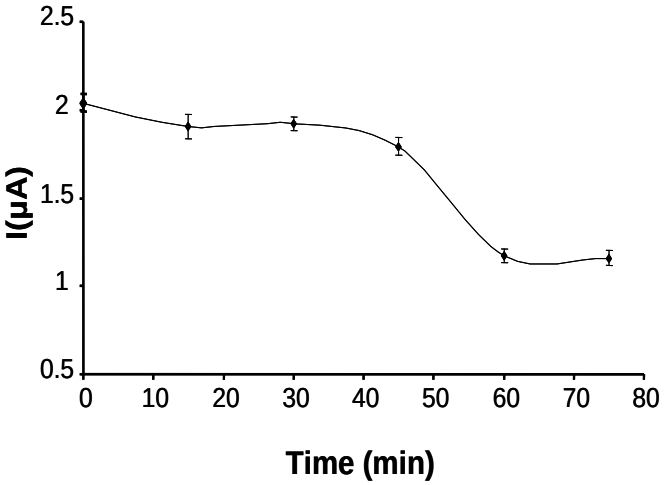

Figure 3. Source-drain current (at Vg=-5 V) obtained for a functionalised device after being exposed to 50 cfu/mL *C. albicans* for 75 minutes. The source-drain current was measured every 15 minutes.

Next, we took a functionalised CNTFET and exposed it to increasing concentrations of *C. albicans.* For each concentration, the CNTFET was immersed for one hour at 37 ºC, rinsed thoroughly with distilled water, dried with nitrogen and electrically characterised by measuring three times the I–V characteristics and plotting the mean value and range of the measurement values. Figure 4 shows the I-V characteristics (for a bias=0.25 V) of a functionalised CNTFET before exposure to *C. albicans* and after exposure to 50, 200 and 400 cfu/mL of the yeast. This figure shows that the adsorption of *C. albicans* significantly decreases the electrical current at negative gate voltages (i.e. from an 18 % decrease for 50 cfu/mL to a 50 % decrease for 400 cfu/mL). The error bars (which correspond to the range of the electrical current measured for the three replicates) show that the variability of the electrical current was less than 10 %.

UNIVERSITAT ROVIRA I VIRGILI
BIOSENSORS BASED ON CARBON NANOTUBE FIELD EFFECT TRANSISTORS (CNTFETs) FOR DETECTING PATHOGENIC MICROORGANISMS
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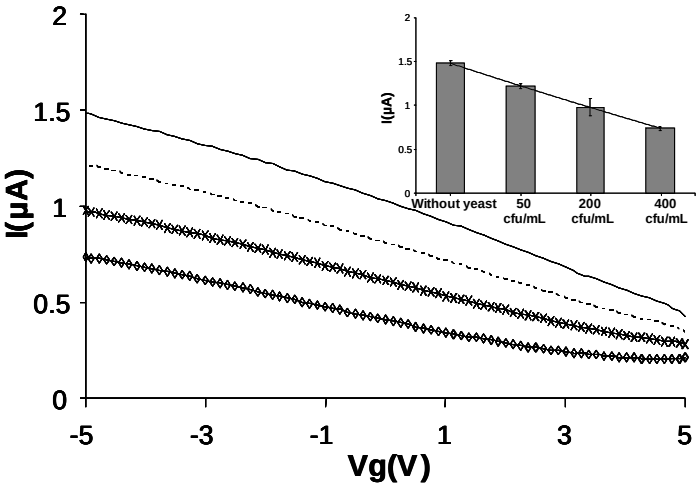

Figure 4. Gate voltage dependence of the source-drain current of a typical CNTFET before exposure to *C. albicans* (—) and after exposure to 50 cfu/mL (----); 200 cfu/mL (-X-) and 400 cfu/mL (-◊-) of *C. albicans*. The inset shows the behaviour of the source-drain current at Vg= -5 V.

The exposure of the devices to *C. albicans* decreased significantly the conductance of the functionalised CNTFET. Two mechanisms can explain this decrease. The first one is a possible distortion of the CNTs as a consequence of the bound yeast [28, 33]. The second one is the charge transfer process in which the analyte provide electrons to the SWCNTs [30, 33]. In this way, we assume that once anti-*Candida* antibodies interact with the antigen "mannan" present in the outer cell wall layer of *C. albicans* there is a charge transfer process. Mannans are molecules composed by homopolymers of D-mannose, which are linked to proteins by N-glycosidic bonds to asparagine residues and by O-glycosidic, alkali-labile linkages to threonine or serine residues [1]. Therefore, the charge transfer process could be mainly due to electron donating from the amino groups of the mannoproteins present in the cell wall of the yeast to the CNTs. The inset in figure 4 shows that the higher the concentration of *Candida*, the more the electrical current decreases. SEM confirmed that the number of yeasts attached to the CNTs was proportional to the concentration of *Candida* in the solution. A functionalised CNTFET was exposed to different yeast concentrations for 1 h each, thoroughly rinsed with distilled water and dried with nitrogen. For each concentration we scanned the area

ISBN:978-84-692-9761-2/DL:T-203-2010

between the electrodes (500 μm^2) with SEM to count the number of yeast attached to the CNTs. We counted 13, 24 and 35 yeasts after exposing the CNTFET to 50, 200 and 400 cfu/mL, respectively.

As a control experiment, we took five CNTFETs devices without having first absorbed the anti-*Candida* antibodies on the CNTs. Each device was immersed into a solution containing Tween 20 at different concentrations (i.e. 0.05 %, 0.1 %, 0.3 %, 0.4 % and 0.5 %) during 2 hours at 37 ºC. Then, the CNTFETs were exposed to 400 cfu/mL of *C. albicans* for 1 hour at 37 ºC. The devices were thoroughly rinsed with distilled water, dried with nitrogen and electrically characterized after each step. Figure 5 shows that the change of the electrical current of the devices was about 37 %, 24 %, 22 %, 11 % when they were protected with Tween 20 at 0.05 %, 0.1 %, 0.3 % and 0.4 % respectively. The error bars correspond to the range of the current values. The CNTFET coated with Tween 20 at 0.5 % shows a slight change in the electrical current due to variability in the electrical current. Thus, we proved that Tween 20 at 0.5 % was effectively protecting the CNTs against the NSB of yeasts and that the decrease in the electrical current was due solely to the antigen–antibody interaction.

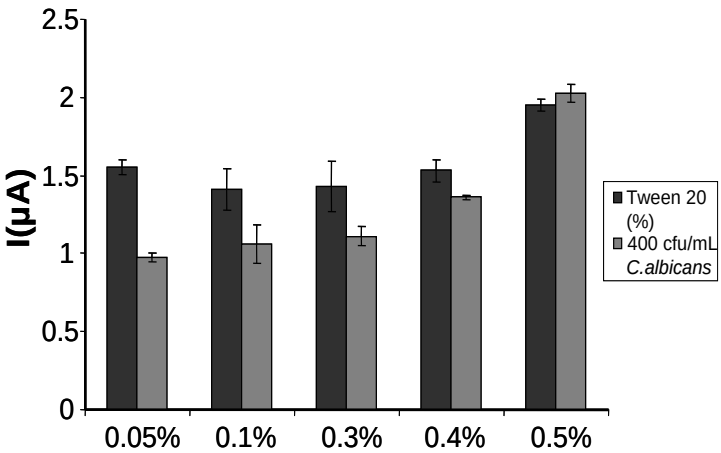

Figure 5. Source-drain current (at Vg=-5 V) obtained for five different Tween 20 modified-CNTFETs before and after exposure to 400 cfu/mL of *C. albicans.* Each electrical current plotted corresponds to the mean value of three replicates.

ISBN:978-84-692-9761-2/DL:T-203-2010

The effectiveness of Tween 20 at 0.5 % against NSB was also checked using SEM. A CNTFET (functionalised with Tween 20 but not with anti-*Candida*) was exposed to 50, 200 and 400 cfu/mL *C. albicans* for 1 h each, thoroughly rinsed with distilled water and dried with nitrogen. For each concentration we scanned the area between the electrodes (500 μm^2) with SEM. No yeasts were bound when the device was exposed to 50 cfu/mL of *C. albicans* whereas 2 yeasts were observed when it was exposed to 200 and 400 cfu/mL of *C. albicans.*

Selectivity of our devices was tested in the presence of two yeasts: *C. albidus and S. cerevisiae. C. albidus* is an emerging pathogen, ubiquitously found in the environment infecting and causing human disease as well as *Candida* [34]. However, it should not have any cross reaction with the anti-*Candida* antibody because it is antigenically different from *Candida*. On the other hand, *S. cerevisiae* is an emerging opportunist pathogen and can be found along with *C. albicans* in clinical cases like cutaneous or vaginal infections [35]. The mannoproteins of *C. albicans* share several general features with the mannoproteins of *S. cerevisiae* [1] as they are antigenically similar.

A functionalised CNTFET was first immersed in a yeast solution containing 400 cfu/mL of *C. albidus* for one hour at 37 ºC, thoroughly rinsed with distilled water, dried with nitrogen and electrically characterised. The device was then exposed to 400 cfu/mL of *C. albicans* under the same conditions mentioned above. We follow the same procedure with another functionalised CNTFET for 400 cfu/mL of *S. cerevisiae*. Figure 6A shows that the electrical current did not change after exposing the CNTFET to 400 cfu/mL of *C. albidus* whereas it decreased by about 30 % after exposure to *C. albicans.* Figure 6B shows that the electrical current changed slightly (i.e. 10 %) after exposing the CNTFET to 400 cfu/mL of *S. cerevisiae,* whereas it decreased in the range of about 36 % after exposure to *C. albicans*. The change in the electrical current after exposing the devices to *S. cerevisiae* are probably due to the antigenic similarity between the two yeasts, which may cause a slight cross-reactivity of the commercially available antibody. The electrical current plotted corresponds to the mean value of the three

UNIVERSITAT ROVIRA I VIRGILI
BIOSENSORS BASED ON CARBON NANOTUBE FIELD EFFECT TRANSISTORS (CNTFETs) FOR DETECTING PATHOGENIC MICROORGANISMS
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measurements. The error bars of the inset correspond to the range of the current values.

Selectivity was also checked with SEM. A functionalised CNTFET was exposed to 50, 200 and 400 cfu/mL of *C. albidus* for 1 h each, thoroughly rinsed with water and dried with nitrogen. For each concentration, we scanned the area between the electrodes (500 μm^2) with SEM. We did not observe any yeast after the device had been exposed to 50 cfu/mL whereas only one yeast was attached after the CNTFET was exposed to 200 and 400 cfu/mL of *C. albidus*. The same procedure was followed for S*. cerevisiae*. In this case, we observed 2, 5 and 7 yeasts after the device had been exposed to 50, 200 and 400 cfu/mL of *S. cerevisiae,* respectively (thus showing, the slight cross-reaction of the antibody with *S. cerevisiae*). With *C. albidus* the sensor displayed high selectivity; however, due to the antigenic similarity between *C. albicans* and *S. cerevisiae* there was a slight cross-reactivity of the commercially available antibody. Therefore, a more specific antibody against *C. albicans* would be able to overcome this problem.

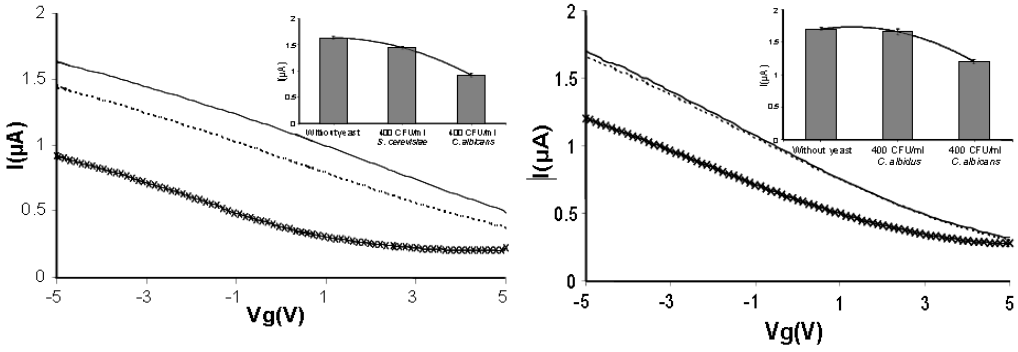

Figure 6. A) Gate voltage dependence of the source-drain current of a functionalised CNTFET before exposure to the bacteria (—) and after exposure to 400 cfu/mL of *C. albidus* (----); and 400 cfu/mL of *C. albicans* (-X-). B) Gate voltage dependence of the source-drain current of a functionalised CNTFET before exposure to the yeast (—) and after exposure to 400 cfu/mL of *S. cerevisiae* (----); and 400 cfu/mL of *C. albicans* (-X-). The inset shows the behaviour of the source-drain current after each functionalisation step at Vg= -5 V.

ISBN:978-84-692-9761-2/DL:T-203-2010

Our devices were also proven against possible interferences that can be found out in real samples like blood or serum of diseased patients. Serum albumin is the most abundant protein present in the circulatory system and could interfere in the performance of our sensor devices. To prove it, we used bovine serum albumin (BSA), like interference protein which has an amino acid sequence very similar to human serum albumin (HAS) [36-37]. Figure 7 shows that there was a not significative change in the electrical current of the device at concentrations below to 100 mg/L of BSA. In presence of higher concentrations (i.e. 1000 mg/L and 10000 mg/L) of the protein, the electrical current decreased about 12 % to 18 % respectively. Through this assay, we have proven that our sensor devices could be used to detect selectively *C. albicans* in samples containing albumin concentration below to 100 mg/L.

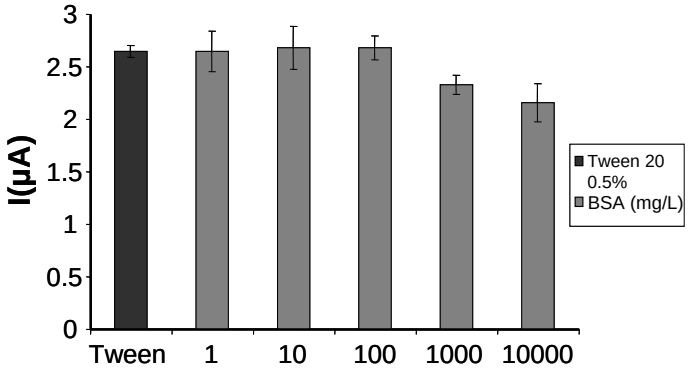

Figure 7. Source-drain current (at Vg=-5 V) obtained for a functionalized CNTFET before and after exposure to increasing concentrations of BSA. Each electrical current plotted corresponds to the mean value of three replicates.

Since this paper reports the first CNTFET to detect yeasts, we have compared our results with those obtained by other techniques. The limit of detection enhanced by our devices (50 cfu/mL) is lower than the obtained for *Candida* with other kind of biosensor based on a quartz piezoelectric crystal (10^3-10^5 cell/mL) [8].

Comparing with traditional methods, our CNTFETs only need 1 hour to detect *C. albicans* while conventional media requires 24-48 h for the interpretation of the results [38-40]. In addition, in our sensor devices the recognition element was a

ISBN:978-84-692-9761-2/DL:T-203-2010

specific antibody anti-*C.albicans* displaying high selectivity against other yeasts both antigenically similar and different and tween 20 avoids effectively the non-specific binding of the yeast or proteins. This is an advantage comparing for instance with chromogenic culture media, which contain chromogenic or fluorogenic substrates for a rapid identification of *C. albicans* on the basis of colony colour. Although some of them present high sensitivity and specificity (97 % and 92 % respectively), false positive results are frequently obtained [38, 40].

CONCLUSIONS

In this paper we have shown that carbon nanotube field effect transistors (CNTFETs) functionalised with monoclonal anti-*Candida* antibodies can be used to detect 50 cfu/mL of the yeast in only one hour. The selectivity of the sensor was determined by exposing the devices to *C. albidus* and *S. cerevisiae.*

The results suggest that CNTFETs biosensors might be a valuable screening tool addressed to future routine analysis of real samples like blood, serum, infected tissues, vaginal smears, swabs samples of diseased patients. On the other hand, it is also possible to detect in human milk clinical cases of mastitis caused by *C. albicans* and using an adequate molecular receptor other pathogenic *Candida* species can be detected in samples like mastitic bovine milk. In addition, our biosensor could help to obtain an early diagnostic of sick patients which would improve the administration of adequate drugs and treatments.

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ISBN:978-84-692-9761-2/DL:T-203-2010

References

[1]. J. P. Martinez, M.L. Gil, J.L. López-Ribot, W.L.Chaffin, Serologic Response to Cell Wall Mannoproteins and Proteins of *Candida albicans,* Clin. Microbiol. Rev, 11, (1998) 121–141.

[2]. R. A. Cavalheiro, F. Fortes, J. Borecký, V. C. Faustinoni, A. Z. Schreiber, A. E.Vercesi, Respiration, oxidative phosphorylation, and uncoupling protein in *Candida albican,* Braz. J. Biol. Biomed. Res, 37 (2004) 1455-1461.

[3]. I. F. Salkin, G. A. Land, N. J. Hurd, P.R. Goldson, M. R. McGinnis, Evaluation of YeastIdent and Uni-Yeast-Tek Yeast Identification Systems, J. Clin. Microbiol, 25 (1987) 624-627.

[4]. J. F. Okulicz, R. G. Rivard, N. G. Conger, M. X. Nguyen, D. R. Hospenthal, Primary isolation of Candida species from urine specimens using chromogenic medium, Mycoses, 51(2007)141–146.

[5]. E. Eraso, M. D. Moragues, M. Villar-Vidal, I. H. Sahand, N. González, J. Pontón, G. Quindós, Evaluation of the New Chromogenic Medium Candida ID 2 for Isolation nd Identification of *Candida albicans* and Other Medically Important *Candida* Species, J. Clin. Microbiol, 44 (2006) 3340-3345.

[6]. W.M. Scheld, R.S. Brown, S.A. Harding, M.A. Sande, Detection of Circulating Antigen in Experimental *Candida albicans* Endocarditis by an Enzyme-Linked Immunosorbent Assay, J. Clin. Microbiol, 12 (1980) 679-683.

[7]. H. Xiang, L. Xiong, X. Liu, Z.Tu, Rapid simultaneous detection and identification of six species *Candida* using polymerase chain reaction and reverse line hybridization assay, J. Microbiol. Methods, 69 (2007) 282–287.

[8]. H. Muramatsu, K. Kajiwara, E. Tamiya, I. Karube, Piezoelectric immunosensor for the detection of *Candida albicans* microbes, Anal. Chim. Acta, 188 (1986) 257-261.

[9]. N. Nugaeva, K.Y. Gfeller, N. Backmann, H.P.Lang, M. Duggelin, M. Hegner, Micromechanical cantilever array sensors for selective fungal immobilization and fast growth detection, Biosens. Bioelectron, 21 (2005) 849–856.

[10]. J.W. Yi, W.Y. Shih, R. Mutharasan, W. H. Shih, In situ cell detection using piezoelectric lead zirconate titanate-stainless steel cantilevers, J. Appl. Phys, 93 (2002) 619-625.

[11]. F. L. Dickert and O. Hayden, Bioimprinting of Polymers and Sol-Gel Phases Selective Detection of Yeasts with Imprinted Polymers, Anal. Chem, 74 (2002) 1302-1306.

[12]. H. Chen, C.K. Heng, P.D. Puiu, X.D. Zhou, A.C. Lee, T.M. Lim, S.N. Tan, Detection of *Saccharomyces cerevisiae* immobilized on self-assembled monolayer (SAM) of alkanethiolate using electrochemical impedance spectroscopy, Anal. Chim. Acta, 554 (2005) 52–59.

[13].Heller, I., A.M. Janssens, J. Männik, E. D. Minot, S.G. Lemay, C. Dekker, Identifying the Mechanism of Biosensing with Carbon Nanotube Transistors, Nano Lett, 8 (2008) 591-595.

[14]. S. J. Tans, A. R. Verschueren, C. Dekker, Room-temperature transistor based on a single carbon nanotube, Nature, 393 (1998) 49-52.

[15]. E.L. Gui, L.J. Li, K. Zhang, Y. Xu, X. Dong, X. Ho, P.S. Lee, J. Kasim, Z.X. Shen, J.A. Rogers, S.G. Mhaisalkar, DNA Sensing by Field-Effect Transistors Based on Networks of Carbon Nanotubes, JACS, 129 (2007) 14427-14432.

[16]. A. Star, E. Tu, J. Niemann, J.C.P. Gabriel, C.S. Joiner, C. Valcke, Label-free detection of DNA hybridization using carbon nanotube network field-effect transistors, PNAS, 103 (2006) 921-926.

[17]. H. M. So, K. Won, Y. H. Kim, B. K. Kim, B. H. Ryu, P. S. Na, H. Kim, A. Star, E. Tu, E. J. Niemann, J.C.P. Gabriel, C.S. Joiner, C. Valcke, Label-free detection of

DNA hybridization using carbon nanotube network field-effect transistors, PNAS, 103 (2006) 921-926.

[18]. H. M. So, K. Won, Y. H. Kim, B. K. Kim, B. H. Ryu, P. S. Na, H. Kim, O.J. Lee, Single-Walled Carbon Nanotube Biosensors Using Aptamers as Molecular Recognition Elements, JACS, 127(2005) 11906-11907.

[19]. K. Maehashi, T. Katsura, K. Kerman, Y. Takamura, K. Matsumoto, Tamiya, E, Label-Free Protein Biosensor Based on Aptamer-Modified Carbon Nanotube Field-Effect Transistors, Anal. Chem, 79 (2007) 782-787.

[20]. C. Cid, J. Riu, A. Maroto, F.X. Rius, Carbon nanotube field effect transistors for the fast and selective detection of human immunoglobulin G., Analyst, doi: 10.1039/b805301b,(2008).

[21]. T. Dastagir, E.S. Forzani, R. Zhang, I. Amlani, L. A. Nagahara, R. Tsui, N. Tao, Electrical detection of hepatitis C virus RNA on single wall carbon nanotube-field effect transistors, Analyst, 132 (2007) 738-740.

[22]. R.A. Villamizar, A. Maroto, F.X. Rius, I. Inza, M.J. Figueras, Fast detection of *Salmonella* Infantis with carbon nanotube field effect transistors, Biosens. Bioelectrons, 24 (2008) 279-283.

[23]. H.M. So, D. W. Park, E. K. Jeon, Y. H. Kim, S.K. Kim, C.K. Lee, S. Y. Choi, S.C. Kim, H. Chang, J.O. Lee, Detection and Titer Estimation of *Escherichia coli* Using Aptamer-Functionalized Single-Walled Carbon-Nanotube Field-Effect Transistors, Small, 4 (2008) 197-201.

[24]. H. R. Byon and H.C. Choi, Network Single-Walled Carbon Nanotube-Field Effect Transistors (SWNT-FETs) with Increased Schottky Contact Area for Highly Sensitive Biosensor Applications, JACS, 128 (2006) 2188-2189.

[25]. R. J. Chen, S. Bangsaruntip, K. A. Drouvalakis, N. W. Kam, M. Shim, Y. Li, W. Kim, P. J. Utz, H. Dai, Noncovalent functionalization of carbon nanotubes for highly specific electronic biosensors, PNAS, 100 (2003) 4984-4989.

[26]. H. C. Chang, J. J. Chang, A. H. Huang, T. C. Chang, Evaluation of a Capacitance Method for Direct Antifungal Susceptibility Testing of Yeasts in Positive Blood Cultures, J. Clin. Microbiol, 38 (2000) 971-976.

[27]. B. Ilic, D. Czaplewski, G. Craighead, Mechanical resonant immunospecific biological detector, Appl. Phys. Lett, 77 (2000) 450–452.

[28]. A. Star, J.C. Gabriel, K. Bradley, G. Gruner, Electronic Detection of Specific Protein Binding Using Nanotube FET Devices, Nano Lett, 3 (2003) 459–463.

[29]. K. Teker, E. Wickstrom, B. Panchapakesan, Biomolecular Tuning of Electronic Transport Properties of Carbon Nanotubes via Antibody Functionalization, IEEE Sens. J, 6 (2006) 1422-1428.

[30]. B. L. Allen, P. D. Kichambare, A. Star, Carbon Nanotube Field-Effect-Transistor Based Biosensors, Adv. Mater, 19 (2007) 1439–1451.

[31]. M. Shim, N.W.S. Kam, R.J. Chen, Y. Li, H. Dai, Functionalization of Carbon Nanotubes for Biocompatibility and Biomolecular Recognition, Nano Lett, 2(2002) 285–288.

[32]. K. Bradley, M. Briman, A. Star, G. Gruner, Charge Transfer from Adsorbed Proteins, Nano Lett, 4 (2004) 253–256.

[33]. G. Grüner, Carbon Nanotube Transistors for Biosensing Applications, Anal. Bioanal. Chem, 384 (2006) 322–335.

[34] J. Schutzbach, H. Ankel, I. Brockhausen, Synthesis of cell envelope glycoproteins of Cryptococcus laurentii, Carbohydr.Res, 342 (2007) 881–893.

[35]. A.L. Goldstein and J.H. McCusker, Development of *Saccharomyces cerevisiae* as a Model Pathogen: A System for the Genetic Identification of Gene Products Required for Survival in the Mammalian Host Environment, Genetics, 159 (2001) 499–513.

ISBN:978-84-692-9761-2/DL:T-203-2010

[36]. S. Aoyagi, M. Dohi, N. Kato, M. Kudo, S. Iida, M. Tozu, N. Sanada. Structure analysis of immobilized-bovine serum albumin by means of TOF-SIMS, e-J. Surf. Sci. Nanotech, 4 (2006) 614-618.

[37]. N. E. Kadi, N. Taulier, J. Y. Le Huerou, M. Gindre, W. Urbach, I. Nwigwe, P. C. Kahn, M. Waks, Unfolding and Refolding of Bovine Serum Albumin at Acid pH: Ultrasound and Structural Studies, Biophys. J, 91(2006), 3397–3404.

[38]. C.D. Cárdenes, A.J. Carrillo, A. Ariasa, C. Rodríguez, A. Torres, A. Sierra, M.P. Arévalo, Comparative evaluation of four commercial tests for presumptive identification of Candida albicans, J. Microbiol. Methods, 59 (2004) 293– 297.

[39]. M. Ilkit, S. Hilmioglu, M. Tasbakan, S. Aydemir, Evaluation of Albicans ID2 and Biggy agar for the isolation and direct identification of vaginal yeast isolates, J. Med. Microbiol, 56 (2007) 762–765.

ISBN:978-84-692-9761-2/DL:T-203-2010

5.3. Supplementary experimental section

5.3.1. Microbiological test

Microbiological cultures were performed in order to confirm the viability of *Candida albicans* strains. Figure 5-1A shows an image of the typical growth of *Candida albicans* in sabouraud agar. This media is used for cultivation of pathogenic and nonpathogenic fungi. The low pH of approximately 5.6 is favorable for the growth of fungi, especially dermatophytes, and slightly inhibitory to contaminating bacteria in clinical specimens (Chung, K.J. and Bennett, J.E. 1992). The typical grow of *Candida albicans* is characterized for white colonies, circular and soft. Only 24 h at 30 ºC were required to observe the first typical colonies of the yeast over the media. It showed the viability of the lyophilized reference strain. Moreover, in order to preserve the culture pure, the colonies were plated on oxytetracycline gentamicine yeast agar (OGY). The oxytetracycline is used as the selective agent suppressing the bacterial growth.

Additionally to the culture plate, a sample was analyzed using SEM. It can be seen in figure 5-1B typical budding yeast (blastoconidia), ovals, with a diameter from 2 to 4 µm.

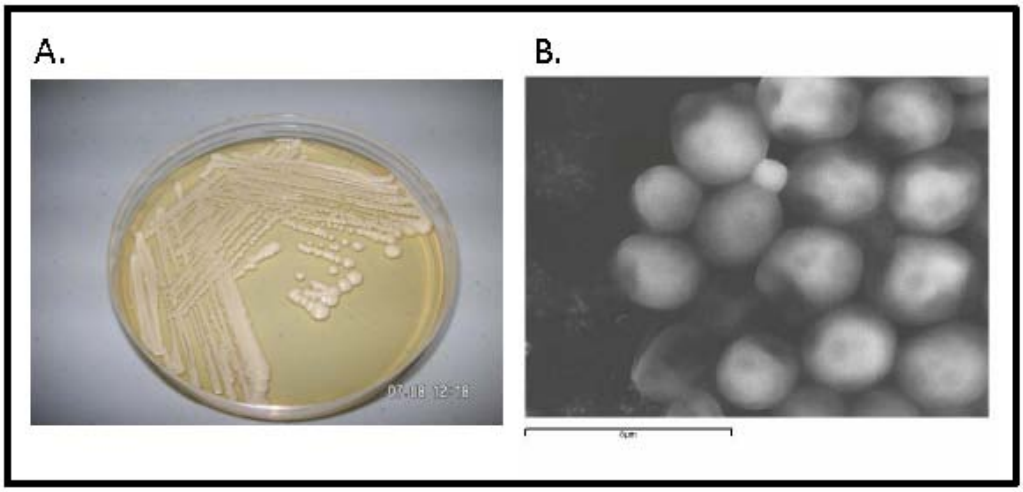

Figure 5-1. A) Typical grow of *Candida albicans* in sabouraud agar. B) SEM image of the yeast.

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5.3.2. Selectivity test

To improve the selectivity of the CNTFET devices, a solution of Tween 20 was used as a blocking agent to protect the sidewalls of the CNTs, thus avoiding non-specific binding (NSB) of the yeast or interferences from the sample. In this way we assure that the changes in the electrical current of the devices is only due to the antigen-antibody interaction.

First, we check if Tween 20 was really avoiding the NSB of *Candida albicans*. For this purpose, a non-functionalized device, that is, without containing adsorbed antibodies, was immersed in a solution of Tween 20 at 0.5 % for 2 h. After that, the device was exposed to the NaCl solution 0.85 % used to dilute the yeast for 1 hour. Finally, the same device was immersed in a sample of saline solution 0.85 % containing 400 cfu/mL of this yeast for 1 hour at 30 ºC. The device was rinsed thoroughly and electrically characterized after each step. Same procedure was followed with the competing yeast *S. cerevisiae* and *C. albidus.* It can be seen in figures 5-2, 5-3 and 5-4 that no significant changes were appreciable when the devices were exposed to both the 0.85 % NaCl solution and to the 0.85 % saline solution containing the yeasts. The slight changes are due to instrumental variability. Thus, we confirmed that Tween 20 at 0.5 % was an effective blocking agent against the saline solution (blank sample) and both, the target and the competing yeasts.

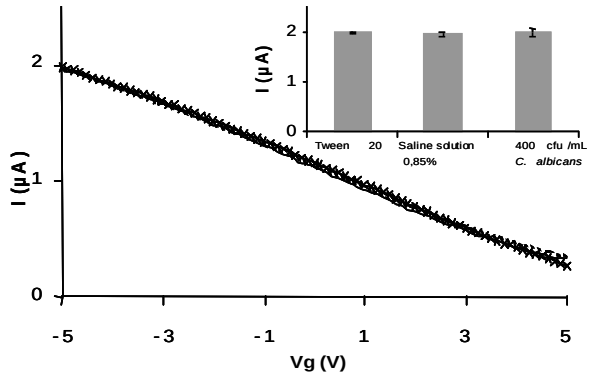

Figure 5-2. Gate voltage dependence of the source-drain current of a non-functionalized CNTFET before (—) and after being exposed to the saline solution (----) and the saline

solution containing 400 cfu/mL of *C. albicans* (—X—). The inset shows the behaviour of the source-drain current after each functionalisation step at Vg= -5 V.

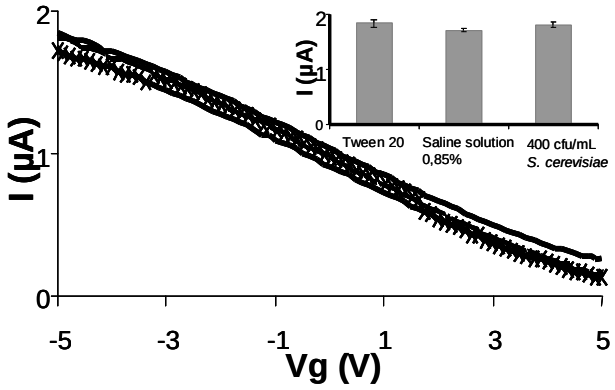

Figure 5-3. Gate voltage dependence of the source-drain current of a non-functionalized CNTFET before (—) and after being exposed to the saline solution (----) and to the saline solution containing 400 cfu/mL of *S. cerevisiae* (—X—). The inset shows the behaviour of the source-drain current after each functionalisation step at Vg= -5 V.

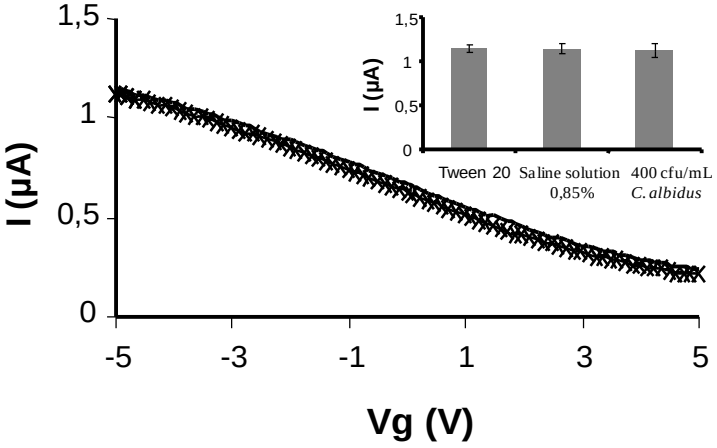

Figure 5-4. Gate voltage dependence of the source-drain current of a non-functionalized CNTFET before (—) and after being exposed to the saline solution (----) and to the saline solution containing 400 cfu/mL of C. *albidus* (—X—). The inset shows the behaviour of the source-drain current after each functionalisation step at Vg= -5 V.

ISBN:978-84-692-9761-2/DL:T-203-2010

5.4. Complementary Conclusions

This study shows another application of the CNTFETs in the biosensing of higher, more complexed organism like yeast. An effective blocking agent (Tween 20) was used to avoid non-specific binding, thus the change in the electrical current was due only to antigen-antibody interaction.

We proved that our sensor devices could be used to detect *C. albicans* selectively in samples containing albumin concentrations below 0.1 g/L. However, the concentration of albumin in a normal serum sample is about 35 to 55 g/L. Therefore, it is necessary to dilute the sample (as is also required in conventional clinical assays) and use another type of blocking agent that can protect the sidewalls more effectively against proteins present in real samples. An option that could have been explored is the use of PBS containing Tween 20 at 0,05 % and gelatin 0,8 % reported by Sanchez -Acevedo *et al*, (Sanchez Acevedo, Z.C. et al, 2009) as an effective blocking agent for small molecules.

We developed an improved biosensor that represents an alternative method than can be used to detect yeast with medical importance in short time. Nevertheless until today, molecular biology based methods like PCR in real time is the most sensitive assay to detect this pathogenic yeast. Our devices must be proven with real sample and must be validated with traditional methods, thus establishing the possibility of using them on the market. Moreover, our devices allow us to make a qualitative detection. This is mainly due to the CNTFETs present different sensitivities because the synthesis process of the CNT is irreproducible generating networks with diverses densities and electrical behaviours.

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5.5. References

Chen, H., Heng, C.K., Puiu, P.D., Zhou, X.D., Lee, A.C., Lim, T.M., Tan, S.N. Analytica Chimica Acta. **2005.** 554, 52–59.

Chunchanur, S.K., Nadgir, S.D., Halesh, L.H., Patil, B.S., Kausar, Y., Chandrasekhar, M.R. Indian Journal of Pathology and Microbiology. **2009.** 52, 501-504.

K.J. Chung, J. E. Bennett, Medical Mycology, Lea & Febiger, Philadelphia, **1992**

Cooke, V.M., Miles, R.J., Price, R.G., Midgley, G., Khamri, W., Richardson, A.C. Applied and Environmental Microbiology. **2002**. 68, 3622-3627.

Horvath, L.L., George, B.J., Murray, C.K., Harrison, L.S., Hospenthal, D.R. Journal of Clinical of Microbiology. **2005**. 42, 115-118.

Maaroufi, Y., Heymans, C., De Bruyne, J.M., Duchateau, V., Rodriguez-Villalobos, H., Aoun, M., Crokaert, F. Journal of Clinical Microbiology. **2003.** 41, 3293–3298.

Marcilla, A., Monteagudo, C., Mormeneo, S., Sentandreu, R. Microbiology. **1999**. 145, 695–701.

Matsui, H., Hanaki, H., Takahashi, K., Yokoyama, A., Nakae, T., Sunakawa, K., Omura, S. Clinical and Vaccine Immunology. **2009**. 16, 1366–1368.

McMullan, R., Metwally, L., Coyle, P.V., Hedderwick, S., McCloskey, B., O'Neill, H.J., Patterson, C.C., Thompson, G., Webb, C.H., Hay, R.J. Clinical Infectious Diseases. **2008**. 46, 890-896.

Moudni, B.L., Rodier, M.H., Daniault,G., Jacquemin, J.L. Clinical Diagnostic of Laboratory Immunology. **1998**. 5: 823–825.

ISBN:978-84-692-9761-2/DL:T-203-2010

Mulero, R., Lee, D.H., Kutzler, M.A., Jacobson, J.M., Kim, M.J. Sensors. **2009.** 9, 1590-1598.

Munin, E., Giroldo, L.M., Alves, L.P., Silva, M. Journal of Photochemistry and Photobiology B: Biology. **2007**. 88, 16–20.

Pontón, J. and del Palacio, A. Revista Iberoamericana de Micología. **2007.** 24, 181-186.

Sánchez-Acevedo, Z.C., Riu, J., Rius, F. X. Biosensors and Bioelectronics. **2009**. 24, 2842–2846.

Trama, J.P., Mordechai, E., Adelson, M.E. Molecular and Cellular Probes. **2005**. 19, 145–152.

Wellinghausen, N., Siegel, D., Winter, J., Geber, S. Journal of Medical Microbiology. **2009.** 58, 1106-1111.

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Chapter 6

Improvement of CNT based FETs to detect microorganisms

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6.1. Introduction

There are a number of parameters used to characterize the performance of biosensors. Among the most relevant are the specificity, sensitivity, stability, limit of detection, time response, life time or recovery time. In the development of an immunosensor the specificity and sensitivity depend greatly on the kind of antibody and its immobilization method (Corry, B. et al, 2003). The denaturation or the conformational changes an antibody can undergo during or after its immobilization is one of the main reasons in the reduction of the immunosensor sensitivity. These types of changes are usually associated with the non-specific adsorption of antibodies on solid supports (Jung, Y. et al, 2008).

A judicious approach to improve the sensitivity of an immunosensor would therefore be to increase the density of the binding sites on the sensor surface. This density mainly depends on the adequate orientation of the bioreceptor (Van Regenmortel, M.H.V. et al, 2002). Several research groups have tried to control the orientation of antibodies. One of the most frequent techniques is the use of bacterial proteins which can bind to the Fc constant fraction of antibodies (Bae, Y.M. et al, 2005.; Kanno, S. et al, 2000). As a result, the antigen binding sites of the immunoglobulins maximize the access to the target.

Additionally to the binding specificity of the bacterial protein for the antibodies, the most important property is their ability to remove the active antibody layer and replace it with a different one under relatively similar conditions. As a result, a surface modified with bacterial proteins can act as a generic platform to build immunosensors, where the selectivity can change by using different antibodies as receptors. Moreover, the sensor surface can also be regenerated by desorbing the antibody from the binding protein, process easier to achieve than trying to break the Ag/Ab interaction. Therefore, the use of bacterial proteins as linkers generate more reproducible surfaces where the antibody can be replaced each time without the use of aggressive conditions thus preserving the surface or biomolecule configuration (Van Regenmortel, M.H.V. et al, 2002).

Nevertheless, the use of linkers between the transducer (CNTs) and the analyte affect the electrical behaviour of the CNTFETs devices. For this reason, an optimal concentration of the protein should be used in order to reduce side effects (electron donating or scattering effects) from the protein to the CNTs, thus preserving the electrical characteristics of the devices for the antigen-antibody interaction.

In this chapter we describe the morphology and electrical characteristics of protein layers non-covalently adsorbed onto an irregular surface of a network of carbon nanotubes (CNT). The specific adsorption of antibodies through the recombinant protein G, previously adsorbed on the CNTs surface, provides enhanced properties of the device, such as a high degree of IgG molecular orientation and adjustable molecular density of the IgG monolayer. Spectroscopy ellipsometry is used to investigate aspects of the structures of the protein layers such as their thickness. Together with atomic force microscopy, these two complementary characterization techniques provide the overall morphology of the functionalized layer system. Furthermore, electrical characterization is performed in order to determine the adsorption dynamics of the proteins.

Finally, this chapter provides supplementary experimental information to that included in the published paper, general conclusions and references.

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6.2. Article:

"Morphological and electrical characteristics of biofunctionalized layers on carbon nanotubes". Biosensors and Bioelectronics, 2009.25, 161-166

Raquel A. Villamizar,[1τ] Julia Braun,[2] Bruno Gompf,*[2] Martin Dressel,[2] and F. Xavier Rius[1]

[1] Department of Analytical and Organic Chemistry. Universitat Rovira i Virgili. Marcel.lí. Domingo, s/n. 43007. Tarragona, Spain.

[2] Physikalisches Institut, Universität Stuttgart, Pfaffenwaldring 57, 70550 Stuttgart, Germany.

[τ] On leave from the Department of Microbiology. University of Pamplona. Km 1, Vía Bucaramanga, N. de S. Colombia.

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ABSTRACT

In this study we have investigated the morphology and electrical characteristics of protein layers non-covalently adsorbed onto an irregular network of carbon nanotubes (CNT). The layer-system presents a prototype for an ion-sensitive field-effect transistor based on CNT-networks. The complementary characterization techniques AFM and ellipsometry give the overall morphology of the functionalized layer system and in combination with concentration dependent measurements a detailed image of the adsorption dynamics. The advantage of CNT-based FETs is their huge surface area, which make them extremely sensitive even to weak adsorption processes. The here presented comparative investigations cleary show that significant changes in the transport properties of the CNTs occur much below one monolayer. This sensitivity is an important condition for the future development of efficient biodevices with optimal performance parameters for the detection of pathogenic microorganisms.

KEYWORDS: Biosensor, Carbon nanotubes, AFM, spectroscopy ellipsometry, proteins.

INTRODUCTION

Biosensors based on carbon nanotubes functionalized with antibodies have been widely studied (Allen et al., 2007; Kim et al., 2008; Sanchez et al., 2008; Cid et al., 2008; Villamizar et al., 2009). However, a critical point for the development of this kind of sensors is the stable immobilization of the biomolecules to the CNT surface (Cosnier 1999; Corry et al., 2003; Bae et al., 2005; Chung et al., 2006). The immobilization process should on the one hand not influence the biological activity of the proteins, on the other hand it should preserve the electrical properties of the CNT. Developing immobilization processes, which fullfill both conditions is an important step towards efficient biodevices for advanced analytical, environmental or medical applications. Carbon nanotubes (CNTs) are one-dimesional nanostructures with a large pure carbon surface, allowing optimal interaction with biomolecules in their natural environment (Heller et al., 2008). They can be

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functionalized in two ways, leading to covalent or non-covalent binding sides (Agüí et al., 2008). The first one, provide higher stability. However, the activity of the immobilized biomolecules may be affected due to steric hindrance by covalent binding (Wang and Lin., 2008). In addition, the electrical properties of the CNTs can be changed (Vaseashta et al., 2005). The second one, involve the formation of supramolecular interactions between the molecular receptor and the transducer. The advantage of the non-covalent immobilization is that it preserves the sp^2-hybridization of the C-atoms and therefore the electronic properties of the CNTs. The disadvantage of this method is that it can lead to ramdonly immobilized biomolecules producing loss of the activity and therefore, less-efficient biodevices. Many approaches to find out an adequate immobilization of antibodies on different surfaces have been made. The use of bacterial proteins like protein A and protein G seems to be a good way to obtain well orientated antibodies.

These proteins have affinity for the constant fraction “Fc” of immunoglubulins assuring full antigen-binding capacity. Therefore, they can be used as cross-linking elements between the CNT and the immunoglobulins. Chen *et al* (Chen et al., 20003) have proven that protein A is absorbed onto the CNTs through hydrophobic interaction after one hour of incubation, while Byon *et al* (Byon et al., 2006) have shown that physics absorption of protein A onto CNTs provide an anchor point to the IgGs, producing changes in the electrical current of the CNTs. However, a disadvantage of protein A is its present affinity to a narrow spectrum of IgGs and that it also can bind to the Fab fragment of the human IgM (Aybay 2003) which reduces the specificity.

In this study, we report the characteristics of the immobilization process of immunoglubulin G against *Aspergillus* spp*.* The antibody has been adsorbed on protein G which had been previously adsorbed on top of a single wall carbon nanotube (SWCNTs) network. Protein G has at least 2 molecule binding sites for IgG and the equilibrium constant of the reactions between protein G and rabbit polyclonal IgG is in a range of $1x10^{10}$ and $7x10^{10}$ M^{-1} (Åkrestöm et al.,1986). In addition, the protein G used in our assays is a recombinant protein, produced by *Streptococcus* spp., genetically truncated, which retains its affinity only for the

constant fraction “Fc“ of the IgG and lacks Fab-binding sites, assuring a well oritentation process of the antibodies.

The adsorption process of the proteins preserves the electrical properties of the CNTs (Byon et al., 2006). In addition, the CNTs acts as transducer elements within a field effect transistor (FETs) configuration. This enables the development of selective biosensors based on the principles of molecular recognition. It is shown that only a small amount of proteins attached to the target give rise to measureable electrical signals in the carbon-nanotube FETs. Therefore, this paper is to our knowledge, the first report about morphological and electrical characteristics of protein-modified carbon nanotubes grown by chemical vapour deposition on SiO_2 surfaces.

The morphology of the layer system was studied by Atomic Force Microscopy (AFM) and spectroscopic ellipsometry (SE), two complementary techniques which together give detailed information on the layered structure (i.e. the thickness and roughness of the individual layers as well as their optical properties). Whereas AFM is a well established technique to investigate protein interaction with different kinds of surfaces (Kuhlmeier et al., 2003; Ouerghi et al., 2002; Lin et al., 1990), only recently it was shown that SE is also a powerfull tool to investigate protein modiefied surfaces (Bae et al., 2005; Yu et al., 2005; Kozma et al., 2008).

However, these studies have been performed on flat surfaces like gold or glass. In this study we demonstrate that SE is also a usefull technique to characterize immobilized proteins on an irregular CNT network. Additionally the effect of protein adsorption on CNTs was studied by electrical measurements.

MATERIALS AND METHODS

Antibodies and biochemicals

Anti-*Aspergillus* (i.e. a polyclonal rabbit anti-*Aspergillus* (1 mg/mL) was purchased from Oxford Biotechnology Ltd (Oxford, U.K.). Protein G from *Streptococcus* spp.

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recombinant, expressed in *Escherichia coli* (1 mg/mL), PBS containing Tween 20 pH 7.4 and gelatine from cold water fish skin were obtained from Sigma-Aldrich.

Synthesis of the SWNTs and development of the CNT-FETs

The CNT network were synthesized by a CVD process previously described (Villamizar et al., 2008) on top of a 500 nm thick SiO_2 layer thermally grown on highly n-doped silicon wafers (total area 0.5 cm x 0.5 cm). Briefly, in the process a solution of 100 ppm iron nitrate in isopropanol is used as catalyst. The CVD process itself is performed at 900 ºC for 20 minutes with 600 sccm of methane and 200 sccm of hydrogen. After preparation source and drain electrodes are screen-printed with silver ink, and as gate electrode an aluminium layer on the back side of wafer is used. In the so obtained field effect transistors (FETs) the CNTs act as the transducer elements. The gap between source and drain was about 0.5 mm and the size of the electrodes was 200 µm x 200 µm.

Functionalization process

The CNT-FET structures obtained in this way were immersed into a solution of protein G at 5 µL/mL for 1 hour at 37 ºC and after that for 3 hours in a solution of PBS containing Tween 20 at 0.05 % and gelatin 0.8 % (PBSTG). Protein G is a monomeric globular biomolecule, that consists of four stranded β-sheet traversed by α-helix containing nearly 600 amino acid residues (Aybay 2003). It adsorbes on the CNTs in a non-specific way via supramolecular interactions, mainly hydrophobic interactions between the aromatic amino acids (i.e., histidine and tryptophan) present in the protein and the CNTs. On the other hand, PBSTG solution is absorbed onto the gaps of the CNTs not covered by protein G to avoid the non-specific absorption of the IgG onto the transducer. It has been proven that Tween 20 is an ideal coating material for hydrophobic surfaces. It contains a long alkyl chain of twelve carbons, which can efficiently interact with other hydrophobic surfaces (in our case CNTs) through twenty ethylene glycol groups and three terminal hydroxy (-OH) groups (Chi et al., 2007). Finally we exposed the samples to 10 µL/mL solution of anti-*Aspergillus* antibody for one additional hour at 37 ºC. After each functionalization step, the samples were thoroughly rinsed with PBS solution,

distilled water and dried with nitrogen. Figure 1 shows a scheme of the functionalization process.

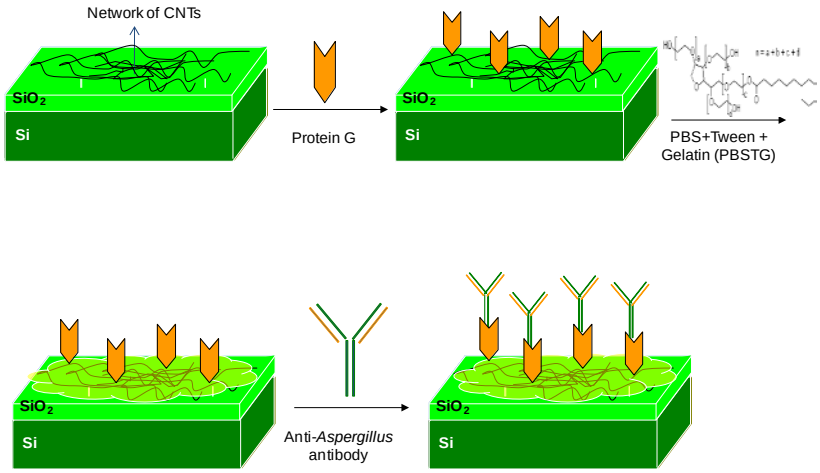

Figure 1. Schematic representation of the non-covalent functionalization process of the CNTs with protein G and IgG. The PBSTG solution avoids the NSB of the proteins and protein G enables the antibodies to be appropriately oriented.

Spectroscopic Ellipsometry

All ellipsometric measurements were carried out at room temperature with a Woollam VASE spectroscopic ellipsometer in the wavelength range between 270 to 1700 nm (0.73–4.59 eV) and at three different angles of incidence: 55º, 65º and 75º. To account for inhomogeneities in the films the ellipsometric angles Ψ and Δ were measured on each sample at three different spots. Data analysis was performed by the commercial software (WVASE32 from J.A. Woollam Co. Lincoln). Prior to the immobilization process the pure wafer and the wafer with the CNT network on top were characterized. Subsequently the ellipsometric angles were recorded after each immobilization step shown in Fig.1

Microscopic characterization

Tapping mode Atomic force microscopy (Pico Plus) was used for the topographic characterization of the CNT network before and after the functionalization process.

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The root mean square roughness (RMS) determined by the software software WSxM 4.0, Develop 13 (Horcas et al., 2007) was utilized for the characterisation of surface irregularities and height distributions.

Electrical characterization

Electrical measurements were carried out using a 4157A Agilent semiconductor parameter analyzer and a Wentworth Laboratories MP1008 probe station. The device characteristics, i.e. the dependence of the source–drain current in dependence of the gate voltage Vg was measured in the range +5 V to −5 V for different steps of the functionalized transistor. The bias voltage, *V*sd was fixed at 250 mV (Villamizar et al., 2009).

RESULTS AND DISCUSSION

Spectroscopy Ellipsometry

Prior to the functionalization process the bare Si/SiO_2 substrates were measured and the obtained parameters were then kept constant in the further fit procedures. The optical properties of single wall carbon nanotubes depend not only on the ratio between conducting and semiconducting CNTs, but are also influenced by the density of the network (Fanchini et al., 2006). It turned out that the here used CNT network with a relative low density appears transparent in the investigated frequency range. Therefore it could be modelled by a Cauchy layer with the two independent fit parameters thickness *d* and refractive index *n*. For protein layers it is known that they are transparent in the visible. Modelling them as Cauchy layers we obtained refractive indices for protein G and IgG in good agreement to literature values (Tronin et al., 1995). One representative stack is shown in Table 1. Layer 0 and 1 represents the Si/SiO_2 substrate. The optical constants for these two materials were taken from literature (Herzinger et al., 1998). Layer 2 represents the CNT network grown on SiO_2. For this layer we obtainded a refractive index of 1.45 and an effective thickness of 18 nm. Layer 3 is the Cauchy layer representing the protein G with a refractive index of 1.3 and an effective thickness of 2.1 nm. The next functionalizing step, the PBSTG treatment, did not lead to any measurable changes in the ellipsometric angles. It seems to absorb only into the voids of the

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CNT network where it is invisible for ellipsometry due to the nearly identical optical properties. Therefore this step was not considered as an additional layer. Finally, the IgG linked through the Fc region to the protein G was modelled with the top Cauchy layer with the two parameters refractive index 1.3 and effective thickness 5.0 nm. In Figure 2 the changes in the measured ellipsometric angles Ψ and Δ cased by the different functionalization steps are shown.

4. Cauchy (IgG)	5.0 nm
3. Cauchy (Protein G)	2.1 nm
2. Cauchy (As-grown CNTs)	18.0 nm
1. SiO_2	490.0 nm
0. Si	0.50 mm

Table 1. Stack structure used to model the thicknesses of the protein G layer onto the as-grown carbon nanotubes and the thicknesses of IgG on the protein G layer. With this model the measured ellipsometric data could be described with MSE values below 4.

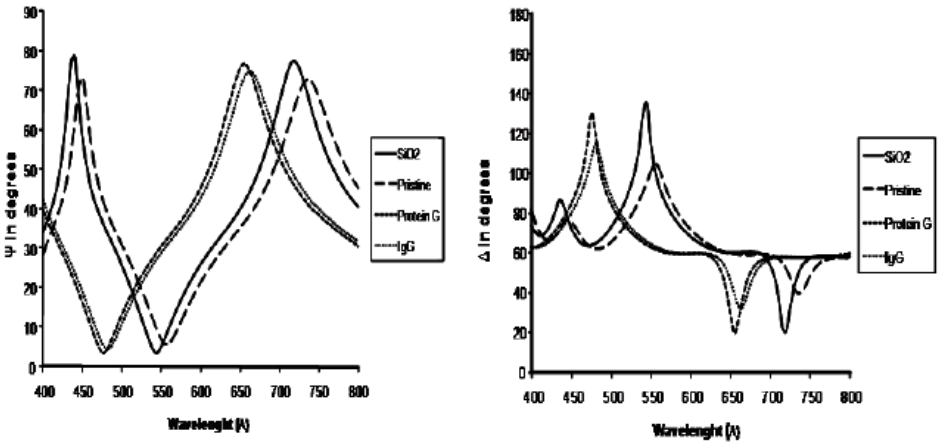

Figure 2: Changes in the measured ellipsometric angles Ψ and Δ caused by the different functionalization steps.

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After the development of a reliable model the thickness of the IgG layers as a function of the IgG concentration was studied. Immunoglobulin G has a height of about 12 nm and a width of 4 nm. Due to its non-spherical structure the thickness of a monolayer should depend on the orientation of the biomolecule on the substrate (Tronin et al., 1995) and the minimum value should be 4 nm (Bae et al., 2005). For our investigations a previously functionalised sample with protein G and coated with a solution of PBSTG was exposed to ascending concentrations of IgG (i.e. 0.02 µg/mL, 0.2 µg/mL, 2 µg/mL and 20 µg/mL). Each sample was incubated for overnight to the solution at 37 ºC, rinsed thoroughly with distilled water, dried with nitrogen and then characterized by ellipsometry.

Figure 3 shows the thickness as a function of the concentration of IgG. As expected the thickness increases rapidly up to about a concentration of 5 µg/mL and then tends to saturate. The maximum thickness obtained in this series of experiments is slightly lower than the one shown in the model in Table 1. This difference is most probably due to dehydration effects which can lead to structural changes in the proteins. We attributed the thickness change to successive steps of washing and drying during the exposition to different protein concentrations which could produce conformational changes in the protein and thereby to a reduced thickness. Additional biochemical tests are required to obtain information about the adhesive stability of the protein on the surface. From the saturation behaviour of the concentration dependence it can be concluded that a IgG concentration between 8 µg/mL and 12 µg/mL forms almost one monolayer of IgG on a previously attached protein G layer on a CNT network.

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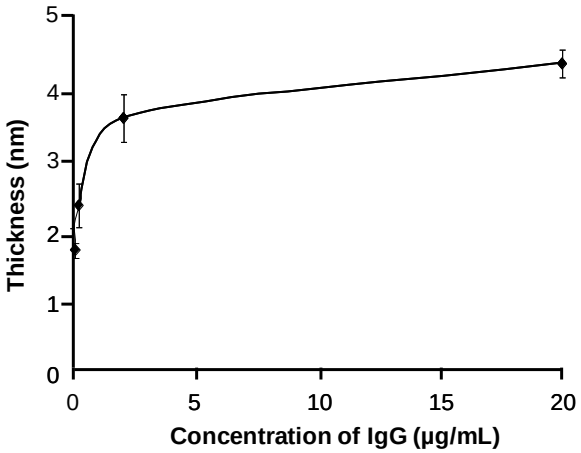

Figure 3. Change in the thickness as a function of the IgG concentration applied to the protein G layer simulated by SE.

AFM analysis

The AFM characterization was performed on dry samples which in principle can lead to some structural changes. All AFM images were taken on an area of 5 µm by 5 µm and for all images the RMS roughness was calculated. For the RMS roughness of the non-functionalised CNT network we obtained 2.7 nm, which is much smaller than the effective thickness obtained by ellipsometry. The protein G layer on top of the CNT network with an RMS roughness of 2.2 nm flattens the surface a little bit. A clear hint, that it is a dense packed layer. As expected from the ellipsometric results, the PBSTG solution has nearly no influence on the surface roughness. Finally, the dependence of the amount of absorbed IgG on the surface roughness was studied. The IgG protein structure has three domains, two Fab and one Fc domain and a hinge, the region between the Fab and Fc, which in principle can lead to lateral and rotational movements of the two antigen binding domains (Tronin et al., 1995).

The different configuration of the antibody could in principle cause different height values; however, the high affinity of the protein G-IgG complex should force the biomolecules all in one orientation. The measured RMS roughness with increasing

antibody concentration (0.02, 0.2, 2 and 20 µg/mL) was 2.5, 2.7, 2.8 and 2.8 nm, respectively and therefore gives clear evidence for this assumption.

At higher concentrations of the antibody (i.e. 20 µg/mL) the surface roughness became constant in accordance with the observed saturation behaviour of the film thickness measured by ellipsometry and in agreement to similar results reported by Bae *et al.* Additionally, we proved that one hour was enough to immobilize the protein since it remains strongly adsorbed on the CNTs after the successive washing and drying process.

In Fig 4 AFM images recorded after each funtionalization step are shown. It can be clearly appreciated that the height of the nanotube increases after the immobilization of protein G on the carbon nanotubes (step B) and after the further immobilization of IgG onto the protein G (step C). The presence of PBSTG does not affect the height of the nanotubes, therefore, it has not been considered in the figure.

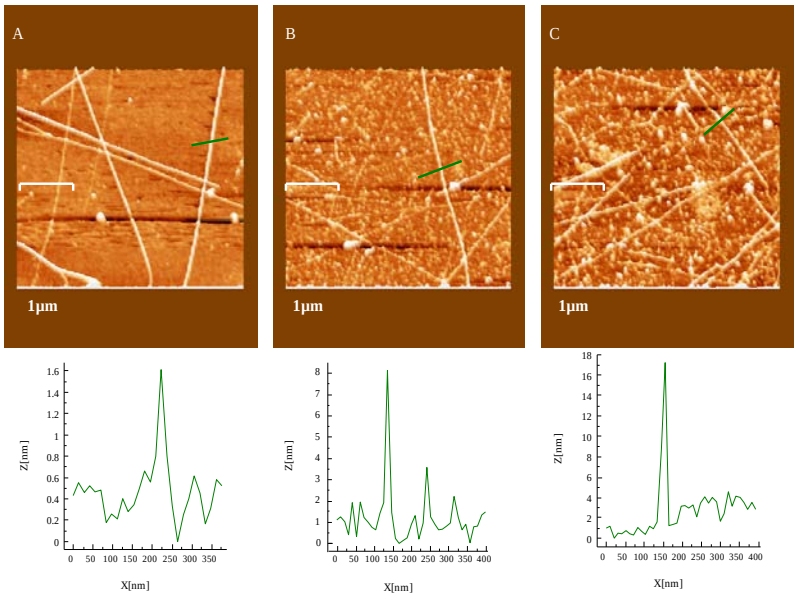

Figure 4. AFM images (above) and height profile (below) recorded after each funtionalization step. A) As-grown CNT network. B) CNT network after the immobilization of protein G onto the as-grown nanotubes. C) CNT network after the immobilization of IgG onto the previously immobilized protein G layer.

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Electrical measurements

For the electrical characterization the dependence of the source-drain current, Isd, on the gate voltage, *V*g, in the range +/-5 V was measured at room temperature under dry conditions. Figure 5A shows how each functionalisation step affects the electrical current of a typical CNTFET. The absorbed proteins cause a change in the electrical properties of the device. It has been proven that when a CNT interact with an analyte two possible mechanisms can take place. The first one is a charge transfer process in which the analyte provide electrons to the CNTs. In the second one, the analyte acts as an additional scatterer at the CNT surface. By using electrical measurements it is possible to distinguish between both effects. In the case of charge transfer, the threshold voltage becomes more positive (electron acceptor) or more negative (electron donor). By contrast, if an additional scattering mechanism occurs the overall conductance drops (Allen et al, 2007). Both mechanisms are present in our stack. Figure 5A shows that after protein G adsorption a general decrease in the conductance is observed (scattering), whereas the binding of IgG clearly leads also to a shift of the threshold (charge transfer).

This doping process is presumable due to amino acids with base-containing residues (i.e. arginine, histidine and lysine) present in the protein that provide electrons to the CNTs (Bradley 2004). The PBSTG process, also barely visible in the AFM and ellipsometry data, causes additional scattering and thereby a slight decrease in the electrical current of the devices as previously observed by So et al. Recently it was shown that with CNT-based FETs it is possible to detect specific protein-protein interactions between the complex protein A-IgG at pM concentrations (Byon et al., 2006). Control experiments for the different functionalization steps were performed in order to check if the cleaning process could affect the electrical current. The absence of any significant change in the device characteristics after the cleaning step proved that the change was only due to the absorbed proteins and the components of the PBSTG solution onto the carbon nanotubes.

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To test the performance of our device we finally studied the effect of increasing concentrations of IgG on the I-V characteristics. Therefore a protein G modified-CNTFET was immersed in a solution containing 0.02 µg/mL of IgG at 37 ºC for 15 minutes. The CNTFET was thoroughly rinsed with water, dried with nitrogen, and then electrically characterised. This process was repeated for increasing concentration on the same device. Figure 5B shows that even the lowest concentration of 0.2 µg/mL is enough to produce a change in the electrical current of the device (about 32 %) in only 15 minutes of incubation. CNTs are extremely sensitive to surface modification due the the fact that they consist only of surface; there are no bulk C-atoms. Figure 5B also clearly shows that saturation in the electrical behaviour sets in at around 4 µg/mL, prior to the formation of a monolayer.

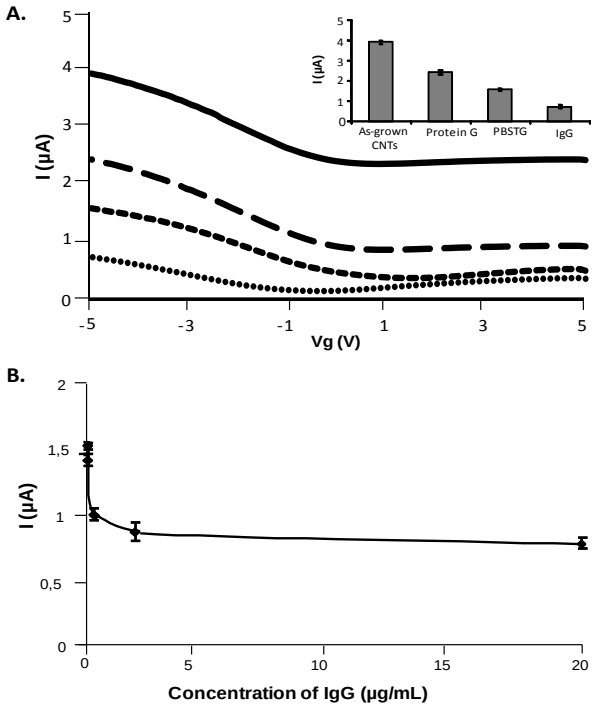

Figure 5. A). Source-drain current vs. gate voltage of a typical CNTFET after each functionalization step. As-grown CNTs (—); protein G adsorbed on the CNTs (— —); CNTs coated with protein G and PBSTG solution (- - - -); and anti-*Aspergillus* antibodies linked to the protein G previously absorbed on the CNTs (……).The inset shows the behaviour of the

source-drain current after each functionalization step at V_g= −5 V. B). Source-drain current (at Vg= -5 V) obtained for a protein G-coated CNTFET after being exposed to ascending concentrations of IgG.

CONCLUSIONS

In summary, we have presented the first comprehensive study on CNT-based FET biosensors where topography and transport properties were linked to a consistent image of the detection mechanism. The different complementary characterization techniques AFM and ellipsometry provide the overall morphology of the layer system. In combination with concentration dependent measurements a detailed image of the adsorption dynamics can be achieved. Functionalising the CNT network with 5 µg/mL of protein G enables the building of almost one monolayer IgG at concentrations above about 8 µg/mL. Once the two available binding sites of the protein G layer are filled no more IgG can be attached to the system. The thickness as well as the RMS roughness saturates. Compared to conventional ChemFETs, CNT-based FETs have the advantage of a much larger surface. Therefore not only charge transfer processes, which will be weak in the case of non-covalent bonding sites, can contribute to the detection mechanism, but also surface scattering. How sensitive this detection mechanism can be, is demonstrated in our study.

The comparative investigations clearly show that significant changes in the transport properties of the CNTs occur much below one monolayer. At least an incubation time of 12 hours are required to obtain a film that can be measured by spectroscopic ellipsometry, while 15 minutes are enough to detect a signal in the I-V characteristic of the CNT-based FET. The here presented results are the basis for the future development of CNT-based FETs for the detection of micotoxigenic mould *Aspergillus flavus* in real samples.

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REFERENCES

Agüí, L., Yañez, P., Pingarrón, J.M., 2008. Analytica Chimica Acta. 622, 11–47.

Akerstroms, B., Bjorck, L., 1986. Journal of Biological Chemistry. 261 (22), 10240-10247.

Allen, B. L., Kichambare, P. D., Star, A., 2007. Advanced Materials. 19, 1439–1451.

Aybay, C., 2003. Immunology Letters. 85, 231-235.

Bae, Y.M., Oh, B.K., Lee, W., Lee, W.H., Choi, J.W., 2005. Biosensors and Bioelectronics. 21,103-110.

Bradley, K., Briman, M., Star, A., Gruner, G., 2004. Nano Letters. 4(2), 253-256.

Byon, H. R., Choi, H.C., 2006. Journal of American Chemical Society. 128, 2188-2189.

Chen, R.J., Bangsaruntip, S., Drouvalakis, K.A., Shi, N.W., Shim, M., Li, Y., Kim, W., Utz, P.J., Dai, H., 2003. Proceedings of the Natural Academy of Sciences. 100 (9) 4984-4989.

Chi, Y.S., Byon, H.R., Choi, H.C., Choi, I. S., 2007. ChemBioChem. 8, 1380 – 1387.

Chung, J.W., Park, J.M., Bernhardt, R., Pyun, J.C., 2006. Journal of Biotechnology. 126, 325–333.

Cid, C., Riu, J., Maroto, A., Rius, F. X., 2008. Analyst. 133, 1005 – 1008.

Corry, B., Uilk, J., Crawley, C., 2003. Analytica Chimica Acta. 496, 103–116.

ISBN:978-84-692-9761-2/DL:T-203-2010

Cosnier, S., 1999. Biosensors and Bioelectronics. 14, 443–456.

Fanchini, G., Unalan, H.E., Chhowalla, M., 2006. Applied Physics Letters. 88, 19191.

Gruner, G., 2006. Analytical Bioanalytical Chemistry. 384, 322–335.

Heller, I., Janssens, A.M., Männik, J., Minot, E.D., Lemay, S.G., Dekker, C., 2008. Nano Letters. 8, 591–595.

Herzinger, C.M., Johs, B., McGahan, W.A., Woollam, J.A., Paulson, W., 1998. Journal of Applied Physics. 83, 3323.

Horcas, I., Fernández, R., Gómez, J. M., Colchero, J., 2007. Review of Scientific Instruments. 78**,** 013705.

Kim, J. P., Lee, B. Y., Hong, S., Sim, S.J., 2008. Analytical Biochemistry. 381, 193–198.

Kozma, P., Nagy, N., Kurunczi, S., Petrik, P., Hámori, A., Muskotál, A., Vonderviszt, F., Fried, M., Bársony, I., 2008. Physica Status Solidi (c). 5(5), 1427–1430.

Kuhlmeier, D., Rodda, E., Kolarik, L.O., Furlong, D.N., Bilitewski, U., 2003. Biosensors and Bioelectronics. 18, 925-936.

Lin, J.N., Drake, B., Lea, A.S., Hansma, P.K., Andrade, J. D., 1990. Langmuir. 6, 509-511.

Martensson, J., Arwin, H., 1995. Langmuir. 11, 963-968.

Ouerghi, O., Touhami, A., Othmane, A., Ouada, H., Martelet, B., Fretigny, C., Renault, N.R., 2002. Biomolecular Engineering. 19, 183-188.

Sánchez, S., Roldán, M., Pérez, S., Fàbregas, E., 2008. Anaytical Chemistry. 80, 6508–6514.

Sánchez, Z.C., Riu,J., Rius, F. X., 2009. Biosensors and Bioelectronics. 24, 2842-2846.

Star, A., Gabriel, J.C., Bradley, K., Gruner, G., 2003. Nano Letters. 3, 459–463.

Tronin, A., Dubrovsky, T and Nicolini, C., 1995. Langmuir. 11 (2)*, 385-389.*

Vaseashta, A., Malinowska, D.D., 2005. Science and Technology of Advanced Materials. 6, 312-318.

Villamizar, R.A., Maroto, A., Rius, F. X. I. Inza., M.J. Figueras., 2008. Biosensors and Bioelectronics. 24, 279-283.

Villamizar, R.A., Maroto, A., Rius, F.X., 2009. Sensors and Actuators B: Chemical. 136 (2), 451-457.

Wang, J., Lin, Y., 2008. Trends Analytical Chemistry. 27(7), 619–626.

Woollam, J.A., Johs, B., Herzinger, C.M., Hilfiker, J., Synowicki, R., Bungay, C.L., 1999. Critical Reviews of Optical Science and Technology. Vol. CR72, 3-28.

Yu, Y., Jin, G., 2005. Journal of Colloid and Interface Science. 283, 477–481.

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6.3. Supplementary experimental section

6.3.1. Effect of the temperature on the adsorption of the protein G

Protein adsorption has been extensively investigated because it is an important aspect for the improvement of many applications including the design of biosensors. The adsorption is the simplest method of antibody immobilization involving hydrophilic, hydrophobic or both types of interactions between the antibody and the substrate (Jung, Y. et al, 2008). Nevertheless, it can be affected by several parameters like pH, temperature or protein concentration among others (Kondo, A. and Fukuda, H. 1998).

In this section we studied the effect of the temperature on the adsorption of protein G onto the CNTs. We took three different CNTFET devices and each of them was immersed in a solution containing 5 µg/mL of protein G. Then, they were incubated at 4 ºC, room temperature and 37 ºC respectively for 90 minutes. Subsequently, the devices were dried with PBS and distilled water and electrically characterized each 30 minutes. Figures 6-1 and show that protein G is adsorbed after 30 minutes of incubation causing a strong change in the electrical current of the device at all temperatures assayed (i.e 38 % at 4 ºC, 35 % at room temperature and 44 % at 37 ºC).

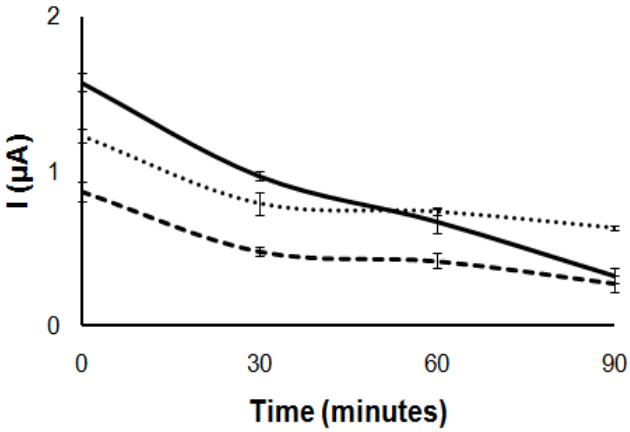

Figure 6-1. Source-drain current (at Vg=-5 V) obtained for three different functionalized devices after being exposed to 5 µg/mL of protein G for 90 minutes at 4 ºC (—), room

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temperature (- - -); and 37 ºC (**- - -**). The source-drain current was measured every 30 minutes. Each electrical current plotted corresponds to the mean value and range of three replicates.

6.3.2. Effect of the temperature on the protein G - anti-*Aspergillus* antibody interaction

The antigen-antibody interaction is a well known process. In this section we studied the effect of the temperature on the interaction between the protein G and the anti-*Aspergillus* antibodies. We took three different protein G-functionalized devices previously coated with PBSTG and exposed them to a solution containing 10 µg/mL of IgG. Then, the devices were incubated at 4 ºC, room temperature and 37 ºC respectively for 30 minutes. After that, they were dried with PBS and distilled water and electrically characterized. Figures 6-2 shows that the binding of the IgG cause a change in the electrical current (i.e. 11 % at 4 ºC, 18 % at room temperature and 29 % at 37 ºC) leading to a shift of the threshold voltage due to a charge transfer process. It can be seen that at low temperature and at room temperature it is required more than 30 minutes to produce a change similar to those obtained at 37 ºC. As a consequence we choose 37 ºC to perform all incubation process including the adsorption of the protein-G.

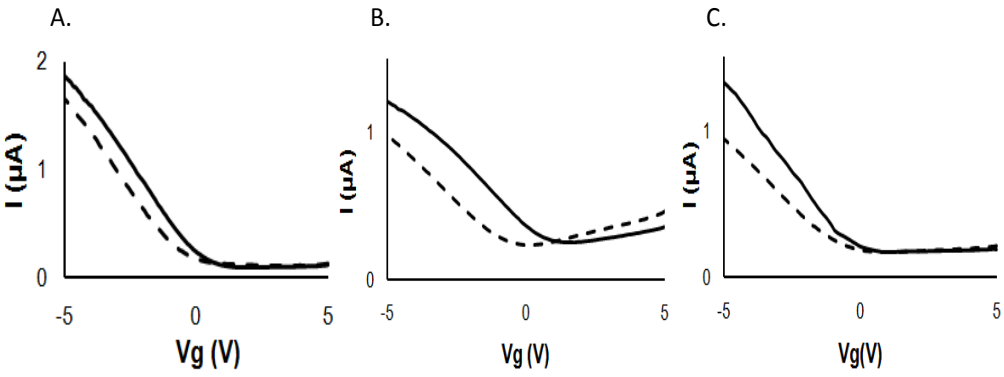

Figure 6-2. Source-drain current vs. gate voltage of three different protein G-modified CNTFETs before (—) and after being exposed to anti-*Aspergillus* antibodies (— —). A) device exposed to the IgG at 4 ºC; B) device exposed to the IgG at room temperature; C) device exposed to the IgG at 37 ºC.

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6.3.3. Non-specific binding test

To improve the selectivity of the CNTFET devices, a solution of Tween 20 at different concentrations was used in order to find out the optimal blocking agent to protect the sidewalls of the CNTs, thus avoiding non-specific binding (NSB) of proteins and other interferences from the sample. In this way we assure that the changes in the electrical current of the devices is only due to the antigen-antibody interaction.

We took three different CNTFETs devices without having adsorbed protein G on the SWCNTs. Each device was immersed into a solution containing Tween 20 at different concentration (i.e. 0.5 %, 1 %, 2 %) during 2 hours at 37 ºC. Subsequently, the CNTFETs were exposed to a solution containing 10 µg/mL of anti-*Aspergillus* antibody for 30 minutes at 37 ºC. The devices were thoroughly rinsed with distilled water, dried with nitrogen and electrically characterized after each step. Figure 6-3 shows that the electrical current changed significantly (i.e. about 66 %, 22 % and 14 %) when devices were protected with Tween 20 at 0.5 %, 1 % and 2 % respectively.

Therefore, we modified a fourth device with a solution of PBS containing Tween 20 at 0.05 % and gelatine at 0.8 % (PBSTG) as effective blocking agent for small molecules (Piao, M.H. et al, 2008). It can be seen in Figure 3 that the PBSTG solution effectively protects the carbon nanotubes from the non-selective binding. The slight change in the electrical current of the fourth device is within the random variability of the electrical current. The error bars correspond to the range of the current values. In this way, we proved that PBSTG was effectively protecting the SWCNTs against the NSB of the antibody and other proteins.

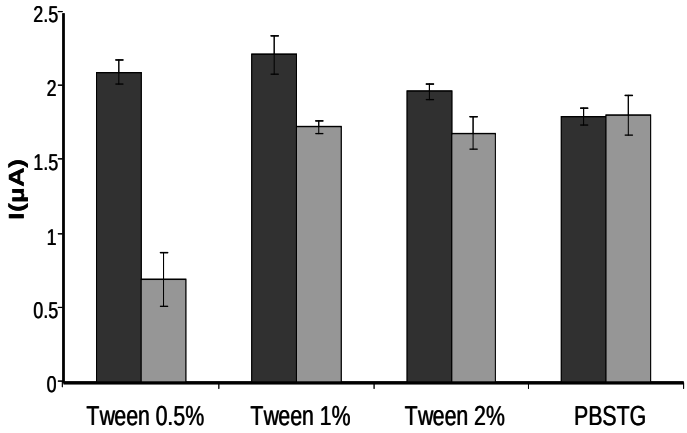

Figure 6-3. Source-drain current (at *V*g=−5 V) obtained for four different CNTFETs coated with different blocking agents before and after exposure to 10 μg/mL of IgG. Each electrical current plotted corresponds to the mean value of three replicates.

6.3.4. Further control experiments

The mechanism of the protein adsorption onto the sidewalls of the carbon nanotubes and their effects on the electrical properties of the CNTFETs has been well studied (Bradley, K. et al, 2004). Nevertheless, we assessed that the rinsing process with PBS and/or distilled water does not affect the device characteristics and that the change in the electrical current is only due to the protein adsorption. We took a CNTFET to perform the control experiments for the different functionalization steps. The device was electrically characterized in dry conditions at room temperature.

Step 1: As-grown carbon nanotubes before and after being rinsed with distilled water and dried with nitrogen.

Step 2: As-grown carbon nanotubes just after being functionalised with 5 μg/mL protein G and after being rinsed with PBS and distilled water and dried with nitrogen.

Step 3: As-grown carbon nanotubes functionalised with 5 μg/mL of protein G just after being covered with PBSTG solution and after being rinsed with distilled water and dried with nitrogen.

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Step 4: As-grown carbon nanotubes functionalised with 5 µg/mL protein G coated with PBSTG just after being exposed to 10 µg/mL of IgG and after being rinsed with distilled water and dried with nitrogen.

Figure 6-4 shows that not significant modifications occurred in the electrical characteristics of the device after the rinsing process. Therefore, these results proved that the changes in electrical current were only due to the protein adsorption onto the carbon nanotubes.

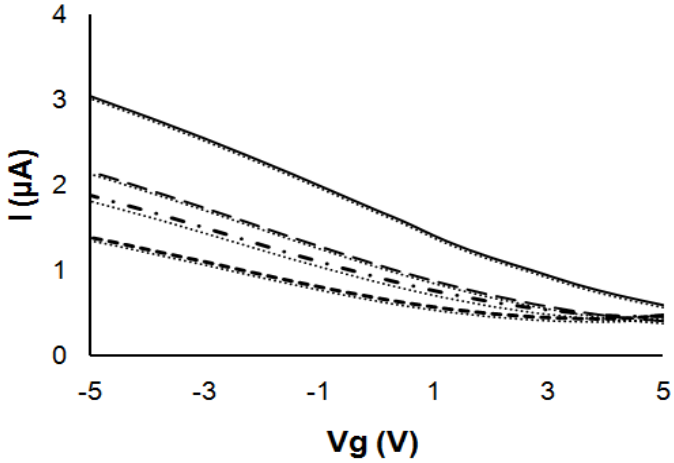

Figure 6-4. Source-drain current vs. gate voltage of a typical CNTFET after each functionalization step. As-grown CNTs (——); protein G adsorbed on the CNTs (— —); CNTs coated with protein G and PBSTG solution (‾ ·) and anti-*Aspergillus* antibodies linked to the protein G previously absorbed on the CNTs (**- - -**). The change in the electrical current after the rinsing process is always represented by (**……**).

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6.4. Complementary conclusions

Combining the results obtained by spectroscopy ellipsometry and atomic force microscopy for the protein immobilization process, enables obtaining detailed information about the protein layers, thus contributing to the understanding the surface characteristics of the biosensor.

Numerous studies have proven that properly oriented antibodies, exhibit higher antigen binding capacities compared to those randomly oriented. In our study the orientation of the antibody was controlled by using a recombinant protein G non-covalently adsorbed on the CNT. This allowed the improvement of the surface density of binding sites over the surface of the sensor and therefore their sensitivity.

Based on the results of the effect of the temperature on the protein adsorption and the protein-protein interaction, it can be concluded that the adsorption of the protein G is not significantly influenced by the temperature, causing considerable changes in the electrical properties of the devices at all temperatures tested. By contrast, the kinetics of the interaction between protein G – anti- *Aspergillus* antibody is clearly slower at low temperatures. Therefore, a temperature close to that of the human body is ideal to carry out these types of interactions.

Regarding the electrical characterization, it can be concluded that our devices are highly sensitive and selective. Low concentrations of the proteins are enough to produce a change in the characteristics of the devices. In addition, these changes are due only to the interaction between the protein G and the anti-*Aspergillus* antibody. This enabled proving the protective effect of the PBSTG solution on the sidewalls of the CNTs.

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6.5. References

Bae, Y.M., Oh, B.K., Lee, W., Lee, W.H., Choi, J.W. Biosensors and Bioelectronics. **2005**. 21, 103–110.

Bradley, K., Briman, M., Star, A., Grüner, G. Nanoletters. **2004**. 4, 253-256.

Corry, B., Uilk, J., Crawley, C. Analytica Chimica Acta. **2003**. 496,103–116.

Jung,Y., Young, J., Chung, B.H. Analyst. **2008**. 133, 697–701.

Kanno, S., Yanagida, Y., Haruyama, T., Kobatake, E., Aizawa, M. Journal of Biotechnology. **2000**. 76, 207–214.

Kondo, A. and Fukuda, H. Journal of Colloid and Interface Science. **1998**. 198, 34-41.

Piao, M.H., Noh, B.H., Rahman, M.A., Won, M.S., Shim, Y.B. Electroanalysis. **2008.** 20, 30–37.

M.H.V. Van Regenmortel, D. Altschuh, G. Schreiber, Biomolecular Sensors. Taylor & Francis Inc, London, **2002**.

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Chapter 7

Application of CNTFETs to the analysis of real samples

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7.1. Introduction

Yeast and moulds are ubiquitously distributed microorganisms that can be found in diverse environments. They have the capacity to grow in a variety of substrates being tolerant to extreme conditions such as low pH, low water activity (a_w)*, and low temperature. Foods are a very suitable culture media for fungal growth because they have essential nutrients (Dantigny, P. et al, 2005). Therefore, food safety is one of the main aspects which have led to an increasing interest regarding contamination of foods and feed with toxigenic fungi, mainly *Aspergillus, Penicillium* and *Fusarium* genera as well as their mycotoxins (Logrieco, A. et al, 2005) which can be a hazard for human health.

Monitoring of agricultural commodities, foods and animal feed for the presence of fungal contamination include conventional culture and taxonomic identification at morphological level. However, this method is time-consuming (about one week to obtain results) and expert mycologists are needed (Konietzny, U. and Greiner, R. 2003).

Enzyme-linked immunosorben assays (ELISA) are also widely used to detect the presence of moulds and mycotoxins in foods with high sensitivity (Tsai, G.J. and Yu, S.C., 1999.; Yong, R.K. and Cousin, M.A., 2001). This technique is based on the detection of extracellular antigens secreted by moulds or mycelial antigens. Yong R.K. and Cousin, M.A. (Yong, R.K. and Cousin, M.A., 2001), reported an ELISA to detect aflatoxigenic moulds in naturally and artificially contaminated maize and peanut samples. Both samples were inoculated with 10^2 spores/mL of *A. parasiticus* and incubated at 15 ºC and 22 ºC for several days. *A. parasiticus* antigens were detected in maize with a detection limit beween 50 cfu/g to $4.9x10^5$ cfu/g at 15 ºC and between 50 cfu/g to $3.6\ x10^5$ cfu/g at 21 ºC. In peanuts, the detection limit was in a range <100 cfu/g to $3x10^3$ cfu/g at 15 ºC and <100 cfu/g to $7.2x10^6$ cfu/g at 21 ºC. The selectivity was tested with several moulds and yeast and the results showed more than 50 % cross-reactivity with different *Aspergillus*

* a_w is the amount of avalable water in a food for the microbial growth. It is measures in a scale from 0 to 1, where the pure water has an activity of 1.

species. Although this method allows the detection of mould at an early stage of mould growth, it is reagent and instrumental consuming. Another disadvantage of ELISA assays it that requires a stable source of antibody to ensure continuity of analytical performance and stability (Logrieco, A. et al, 2005).

PCR-based methods to detect mycotoxigenic fungi have provided alternatives to standard methods. The polymersa chain reaction (PCR) amplifies DNA sequences of defined length. The process involves 25 to 40 repetitive cycles of heat denaturation, primer annealing and enzymatic primer extension generating about 32 billion of copies of the DNA sequence. Nowadays, this technique has been improved being possible to perform real time detection. Suanthie, Y. et al, (Suanthie, Y. et al, 2009) developed a multiplex real-time PCR assay to detect and quantify important mycotoxigenic species of *Aspergillus*, *Penicillium* and *Fusarium.* Authors recommend this assay for monitoring fungal growth in distiller's grain (DG) because fungal DNA was quantifiable one day after inoculation with the fungi when no signs of them were visible with a microscope. The linear range of detection was 1 pg to 10 ng of DNA. Both PCR and real-time PCR are accurate and sensitive. However, the second method is less time time-consuming because it avoids analyzing the reaction product by techniques such as southern blotting and agarose gel electrophoresis (Yong, R.K. and Cousin, M.A., 2001). This technique can be applied to the screening of agricultural commodities for the absence of mycotoxin producer before or even after processing (Konietzny, U. and Greiner, R. 2003).

Electrometric methods are applied to detect fungus. Indirect conductance measurement of carbon dioxide produced by yeasts and molds can be performed and correlated with the amount of fungi present in a sample. Although it is sensitive (10 cfu/mL), it takes about 48 h obtain results (Foong-Cunningham, S.C. et al, 2009).

Mass and electrochemical sensors have also been introduced to detect moulds and yeast. However, electrochemical sensors have not been used to detect specifically mycotoxigenic moulds. Most of the research has been focused towards the detection of mycotoxins (Parker, C.O. et al, 2009.; Tan, Y. et al, 2009). Tan, Y. et al,

reported the indirect electrochemical monitoring of the interaction of a mycotoxin with an immobilized recognition system. At the optimal conditions, the working range of the electrochemical immunosensor was from 0.1 to 10 ng/mL with a detection limit of 0.06 ng/mL with good recoveries in spiked rice samples. This sensor is highly sensitive; nevertheless a complex procedure including the use of labeled-antibodies is required.

In this chapter we report the first electrochemical biosensor based on carbon nanotubes field effect transistors for the rapid and label-free detection of *A*. *flavus* in contaminated milled-rice. The developed devices were previously characterized in order to improve the immobilization of the molecular recognition element onto the transducer. The adsorbed protein G onto the carbon nanotubes walls enables the IgG anti-*Aspergillus* antibodies to be well orientated and therefore to display full antigen binding capacity for fungal antigens. An optimized solution of Tween 20 and gelatine was used as effective blocking agent to prevent the non-specific binding of the antibodies and other moulds onto the CNTs but also to protect the transducer against the interferences present in the rice samples. We proved that our devices are useful tools for detecting mycotoxigenic moulds at low concentrations in real samples with high selectivity.

Finally, this chapter supplies supplementary experimental information to that included in the published paper, complementary conclusions and references.

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7.2. Article:

"Rapid detection of *Aspergillus flavus* in rice using biofunctionalized carbon nanotube field effect transistors". Submitted to Analyst, 2009.

Raquel A. Villamizar [1τ], Alicia Maroto [1,2] and F. Xavier Rius[1*]

[1] Department of Analytical and Organic Chemistry. Universitat Rovira i Virgili. Marcel.lí. Domingo, s/n. 43007. Tarragona, Spain.

[2] École Supérieure de Chimie Organique et Minérale (ESCOM), 1 allée du réseau Jean-Marie Buckmaster, 60200 Compiègne, France

[τ] On leave from the Department of Microbiology. University of Pamplona. Km 1, Vía Bucaramanga, N. de S. Colombia.

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Summary

In the present study we have used carbon nanotube field effect transistors that have been functionalised with protein G and IgG to detect *A*. *flavus* in contaminated milled-rice. The adsorbed protein G on the carbon nanotubes walls enables the IgG anti-*Aspergillus* antibodies to be well oriented and therefore to display full antigen binding capacity for fungal antigens. A solution of Tween 20 and gelatine was used as an effective blocking agent to prevent the non-specific binding of the antibodies and other moulds and also to protect the transducer against the interferences present in the rice samples. Our FET devices were able to detect at least 10 µg/g of *A. flavus* in only 30 minutes. To evaluate the selectivity of our biosensors, *Fusarium oxysporum* and *Penicillium chrysogenum* were tested as potential competing moulds for *A. flavus*. We have proven that our devices are high selective tools for detecting mycotoxigenic moulds at low concentrations in real samples.

1. Introduction

Aspergillus flavus is a mycotoxigenic and filamentous mould widely distributed in nature on a variety of food and agricultural products. It causes a wide range of diseases, ranging from hypersensitive reactions to invasive infections associated with angioinvasion. *A. flavus* is after *A. fumigatus*, the second leading cause of invasive and non-invasive aspergillosis[1]. In addition it is one of the most significant fungi in the spoilage of grain during storage and is therefore responsible for the economic devaluation of the grain through mycotoxin contamination[2]. Rice is one of the most important staple foods for a large part of the world's human population. It can grow in different agro-climatic conditions and is vulnerable to *Aspergillus* infection in the field as well as in storage.

It is difficult to develop a general method for detecting all mycotoxins because they are metabolites that display different chemical structures. A good approach, therefore, is to detect the mycotoxigenic moulds in the early stages of growth before they can produce mycotoxins[3]. Conventional methods for identifying and detecting mycotoxigenic fungi in rice and foods use cultures in different media or immunological methods[4]. Viable plate count method uses specific media which

allow the moulds to grow over a surface. A high mould count indicates possible aflatoxin contamination[3]. Immunological methods rely on the specific binding of an antibody to an antigen[5]. Several studies have reported using antibodies to detect microbial toxins [6-7].

Other methods currently available either analyze the electrical properties (e.g. the impedance) of the contaminated food substratum or they directly detect fungal metabolites such as chitin, ergosterol or ATP[8]. PCR-based methods are specific and have been used to detect aflatoxigenic strains of *A. flavus*[9-10]. However, most of these methods are time consuming and require trained personal.

Another methodology uses electronic noise to detect volatiles and odours produced by the fungi[11-14]. These results can subsequently be correlated with certain parameters such as mycelia development on cereal grain or mycotoxin production[11]. This methodology is able to distinguish between infected and non-infected samples and can sometimes even distinguish between non-mycotoxigenic and mycotoxigenic fungi. However, the methodology is time consuming because the devices have to be previously trained to avoid false positive or false negative results. Moreover, the information obtained is often qualitative.

To overcome this problem, mass and electrochemical sensors have been developed to quickly detect and quantify fungi[15-17]. Nugaeva et al. [16] used protein modified-microcantilevers to detect in situ the growth process of *Aspergillus niger* and *Saccharomyces cerevisiae.* After four hours of incubation the cantilever detected the presence of the fungi and was able to distinguish between alive and dead cells. Despite the emerging methods, some performance parameters such as sensitivity and selectivity must be improved.

Single-walled carbon nanotubes (SWCNTs) are one-dimensional nanostructures that display remarkable physical and mechanical properties. Moreover, they can be incorporated into a field effect transistor (CNTFET) to make a biosensor with improved performance parameters[18-25]. In this study we used a new electrochemical biosensor based on carbon nanotube field effect transistors to quickly and selectively detect *Aspergillus flavus* in rice samples. In order to improve the

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sensitivity of our devices we have used protein G; this allows the proper orientation of the polyclonal anti-*Aspergillus* antibodies and, therefore, optimal antigen binding. Thus, anti-*Aspergillus* antibodies are able to provide two specific binding sites for fungal membrane antigens. A phosphate buffer (PBS) solution containing Tween 20 and gelatine was used as a blocking agent to prevent the non-specific binding (NSB)[26] of the antibodies and other moulds and also to protect the transducer against the interferences present in rice samples. The sensor showed high selectivity in the presence of competing moulds. Our devices are label-free and able to detect 10 µg/g of *A. flavus* with a time response of 30 minutes. All these performance parameters are clearly better than those of the methods currently used to detect mycotoxigenic fungi.

2. Materials and methods

2.1. Proteins and biochemicals

Anti-*Aspergillus* (i.e. a polyclonal rabbit anti-*Aspergillus* (1 mg/mL) was purchased from Oxford Biotechnology Ltd. (Oxford, U.K.). It was dissolved in phosphate buffer saline (PBS) to a final concentration of 8 µg/mL (pH=7.2) and stored at -20 ºC until use. Protein G from *Streptococcus* spp. recombinant, expressed in *Escherichia coli* (1 mg/mL), phosphate buffered saline (PBS, pH 7.4), Tween 20 and gelatin from cold water fish skin were obtained from Sigma-Aldrich. Sabouraud dextrosa agar (SDA) was provided by Oxoid and brain heart infusion broth (BHI) and buffered peptone water were obtained from Scharlau Chemie Microbiology and prepared according to their specifications.

2.2. Apparatus

A mini orbital shaker Stuart was used to prepare the fungal biomass. An environmental-scanning electron microscope (ESEM), Quanta 600, (FEI, Hillsboro, OR, USA) was used to take images of the as-grown networks of SWCNTs and the functionalization process. Electrical measurements were taken using a 4157A Agilent semiconductor parameter analyzer and a Wentworth Laboratories MP1008 probe station.

2.3. Fungal growth and preparation of fungal biomass

Lyophilized strains of *Aspergillus flavus* CECT 2684, *Penicillium chrysogenum* (*P. chrysogenum*) CECT 2307 and *Fusarium oxysporum* (*F. oxysporum)* CECT 2154 were obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain). They were rehydrated with sterile water, subcultured at least three times on SDA and incubated at 30 ºC for 7 days to form single colonies [7]. The spores from the subcultured media were washed from the surface with a PBS solution. Subsequently, flasks containing 100 mL of BHI were inoculated with 1 mL of the spore suspension obtained before and were incubated at 30 ºC and agitated at 120 rpm for 7 days [6]. The cultures were harvested and the mycelium was separated by filtration through Whatman No. 5 filter paper[27]. The mycelium was collected, washed with sterile distilled water and dried in the oven at 105 ºC until its weight was stable[28]. Then, it was diluted in PBS (w/v) to produce the final samples of *A. flavus* at 10 ng/mL, 100 ng/mL and 1000 ng/mL. The same procedure was followed for *P. crysogenum* and *F. oxysporum.*

2.4. Rice sample preparation

Milled-rice samples were obtained from Arrosaires del Delta de l´Ebre, Tarragona, Spain. 25 g of the sample was dissolved in 225 mL of 0.1 % sterile buffered peptone water and sterilized by autoclaving at 121 ºC for 15 minutes. The rice sample was then shaken and diluted in the same solvent for up to 10^5 fold. After that, the resulting samples were artificially contaminated by spiking 1 mL of *A. flavus* at 10, 100 or 1000 ng/mL in 9 mL of the diluted rice sample. In this way, the final concentration of *A. flavus* in the diluted rice samples was 1, 10 or 100 ng/mL. The same procedure was used to contaminate the diluted rice samples with 1, 10 or 100 ng/mL of *P. chrysogenum* and *F. oxysporum* [6]*.*

2.5. Development and functionalization of the CNTFETs.

The SWCNT networks were synthesized on a 500 nm layer of silicon dioxide thermally grown on highly doped n-type silicon chips (total area 0.5 cm x 0.5 cm) using chemical vapour deposition (CVD). This process has been described elsewhere[24]. To obtain the field effect transistor configuration, source and drain

ISBN:978-84-692-9761-2/DL:T-203-2010

electrodes were screen-printed with silver ink over the synthesized SWCNTs. The gap between both electrodes was 0.5 mm and the size of the electrodes was 200 µm x 200 µm. The gate electrode was an aluminium layer on the back side of Si. The CNTFETs were electrically characterized by recording the current vs. the gate voltage. To obtain the instrumental variability, we took all the electrical characterisation measurements three times and plotted the mean value and the range value of the measurements.

The functionalization protocol was similar to that applied in a previous work where we characterized the biofunctionalized layer deposited on the SWCNTs[29]. Briefly, CNTFETs were incubated for 30 minutes at 37 ºC in a 5 µg/mL solution of protein G. This is a small globular cell surface protein produced by *Streptococcus* spp. It is composed of two or three nearly identical domains of 55 amino acids each. The protein G used in our assays is genetically truncated, which means that retains its affinity for the constant fraction “Fc” of the IgG, but lacks the Fab-binding sites.

Subsequently, the devices were immersed for 3 hours in a solution of Tween 20 and gelatine (PBSTG). The amount of Tween 20 and gelatine was optimised to avoid the NSB of the anti-*Aspergillus* antibodies and, more importantly, to avoid that the components of the rice samples could interfere with the determination of *A. flavus*. Next, the CNTFETs were immersed in an 8 µg/mL solution of anti-*Aspergillus* antibodies for 30 minutes at 37 ºC. Finally, the CNTFETs were again thoroughly rinsed with distilled water and ready to be used to detect *A. flavus*. After each functionalization step the devices were dried with nitrogen and electrically characterized. Figure 1 shows the experimental process used to functionalize the CNTFET for the detection of *A. flavus.*

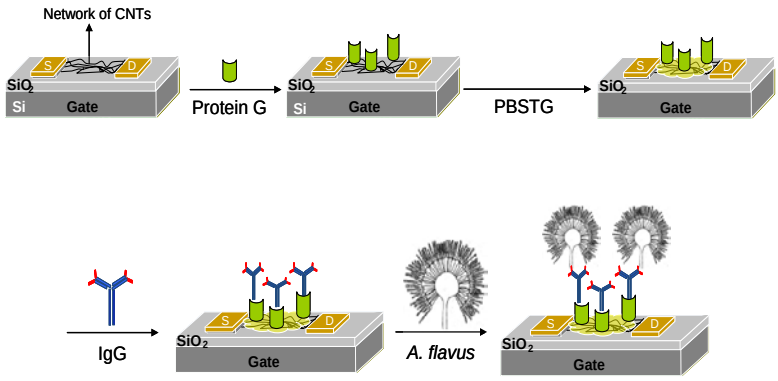

Figure 1. Functionalization process to detect *A. flavus* with CNTFETs. Protein G enables the antibodies to be appropriately orientated while PBSTG avoids the NSB of biomolecules on the transducer. *A. flavus* is detected by means of the antigen-antibody interaction.

2.6. Optimisation of the PBSTG and the dilution factor of rice

Several CNTFET devices were incubated with protein G. Each device was then incubated with a different concentration of Tween 20 and gelatine for 3 hours. After this, the devices were immersed in an 8 µg/mL solution of anti- *Aspergillus* antibodies for 30 minutes at 37 ºC. Finally, the devices were exposed for 1 hour to the diluted rice samples: first to the 10^5-fold diluted rice sample and finally to the 10-fold diluted rice sample.

2.7. Determination of the response time

A functionalized CNTFET (protected with 1.5 % Tween and 2 % gelatine) was immersed at 37 ºC for 1 hour in a 10^3-fold diluted rice solution contaminated with 1 ng/mL of *A. flavus*. Every 15 minutes the CNTFET was thoroughly rinsed with water, dried with nitrogen, electrically characterized, and submerged again in the solution containing 1 ng/mL of *A. flavus* for another 15 minutes.

2.8. Detection of *A. flavus*

Another functionalised CNTFET (protected with 1.5 % Tween and 2 % gelatine) was exposed to 10^3-fold diluted rice samples spiked with increasing concentrations of *A. flavus* (i.e. 1, 10 and 100 ng/mL). For each concentration, the CNTFETs were

immersed for 30 minutes at 37 ºC, rinsed thoroughly with distilled water, dried with nitrogen and electrically characterized. The presence of the moulds in the recognition layer of the biosensor was confirmed microscopically with ESEM.

2.9. Selectivity of the CNTFETs

Selectivity was checked in the presence of two mycotoxigenic mould *P. chrysogenum* and *F. oxysporum*. A functionalised CNTFET (protected with 1.5 % Tween and 2 % gelatine) was exposed to rice samples contaminated with 100 ng/mL of *P. chrysogenum,* for 30 minutes at 37 ºC, thoroughly rinsed with distilled water, dried with nitrogen and electrically characterized. It was subsequently exposed to 100 ng/mL of *A. flavus* under the same conditions mentioned above. The same procedure was followed for *F. oxysporum.* The presence of the *A. flavus* and the interference moulds was also confirmed microscopically with ESEM.

3. Results and discussion

3.1. Electrical characterization of the functionalised CNTFETs.

The electrical behaviour of the CNTFETs was monitored after each functionalization step. We measured the dependence of the source-drain current, I, three times on the back gate voltage, Vg, in the +5 V to -5 V range at room temperature in dry conditions. The bias voltage, Vsd, was fixed at 250 mV. The change in the electrical current as a consequence of the attached proteins has been already studied [29].

3.2. Optimisation of the PBSTG and the dilution factor of rice

It is essential to prevent the non-specific binding (NSB) of the antibodies on the CNTs and, even more important, to protect the CNTs against possible interferences from the rice samples. Avoiding the NSB of the anti-*Aspergillus* antibodies allows having all the antibodies with the correct orientation. In this way, the sensitivity of the devices is improved because all the antibodies are able to provide two antigen binding sites for *A.flavus*. Most importantly, preventing the NSB of the rice compounds onto the CNTs ensures that the electrical characteristics of the devices will only respond to the presence of *A. flavus*. Therefore, we ensure that the devices

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will be selective to *A. flavus* as long as the antibody does not have cross-reactions with other fungi.

Sánchez-Acevedo et al [30] prevented the NSB of small molecules by protecting the CNTs with a Tween 20 at 0.05 % and gelatine at 0.8 % diluted in phosphate buffer solution (PBSTG). Figure 2 shows the results obtained for a CNTFET device protected with this solution and after being exposed to a solution of anti-*Aspergillus* antibodies. It can be seen that in fact the PBSTG effectively protects the sidewalls of the CNTs against the NSB of the proteins. However, we also have to protect the CNTs against the effect of the matrix sample to prevent the rice compounds from interfering with the detection of *A. flavus*. Figure 4 shows that increasing the percentage of gelatine to 2 % and Tween to 1.5 % allows *A.flavus* to be detected in 10^3-fold diluted rice samples. The change in the electrical current when the devices are exposed to higher dilution is attributed to the nature of the sample (i.e. the presence of polysaccharides such as starch). As a result, a 10^3-fold dilution of milled rice was chosen as the medium to be artificially contaminated with *A. flavus* assuring that the matrix sample would not affect the electrical behaviour of devices protected with 2 % of gelatine and 1.5 % of Tween 20.

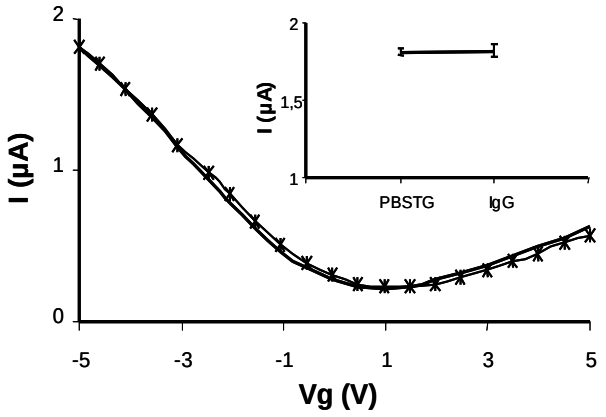

Figure 2. Source-drain current vs. gate voltage of a CNTFET coated with PBSTG (——) and after being exposed to a solution of anti-*Aspergillus* antibodies (—x—). The inset shows the behaviour of the source-drain current after each functionalization step at *V*g = −5 V.

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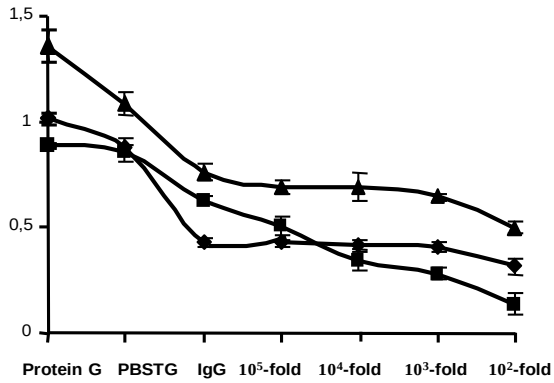

Figure 3. Source-drain current (at Vg=-5 V) obtained for three functionalized CNTFET coated with different concentrations of PBSTG and after being exposed to different dilutions of milled rice. Each electrical current plotted corresponds to the mean value of three replicates.

3.3. Time response

The time response was then optimised by immersing functionalized CNTFETs (protected with 2 % gelatine and 1.5 % Tween 20) in a 10^3-fold milled rice solution containing 1 ng/mL of *A. flavus* at 37 ºC for 1 hour. Every 15 minutes, the CNTFETs were thoroughly rinsed with water, dried with nitrogen and electrically characterized. Figure 4 shows that the highest decrease (i.e. about 12 %) in current intensity was obtained after 30 minutes of incubation. No further decrease was obtained. We chose a response time of 30 minutes for our devices.

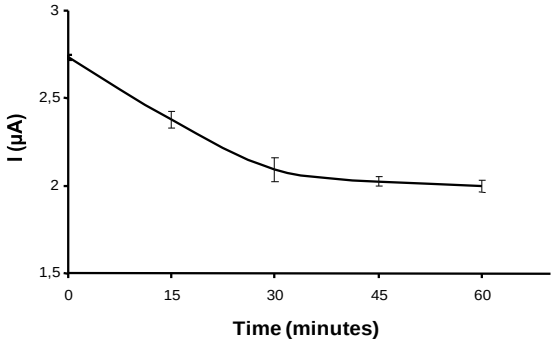

Figure 4. Source-drain current (at *V*g =−5 V) obtained for a functionalised device after being exposed to 1 ng/mL of *A. flavus* for 15, 30, 45 and 60 minutes.

UNIVERSITAT ROVIRA I VIRGILI
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3.5. Detection of *A. flavus* mycelia with CNTFETs in milled rice.

A functionalised CNTFET (protected with 2 % gelatine and 1.5 % Tween 20) was then exposed to 10^3-fold diluted milled rice artificially contaminated with 1, 10 and 100 ng/mL of *A. flavus*. For each concentration, the CNTFET was immersed for 30 minutes at 37 ºC, rinsed thoroughly with distilled water, dried with nitrogen and electrically characterised by measuring the I–V characteristics three times and plotting the mean value and range of the measurement values.

Figure 5 shows the I-V characteristics (for a Vsd=0.25 V) of a functionalised CNTFET before and after exposure to 1, 10 and 100 ng/mL of the mould. It can be seen that the adsorption of *A. flavus* decreases the conductance of the devices (i.e. from a 9 % decrease for 1 ng/mL to a 30 % decrease for 100 ng/mL). This may be because the antigen-antibody interaction causes a deformation on the SWCNTs and thus a scattering effect. The anti-*Aspergillus* antibody is able to recognize the antigen galactomannan (GM). It is the major cell wall component present in *Aspergillus* species and is made up of a main chain of mannose with galactose side groups [31].

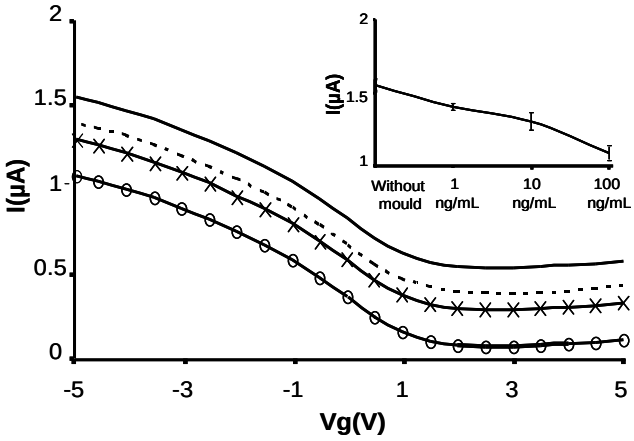

Figure 5. Gate voltage dependence of the source-drain current of a typical functionalized CNTFET before exposure to *A. flavus* (—) and after exposure to 1 ng/mL (- - - -); 10 ng/mL (—x—) and 100 ng/mL (—o—) of milled rice spiked with *A. flavus*. The inset shows the behaviour of the source-drain current after each functionalization step at *V*g = −5 V.

ISBN:978-84-692-9761-2/DL:T-203-2010

SEM confirmed that the mould was attached to the SWCNTs. Figure 6 shows an image of a functionalized device after being exposed to *A. flavus.* We observed that regardless the mycelia filtration process, some spores remained attached. *A. flavus* has hyphas that varies in length and that is rough with conidia globose to subglobose varying from 3.5 to 4.5 µm in diameter. GM antigen is produced at an early stage in the metabolic activation of resting conidia, but it is increasingly expressed during the swollen-conidium and hypha stages. Since moulds are primarily found as asexual spores or dried mycelia on food, the anti-*Aspergillus* antibody used in our assays could recognize the GM in both conidia and hyphas structures [27]

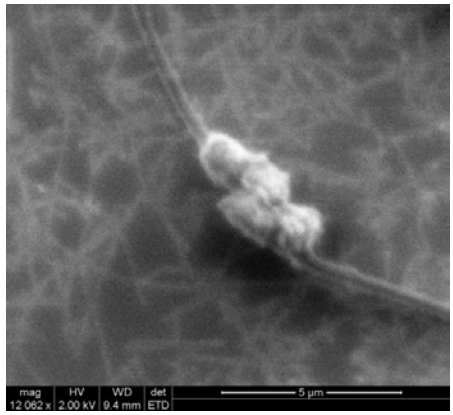

Figure 6. A SEM image of a functionalized CNTFET after exposure to *A. flavus.* The mould is linked to the SWCNT network through antigen–antibody interaction.

3.6. Selectivity of the CNTFETs.

The selectivity of our devices was tested in the presence of two molds: *P. chrysogenum* and *F. oxysporum.* Under favourable environmental conditions, these mycotoxigenic moulds can be found together with *A. flavus* causing disease in grains during plant growth or after harvest in storage. Chemical analyses of the cell wall of *Fusarium oxysporum* have shown that these hyphal walls are composed of (*N*-acetyl)-glucosamine, glucose, mannose, galactose, uronic acid, and proteins[32]. Thus, although this mould shares some general features with *A. flavus*, it should not have any cross reaction with the anti-*Aspergillus* antibody. By contrast the GM is

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present in the cell wall of most *Penicillium* and *Aspergillus* species[33]. Therefore, because these two fungi present similar antigenic epitopes, cross-reactivity would occur with the antibody.

A functionalised CNTFET was first immersed in a rice solution containing 100 ng/mL of *P. chrysogenum* for 30 minutes at 37 ºC, thoroughly rinsed with distilled water, dried with nitrogen and electrically characterized. The device was then exposed to 100 ng/mL of *A. flavus* under the same conditions as mentioned above. We follow the same procedure with another functionalized CNTFET for 100 ng/mL of *F. oxysporum*. Figure 7 shows that the electrical current changes slightly after the CNTFET was exposed to *F. oxysporum* whereas it decreases about 16 % after exposure to *A. flavus*. The slight changes of the electrical current after exposing the devices to *F. oxysporum* are due to the variability of the electrical current. By contrast, when the devices are exposed to *P. chrysogenum*, the electrical current decreases about 3.5 % whereas it decreases by about 20 % after exposure to *A. flavus*.

The change in the electrical current after exposing the devices to *P. chrysogenum* is probably due to the immunological similarity or the identity of the galactomannans in both moulds. Therefore, the anti-*Aspergillus* antibody is able to recognize cross-reacting epitopes on other fungal cell walls that are causing a slight cross-reactivity with the commercially available antibody, as has also been observed by De Vos *et al*[28]. The electrical current plotted corresponds to the mean value of the three measurements. The error bars of the inset correspond to the range of the current values.

ISBN:978-84-692-9761-2/DL:T-203-2010

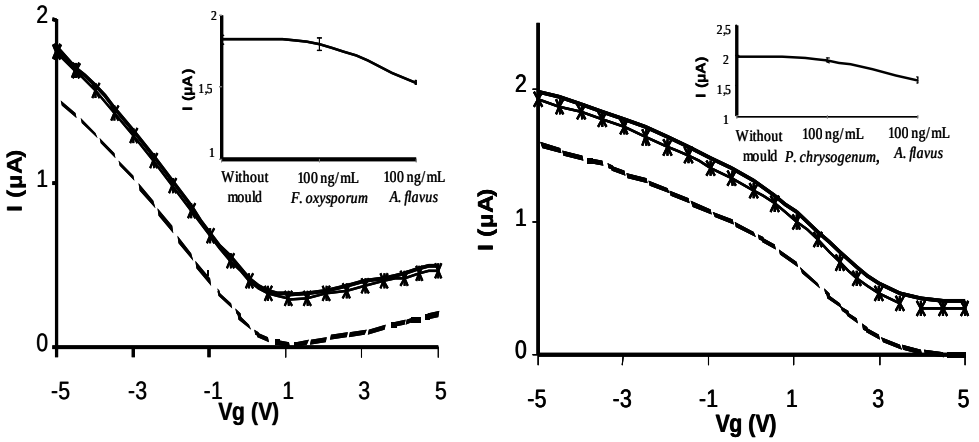

Figure 7. A). Gate voltage dependence of the source-drain current of a functionalised CNTFET before exposure to the mould (—) and after exposure to 100 ng/mL of *F. oxysporum* (–X–); and 100 ng/mL of *A. flavus* (----) . B) Gate voltage dependence of the source-drain current of a functionalized CNTFET before exposure to the mold (—) and after exposure to 100 ng/mL of *P. chrysogenum* (–X–); and 100 ng/mL of *A. flavus* (----). Each electrical current plotted corresponds to the mean value of two replicates. The inset shows the behaviour of the source-drain current at Vg=-5 V. The error bars correspond to the electrical current obtained for the three replicates.

Selectivity was also checked with SEM. A functionalised CNTFET was exposed to 1, 10 and 100 ng/mL of *F. oxysporum* for 30 minutes each, thoroughly rinsed with water and dried with nitrogen. For each concentration, we scanned the area between the electrodes (500 μm^2) with SEM. We did not observe either conidia nor mycelia when the device was exposed to the different mould concentrations. The same procedure was followed for *P. chrysogenum*. In this case, we did not observe any conidia or mycelia when the device had been exposed to 1 ng/mL and 10 ng/mL, whereas 3 conidia were observed when it was exposed to 100 ng/mL *P. chrysogenum,* (thus showing, a slight cross-reaction between the antibody and *P. chrysogenum*). With *F. oxysporum* the sensor displayed high selectivity; however, due to the antigenic similarity between *P. chrysogenum* and *A. flavus* there was a slight cross-reactivity of the commercially available antibody. Therefore, a monoclonal antibody against *A. flavus* would be able to overcome this problem.

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Our CNTFETs are then quite fast, selective and with a minimum detectable level of only 10 µg/g. This value was obtained from the lowest amount of mould detected by the CNTFET (1 ng/mL) and by taking into account all the dilution factors to prepare the artificially contaminated 10^3-fold diluted rice samples (section 2.4). The devices we have developed in this study, have improved some performance parameters like selectivity and sensitivity if we compare with other biosensors applied in the detection of mycotoxigenic moulds. For instance, Paolesse et al. (2006) used an electronic nose and were able to discriminate between non-infected and infected samples with two *Penicillium chrysogenum* and *Fusarium verticillioides*. Although this sensor can detect the early stages of grain spoilage by fungi, the detection process is qualitative. Moreover, the sensor cannot distinguish between different strains of fungi because they produce similar volatile compounds, thus reducing the selectivity of the device. Finally, our CNTFETs only need 30 minutes to detect *A. flavus* whereas conventional media require about 6 days to interpret the results[4].

4. Conclusions

We have developed a CNTFET device that uses the recognition ability of the antibodies and the transduction capacity of carbon nanotubes to detect *A. flavus*. Under adequate conditions (protein concentrations, pH, temperature) the device could selectively detect at least 10 µg/g of *A. flavus* in milled rice. This is probably because the use of protein G permits the IgG antibodies to be properly oriented. The transduction power of the carbon nanotubes makes a special labelling process unnecessary; as a result, no additional reagent is required, thereby reducing costs. The sensor devices only measure the change caused by the antigen-antibody interaction; therefore, the system displays high selectivity and, since it is well protected, it is not affected by possible interferences from the raw food. Using suitable molecular receptors, this type of device would be highly sensitive and could detect any mycotoxigenic mould in grains and foods in a short time.

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References

1. M. T. Hedayati, A. C. Pasqualotto, P. A. Warn, P. Bowyer, D. W. Denning, *Microbiology*., 2007, **153**, 1677–1692.
2. S. H. Gordon, R. W. Jones, J. F. McClelland, D. T. Wicklow, R. V. J. Greene, J, *Agr Food Chem*., 1999, **47**, 5267-5272.
3. R.K. Yong and M.A. Cousin, Int J Food Microbiol., *2001, **65**,* 27–38.
4. K.R.N. Reddy, C.S. Reddy, K. Muralidharan, *Food Microbiol*., 2009, **26**, 27 31.
5. E. de Boer and R.R. Beumer, Int J Food Microbiol., 1999, **50,** 119–130 .
6. G.J. Tsai and S.C. Yu, *Int J Food Microbiol.,* 1999, **50**, 181–189.W. Hao, Y.X. Pan, Y.Q. Ding, S. Xiao, K. Yin, Y.D. Wang, L.W. Qiu, Q.L. Zhang, P.C.Y. Woo, S.K.P. Lau, K.Y. Yuen, X.Y. Che, *Clin Vaccine Immunol.*, 2008, **15**, 2. 194–202.
7. H. Girardin, Sci. Des Aliments., 1997, **17**, 3–19.
8. A. González, T. González, C. Vázquez, B. Patiño, *Food Addit Contam*., 2008, **25**, 6,758–764.
9. G.E.O. Midorikawa, M.R.R. Pinheiro, B.S. Vidigal, M.C. Arruda, F.F., Costa, G.J. Pappas, S.G. Ribeiro, F. Freire, R.N.G. Miller. *Lett Appl Microbiol.*, 2008. **47**,12-18.
10. R. Paolesse, A. Alimelli, E. Martinelli, C. Di Natale, A. D'Amicob, M.G. D'Egidio, G. Aureli, A. Ricelli, C. Fanelli. *Sensor Actuator B Chem.*, 2006, **119**, 425–430.
11. G. Keshri and N. Magan, *J Appl Microbiol.,* 2000, **89**, 825-833.
12. B.P.J. de Lacy Costello, R.J. Ewen, H. Gunson, N.M. Ratcliffe, P.S. Sivanand, P.T.N. Spencer. *Meas Sci Tech*., 2003. **14**, 397-409.
13. M. Falasconi, E. Gobbi, M. Pardo, M. Della Torre, A. Bresciani, G. Sberveglieri. *Sensor Actuator B Chem.*, 2005, **108**, 250–257.
14. H. Muramatsu, K. Kajiwara, E. Tamiya, I. Karube. *Anal Chim Acta.,* 1986, **188**, 257-261.

ISBN:978-84-692-9761-2/DL:T-203-2010

15. N. Nugaeva, K.Y. Gfeller, N. Backmann, H.P. Lang, M. Duggelin, M. Hegner. *Biosens & Bioelectron*., 2005, **21**, 849–856.
16. H. Chen, C.K. Heng, P.D. Puiu, X.D. Zhou, A.C. Lee, T.M. Lim, S.N. Tan, *Anal Chim Acta*., 2005, **554**, 52–59.
17. E.L. Gui, L.J. Li, K. Zhang, Y. Xu, X. Dong, X. Ho, P.S. Lee, J. Kasim, Z.X. Shen, J.A. Rogers, S.G. Mhaisalkar, *JACS.,* 2007, **129**, 14427-14432.
18. A. Star, E. Tu, J. Niemann, J.C.P. Gabriel, C.S. Joiner, C. Valcke, *PNAS.,* 2006, **103, 921-926.**
19. H. M. So, K. Won, Y. H. Kim, B. K. Kim, B. H. Ryu, P. S. Na, H. Kim, J.O. Lee, *JACS.,* 2005, **127**, 11906-11907.
20. K. Maehashi, T. Katsura, K. Kerman, Y. Takamura, K. Matsumoto, E. Tamiya, *Anal Chem***.,** 2007**, 79**, 782-787**.**
21. T. Dastagir, E.S. Forzani, R. Zhang, I. Amlani, L. A. Nagahara, R. Tsui, N. Tao, *Analyst.,* 2007, **132**, **7**38-740**.**
22. H.M. So, D. W. Park, E. K. Jeon, Y. H. Kim, S.K. Kim, C.K. Lee, S. Y. Choi, S.C. Kim, H. Chang, J.O. Lee, *Small.*, 2008, **4**, 197-201**.**
23. R.A. Villamizar, A. Maroto, F. X. Rius, I. Inza., M.J. Figueras, *Biosens & Bioelectron.,* 2008, **24**, 279-283.
24. R.A. Villamizar, A. Maroto, F.X. Rius, *Sensor Actuator B Chem.*, 2009, **136**, 451-457.
25. M.H.Piao, M.H. Noh, M.A. Rahman, M.S. Won, Y.B. Shim, *Electroanalysis*., 2008, **20**, 30–37.
26. S.V. Hetherington, S. Henwick, D.M. Parham, C.C. Patrick, *Clin Diagn Lab Immunol.*, 1994, **1**, 63-67.
27. M.M. De Vos, H.J. Nelis, *J Microbiol Meth*., 2003, **55**, 557–564.
28. R.A. Villamizar, J. Braun, B. Gompf, M. Dressel, F. X. Rius, 2009. *Biosens & Bioelectron.,* 2009, 25, 161-166.
29. Z.C. Sánchez-Acevedo, J. Riu, F. X. Rius, *Biosens & Bioelectron.,* 2009, **24**, 2842–2846.
30. M. Bernard, J.P. Latgé, *Med Mycol.,* 2001, **39**, 9–17.
31. E.A.M. Schoffelmeer, F.M. Klis, J.H. Sietsma, B.J.C. Cornelissen, *Fungal Genet Biol*., 1999, **27**, 275–282.

32. J.P. Latgé, H. Kobayashi, J.P. Debeaupuis, M. Diaquin, J. Sarfati, J.M. Wieruszeski, E. Parra, J.P. Bouchara, B. Fournet, *Infect Immun.,* 1994, *62*, 5424–33.

UNIVERSITAT ROVIRA I VIRGILI
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7.3. Supplementary experimental section

7.3.1. Microbiological test

Microbiological cultures were performed in order to confirm the viability of *Aspergillus flavus* strain. Figure 7-1A shows an image of the typical growth of the mould in sabouraud agar. As it was previously described in chapter 5, section 5.3.1. this media is used to cultivate pathogenic and nonpathogenic fungi. The typical growth of *A. flavus* is characterized for granular colonies, often with radial grooves, yellow at first but quickly becoming green when ageing. After 48 h at 30 ºC we can observe the first typical colonies of the mould over the media. It shows the viability of the lyophilized reference strain. Moreover, in order to preserve the culture pure, the colonies were plated on oxytetracycline gentamicine yeast agar (OGY).

Additionally to the culture plate, a sample was analyzed using SEM. It can be seen in figure 7-1B the conidiophores, whith lengths in the range 400-850 µm, are mostly hyaline and coarsely roughened. The spores also called conidiospores, are typically colorless, globose to echinulate and approximately 3-6 µm in diameter.

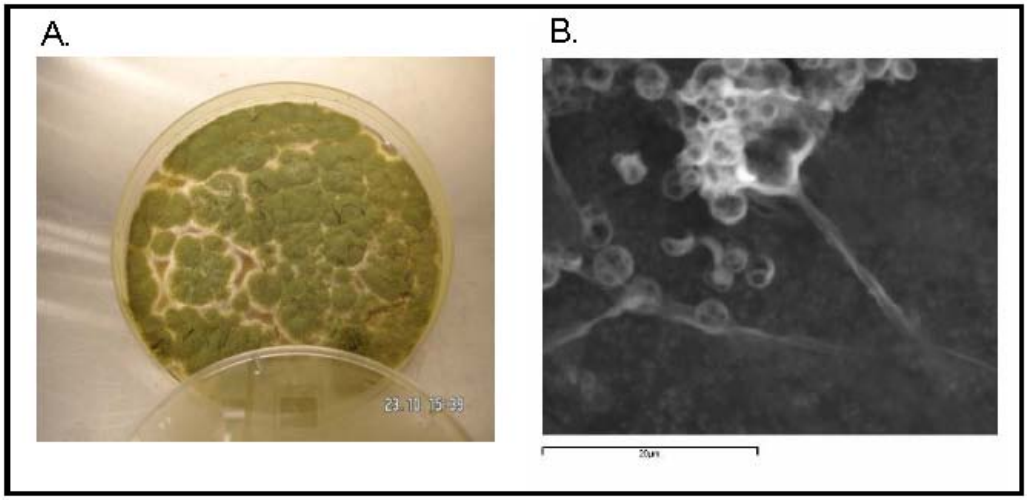

Figure 7-1. A) Typical grow of Aspergillus flavus on sabouraud agar. B) SEM image of the mould showing a) conidiophore; b). vesicle (it is a swollen conidiophore) ; c). conidiospores.

ISBN:978-84-692-9761-2/DL:T-203-2010

7.4. Complementary conclusions

The detection of microorganisms in real food samples remains a huge challenge due to the complexity of the matrix. The presence of proteins, polysaccharides, as well as the presence of a wide range of organic compounds can interfere in the device measurements. For rice samples, we have shown that diluting and using an effective blocking agent is enough to avoid interference. However, analysis of mycotoxigenic moulds by using CNTFETs in more complex matrix like foods with a great deal of fats (i.e. the peanut) would require certain supplementary sample pre-processing.

Analysis of foods requires the dilution process of the sample. The main objective of this step is to avoid interferences in the performance of the assays due to the complexity of the foods. However, when samples are diluted the limit of detection (LOD) decreases. CNTFETs applied to the detection of mycotoxigenic fungi in distilled water has a LOD of about 1ng/mL which is better than the LOD obtained using ELISA assays (1 μg/mL). However, when CNTFET are applied to a real sample the LOD increases 10^3-fold to ~10 μg/g. Therefore, the use of more effective blocking agents could help to overcome interference from the sample, therefore improving the LOD of the CNTFETs.

Finally, it is well known that nowadays there is an increasing interest in the development of fast, accurate, sensitive, user friendly and cost-effective biosensors for the detection of mycotoxigenic moulds in the food industry. The devices developed here, seem to go in the right direction to create a method able to provide some of the above mentioned characteristics.

7.5. References

Dantigny, P., Guilmart, A., Bensoussan, M. International Journal of Food Microbiology. **2005**. 100, 187–196.

Foong-Cunningham, S.C., Vasavada, P.C., Deak, T., Fung, D.Y.C. ARI The Bulletin of the Istanbul Technical University. Volume 54, number 4. http://www.ari.itu.edu.tr/previous_articles5/c.pdf. Dated of access; September, 2009.

Konietzny, U. and Greiner, R. Brazilian Journal of Microbiology. **2003**. 34, 283, 300.

Logrieco, A., Arrigan, D.W.M., Brengel-Pesce, K., Siciliano, P., Tothill, I. Food Additives and Contaminants. **2005**. 22, 335-344.

Parker, C.O., Lanyon, Y.H., Manning, M., Arrigan, D.W.M., Tothill, I.E. Analytical Chemistry. **2009**. 81, 5291–5298.

Tan, Y., Chu, X., Shen, G.L., Yu, R.Q. Analytical Biochemistry. **2009**. 387, 82-86.

Tsai, G.J. and Yu, S.C. International Journal of Food Microbiology. **1999.** 50,181–189.

Suanthie, Y., Cousin, M.A., Woloshuk, C.P. Journal of Stored Products Research. **2009.** 45, 139–145.

Yong, R.K. and Cousin, M.A. International Journal of Food Microbiology. **2001.** 65, 27–38.

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Chapter 8

Conclusions

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This chapter contains the general conclusions extracted from the overall research work. Furthermore, a set of attributes and skills acquired throughout the four years of doctoral research are also presented.

8.1. General conclusions

The present thesis reports the first electrochemical devices based on carbon nanotube field effect transistors applied to the detection of pathogenic microorganisms which can be of high relevance in the food and medical sectors. Experimental results proved that nanomaterials displaying extraordinary properties like carbon nanotubes can be combined with biological compounds to obtain highly sensitive and selective biosensors able to detect bacteria, yeasts and moulds in a very short time.

The small diameter, high aspect ratio and capacity of CNTs to transfer charges from heterogeneous phases make them ideal nanomaterials that can be incorporated in a configuration of field effect transistors, where they also act as the conducting channel. In this architecture the CNTs, acting as transducer element, are directly exposed to their immediate chemical environment, increasing in this way, the sensitivity with respect to the classical FETs. Taking advantage of this FET architecture, specific IgG antibodies were adsorbed as receptors on the surface of the carbon nanotubes to detect different pathogenic microorganisms.

In the first two studies converning the detection of *Salmonella* Infantis and *Candida albicans* there was not a forced orientation control of the antibodies over the CNTs. However, in the last study concerning *Aspergillus flavus*, the orientation of the molecular receptor was forced by adsorbing a recombinant-protein G over the carbon nanotubes previous to the interaction with the IgG. This protein provided stable and permanent anchoring for the antibodies through polar knobs-into-holes interaction. The results enabled the confirmation that the antibodies were well orientated providing high surface density of binding sites for the antigen.

ISBN:978-84-692-9761-2/DL:T-203-2010

The functionalized CNTFETs devices were characterized by both optical and electrical techniques. Scanning electron microscopy (SEM), environmental-scanning electron microscopy (ESEM) and Atomic Force Microscopy (AFM) provided valuable information regarding to the dimensions of the as-grown nanotubes as well as the change of these nanostructures through the different functionalization steps. By means of these techniques it was possible to confirm that IgG antibodies as well as protein G were strongly bound to the surface of the CNTs only after one hour of incubation time. Moreover, spectroscopic ellipsometry (SE) was used to make the first study about morphological characteristics of irregular surfaces like protein-modified carbon nanotubes and established the ideal conditions to obtain monolayers deposited over these nanostructures.

The electrical characterization of the CNTFET enabled the confirmation of the performance of the devices. Furthermore, the molecular recognition process between the antibody-microorganism was followed by recording the gate voltage dependence. The conducting channel of the CNTFET was a network of CNTs. The results showed a variation of the gate voltage dependence among the devices. This phenomenon can be attributed to different chiralities of CNTs as well as to the variation of the density of the CNT network among the devices with resistance ranging from 100 kΩ to 5 MΩ. As a consequence, the sensitivity was variable.

For all the devices, an overall decrease of the conductance was observed after each functionalization step. This decrease is due to the charge transfer process of the proteins adsorbed on the CNTs while the mechanism associated to the antigen-antibody interaction can be attributed to a scattering effect.

The development of the CNTFET devices was quite a simple process. CVD was used as a technique that allows obtaining networks of CNTs with good electrical behaviour in short time. However, it is still a challenge to optimize the process since the CNTs have different diameters and chiralities. Moreover, the CVD is not a completely reproducible process producing a variation in the density of the CNT network among the devices. The configuration of field effect transistors was obtained by depositing silver microelectrodes through a home-made silicon mask.

Compared to lithography, it is a simple, fast and inexpensive technique for electrode fabrication.

The regeneration of the sensor is one of the critical points of our devices. This is mainly due to two reasons: the first one, is related to the proteic nature of our molecular receptors which can undergo a denaturation process through time. However, it could be overcome by using other types synthetic receptors like aptamers or peptides which are more stable. The second one is due to the silver screen-printed electrodes. They were easily removed after being exposed to the regeneration solution. Therefore, a different deposition method of the electrodes like photolithography should be applied. However, this could increase the manufacturing cost.

In summary, the main challenge of this thesis was to develop CNTFETs devices that could be used to detect pathogenic microorganisms with improved performance parameters like sensitivity, selectivity and response time. Analyzing bacteria was the beginning in a simple matrix (0.85 % saline solution) and it was proven for the first time that CNTFET devices were able to detect, at least, 100 cfu/mL of *Salmonella* Infantis in just one hour with high selectivity. Subsequently, the application field was expanded to other type of microorganism, *Candida albicans.* In this study the detection limit of the devices was improved to 50 cfu/mL, but also the selectivity of the CNTFETs against possible interferences that can be present in real samples like serum proteins was proven. As a result, it was found that an effective blocking agent against small molecules was needed in order to enhance the selectivity of the devices.

Finally, taking into account the previous results, the devices were optimized, well characterized and tested in the detection of the mould *Aspegillus flavus* in real samples. In this assay the response time of the devices was 30 minutes and good sensitivity (10 µg of *A. flavus* / 25 g of rice) was obtained. However, other performance parameters such as long term stability, robustness and reusability must be studied further and contrasted with standard methods before to ultimately be used in the commercialization of the device.

ISBN:978-84-692-9761-2/DL:T-203-2010

8.2. Acquisition of attributes and skills

The development of the doctoral thesis provided a great deal of personal and professional experience in addition to the acquisition of a series of skills. A perspective was acquired on key topics in Nanoscience and Nanotechnology during the study of my master degree. It was structured through lectures and practical class sessions that offered a broad overview of the concepts needed to understand this growing field. Moreover, it gave me a large opportunity to ask questions and discuss these topics with mates. Additionally, the research activity provided important tips to help in the management of this current research and future projects.

Furthermore, the ability to master the bibliographical resources, extract the relevant information from them and use them as supporting material to discuss and compare experimental results with those obtained by others researchers was also adhered to the gamut of experience. Also acquired the skill to work in a research group and, to communicate, both orally and in a written form in English was enhanced. Moreover, to be able to develop presentations and to write articles published in scientific journals under ethical principles of originality and truth was also achieved.

As part of personal experiences an internship benefited this research for three months in Germany. It was an extraordinary opportunity to know people from different countries allowing me to share and learn other cultures aspects but even more important to perform a part of my research work in the Physical Institute of the University of Stuttgart. I learned about spectroscopy characterization techniques along with the ability to present the knowledge obtained in the field of carbon nanotube-based biosensors by means of scientific lectures and discussions.

Finally, during these last four years, I was also working with a high level research group together with people of different nationalities, thoughts and backgrounds. It was a great occasion to share knowledge which was very useful in the development of this research.

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Annexes

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Annex 1: Glossary

Ab: Antibody

AFM: Atomic force microscopy

Ag: Antigen

BSA: Bovine serum albumin

BHI: Brain-heart infusion broth

CB: Conduction band

CFU: Colony forming unit

CNT: Carbon nanotube

CNTFET: Carbon nanotube field-effect transistor

CVD: Chemical vapour deposition

DOS: Density of states

EF: Fermy energy

Eg: Energy required to move an electron from the valence band to the conduction band

ELISA: Enzyme-linked immunosorbent assay

E-SEM: Environmental scanning electron microscopy

eV: Electron volt

Fab: Fragment antigen binding

Fc: Constant fraction of the antibodies

FDA: Food and drug administration

FET: Field-effect transistor

GA: Glutaraldehyde

IgG: Immunoglobulin G

I: Electrical current

KDa: Kilodalton

LPS: Lipopolysaccharides

MOSFET: Metal-oxide-semiconductor field-effect transistor

MWCNT: Multi-walled carbon nanotube

NSB: Non-specific binding

NPs: Nanoparticles

OGY: Oxytetracycline, gentamicine yeast agar

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PEG: Polyetylenglycol

PEI: Polietylenimine

PBS: Phosphate buffer solution

PBSTG: Phosphate buffer solution containing Tween 20 and gelatine

PCR: Polimerase chain reaction

QDs: Quantum Dots

rpm: Revolutions per minute

sccm: Standard cubic centimeters per minute

SAM: Self-assembly monolayer

SB: Schottky barrier

SDA: Sabouraud Dextrose Agar

SEM: Scanning electron microscopy

SE: Spectroscopic ellipsometry

SWCNT: Single-walled carbon nanotube

TSA: Trypticase soy agar

TSB: Trypticase soy broth

Vg: Gate-to-source voltage

Vsd: Source-to-drain voltage

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Annex 2: Contributions to the scientific literature

Scientific articles

Authors: Raquel A. Villamizar, Alicia Maroto, F. Xavier Rius, Isabel Inza, Maria J. Figueras
Year: 2008
Title: Fast detection of *Salmonella* Infantis with carbon nanotube field effect transistors
Journal: Biosensors and Bioelectronics
Volume: 24
Pages: 279-283

Authors: Raquel A. Villamizar, Alicia Maroto, F. Xavier Rius
Year: 2009
Title: Improved detection of *Candida albicans* with carbon nanotube field effect transistors
Journal: Sensors and Actuators B
Volume: 136
Pages: 451-457

Authors: Raquel A. Villamizar, Julia Braun, Bruno Gompf, Martin Dressel, F. Xavier Rius
Year: 2009
Title: Morphological and electrical characteristics of biofunctionalized layers on carbon nanotubes
Journal: Biosensors and Bioelectronics
Volume: 25
Pages: 161-166

Authors: Raquel A. Villamizar, Alicia Maroto, F. Xavier Rius
Year: 2009

Title: Rapid detection of *Aspergillus flavus* in rice using biofunctionalized carbon nanotubes field effect transistors
Journal: Submitted to Analyst.

Congresses contributions

Contribution Type: Oral presentation
Authors: Raquel A. Villamizar, Alicia Maroto, F. Xavier Rius
Year: 2007
Title: Determination of *Salmonella* with carbon nanotube field effect transistors
Congress: 4th Nanospain Workshop
Place: Sevilla (Spain)

Contribution Type: Poster
Authors: Raquel A. Villamizar, Alicia Maroto, F. Xavier Rius
Year: 2007
Title: Fast, selective and label free sensor based on carbon nanotubes field effect transistor for Salmonella spp. determination
Congress: I Workshop Nanociencia y Nanotecnología Analíticas
Place: Cordoba (Spain)

Contribution Type: Poster
Authors: Raquel A. Villamizar, Alicia Maroto, F. Xavier Rius
Year: 2008
Title: Improved detection of *Candida albicans* with carbon nanotube field effect transistors
Congress: II Workshop Nanociencia y Nanotecnología Analíticas
Place: Tarragona (Spain)

Contribution Type: Poster
Authors: Raquel A. Villamizar, Julia Braun, Bruno Gompf, Martin Dressel, F. Xavier Rius
Year: 2009

Title: Morphological and electrical characteristics of biofunctionalized layers on carbon nanotubes

Congress: III Workshop Nanociencia y Nanotecnología Analíticas

Place: Oviedo (Spain)

Contribution Type: Poster

Authors: Raquel A. Villamizar, Alicia Maroto, F. Xavier Rius

Year: 2009

Title: Rapid detection of *Aspergillus flavus* in rice using biofunctionalized carbon nanotubes field effect transistors

Congress: III Workshop Nanociencia y Nanotecnología Analíticas

Place: Oviedo (Spain)

ISBN:978-84-692-9761-2/DL:T-203-2010

Annex 3: Popularization of science through the general information media

Interviews

Radio Nacional: Radio Regional de Murcia, España

Local TV Channel: Canal CataláTV (http://www.quimica.urv.es/quimio/nanosensors/)

Local Newspapers

Title: Científics de la URV desenvolupen un sensor que detecta el "Candida albicans" en una hora

Source: Diarimés

National Newspapers

Title: Un biosensor que detecta en una hora el hongo responsable de la candidiasis

Published by: El Mundo.es

Information available at:

http://www.elmundo.es/elmundosalud/2009/05/13/tecnologiamedica/1242208279.html/

National Web sites

Title: Las cándidas se pueden ver ya con transistores

Published by: Servicio de Información y Noticias Científicas (SINC)

Information available at: http://www.plataformasinc.es/index.php/esl/Noticias/Las-candidas-se-pueden-ver-ya-con-transistores/

Title: Transistors used to detect Candida

Published by: Science Centric

Information available at: http://www.sciencecentric.com/news/article.php?q=09050833-transistors-used-detect-candida/

Title: Investigadores de la URV desarrollan un biosensor para detectar el hongo "Candida albicans"

UNIVERSITAT ROVIRA I VIRGILI
BIOSENSORS BASED ON CARBON NANOTUBE FIELD EFFECT TRANSISTORS (CNTFETs) FOR DETECTING PATHOGENIC MICROORGANISMS
Raquel Amanda Villamizar Gallardo
ISBN:978-84-692-9761-2/DL:T-203-2010

Published by: Universitat Rovira I Virgili

Information available at: http://www.urv.cat/es_noticies/161/investigadores-de-la-urv-desarrollan-un-biosensor-para-detectar-el-hongo-candida-albicans/

International Web sites

Title: Sexually Transmitted Infections: Transistors Used To Detect Fungus Candida Albicans

Published by: Science Daily

Information available at: http://www.sciencedaily.com/releases/2009/05/090507094308.htm#/

Title: Nanotechnology Researches Develop Biosensor Capable of Detecting Candida Albicans yeast

Published by: Azonano.com

Information available at: http://www.azonano.com/news.asp?newsID=11355/

Title: Biosensor detects sexually transmitted disease in a jiffy

Published by: Thaindian News

Information available at: http://www.thaindian.com/newsportal/sci-tech/biosensor-detects-sexually-transmitted-disease-in-a-jiffy_100190858.html#/