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Overview of CARDIOSENSOR Project on the Development of a Nanosensor for Assessing the Risk of Cardiovascular Disease

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***Abstract*—**This paper aims at overviewing the topics of a research project (CARDIOSENSOR) on the field of health sciences (biomaterials and biomedical engineering). The project has focused on the development of a nanosensor for the assessment of the risk of cardiovascular diseases by the monitoring of C-reactive protein (CRP), which has been currently considered as the best validated inflammatory biomarker associated to cardiovascular diseases. The project involves tasks such as: 1) the development of sensor devices based on field effect transistors (FET): assembly, optimization and validation; 2) application of sensors to the detection of CRP in standard solutions and comparison with enzyme-linked immunosorbent assay (ELISA); and 3) application of sensors to real samples such as blood and saliva and evaluation of their ability to predict the risk of cardiovascular disease.

***Keywords*—**Carbon nanotubes field effect transistors, cardiovascular diseases, C-reactive protein, sensor.

I. Introduction

BASED on the latest statistics, the cardiovascular diseases are among the major causes of ill health, invalidity and death, both in Europe [1] as well as at the worldwide level [2]. In this field, the C-reactive protein (CRP) has been recognized as one of the most expressed proteins in case of acute phase inflammation, also being the best validated inflammatory biomarker associated to cardiovascular disease.

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CRP is normally found at low levels in serum and an increased production (up to 1000-fold or more) is induced during the acute phase response, i.e., infection, systemic inflammation, and tissue damage. In human serum, the levels of CRP can reach values lower than 1.0 mg/L, the moderate levels are between 1.0 and 3.0 mg/mL, and the high levels can be great than 3.0 mg/L [3] relatively to the risk of the incidence of vascular disease. Elevated levels of CRP are strongly associated to an increased risk of coronary heart disease, incident myocardial infarction, stroke, peripheral arterial disease, and sudden cardiac death [3], [4]. CRP has also been considered as an immunogenic protein due to its high molecular weight (115 kDa), suggesting a high molecular recognition and antibody binding capability with very high specificity [5]. Furthermore, the immunoassays are currently used in clinical analysis for the detection of CRP, i.e., by enzyme-linked immunosorbent assay (ELISA) method with a limit of detection (LOD) of 1 ng/mL, as well as by other analytical techniques such as immunochemiluminometric assays (LOD of 4 ng/mL), immunoturbidimetry (LOD of 0.2 μg/mL), and rapid immunodiffusion [6], [7]; however, such methods based on optical detection principles are time consuming, and very expensive. Besides, they need labels, they require sample pre-treatment procedures, and they are not direct reading methods since they need the development of secondary reactions for detection of the analyte of interest. In terms of biosensing systems for CRP detection, various biosensors based on immunosensing are reported with magnetic and optical transduction principles [8]-[12]; however, the large sample size, the long time required for measurement, the intensive labour processing, and the expensive equipment are pointed out as limitations of such systems.

From such considerations, novel approaches on the development of disposable, accurate and sensitive analytical methodologies, as well as being low-time consuming without sample pre-treatment, have been needed in such field. A research project CARDIOSENSOR (Cardiovascular Disease Risk Nanosensor) was proposed on the field of health sciences (biomaterials and biomedical engineering) by a research team with an established experience on the development of sensors/detectors for environmental [13]-[21] and food applications [22]-[24] and also clinical applications [25]-[29]. The project has been financed by European funds (FEDER) under the Operational Program for Competitiveness Factors (COMPETE) and by Portuguese funds via FCT (Fundação

para a Ciência e a Tecnologia, Portugal), and it consists in the development of a nanosensor for the assessment of the risk of cardiovascular diseases by monitoring the levels of CRP on real samples, such as blood and saliva; the work scope of the project is schematized in Fig. 1.

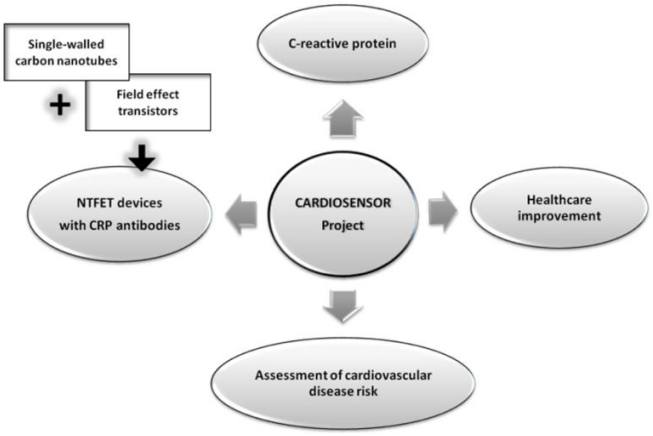

Fig. 1 Schematics of the work scope of the CARDIOSENSOR project for the assessment of the risk of cardiovascular disease

In the present paper, we give an overview of the general topics of CARDIOSENSOR project summarizing its aims and scope, the main results already obtained, and further perspectives on the possible application of nanosensor in suitable diagnosis of cardiovascular disease in subjects.

II. Scope of the Project

On the scope of CARDIOSENSOR project, the developed disposable nanosensors are based on field effect transistors (FET) devices with single-walled carbon nanotubes (SWCNT) for the detection of CRP through immunoreaction. This approach was based on various tasks with respective sub-tasks, and some of them have already been developed, i.e., a) microfabrication of FET devices, and study of the better geometry parameters in order to improve the device performance; b) preparation of SWCNT dispersion in order to obtain individual and dispersed SWCNT (application of an experimental design to found the better experimental conditions for dispersion of SWCNT) and spectroscopic characterization to identify the electronic and vibrational structures of dispersed SWCNT; c) assembly of sensor devices (carbon nanotubes field effect transistors, NTFET) and respective electrical characterization; and, d) application of NTFET devices to CRP standard solutions, and study of their analytical performance. The project also contemplated other tasks which will be developed as near future work, and they will consist in the application of sensors for the detection of CRP in real samples such as blood and saliva, and the comparison of results with standard immunoassays (e.g., enzyme-linked immunosorbent assay, ELISA). The results obtained from saliva and blood will be compared themselves in order to know if saliva can constitute a potential biological fluid to perform non-invasive analysis of CRP, which it is painless and its associated to easy sampling. The collection of blood (invasive sampling) and saliva (non-invasive sampling) will be made in healthy subjects and in persons whose suffer of various diseases including cardiovascular diseases, in order to study the correlation between CRP levels and the incidence of diseases. Questionnaires will be made in all subjects in order to identify their medical story and diary habits that may interfere with CRP levels.

III. Description of the Project Experiments

A. Dispersion of Single-Walled Carbon Nanotubes

The dispersion of single-walled carbon nanotubes (SWCNT) was based on the non-covalent functionalization of commercially available SWCNT (Sigma-Aldrich) in aqueous solutions of sodium cholate (0.2% w/v SC, Sigma-Aldrich). Physical procedures such as sonication and centrifugation were employed to unbundle the dispersed SWCNT, and to remove the impurities (e.g., amorphous carbon and graphite), respectively. The experimental conditions for sonication (time of sonication) and centrifugation (i.e., time and relative centrifugal force) were optimized through the application of an experimental design based on a 2^3 factorial design, as reported elsewhere [21]. The final SWCNT dispersion (0.28 mg/mL) was characterized by Raman spectroscopy and ultraviolet-visible spectrometry for electronic and structural information, and it was applied for the assembly of carbon nanotubes field effect transistors (NTFET) which function as the biosensing systems on this project.

B. Device Fabrication

The fabrication of field effect transistors (FET) devices was based on standard techniques of microfabrication, namely on:

a) Passivation of SiO_2 layer on silicon wafer by plasma enhanced chemical vapour deposition;

b) Deposition of Ti/Au bi-layer (obtained by sputtering) on SiO_2;

c) Coating of a positive photoresist layer by spin coating, and definition of Ti/Au source and drain electrode contacts by optical lithography system, and ion beam etching;

d) Removing of thin SiO_2 layer by reactive ion etching, and deposition of Cr/Au films by ion beam deposition;

e) Cleaning of wafer with isopropanol and distilled water, and drying with a stream of nitrogen gas.

f) Cutting of wafer to obtain individualized FET (~3x2 mm^2), which were fixed to a print circuit board (PCB), wirebonding of each electrode to a soldered pin, and protection of all wirebondings by silicone also for the definition of an open chamber for sensing experiments.

Various combinations of different geometry parameters (distance between electrodes, number of electrodes and thickness of gold layer) of FET devices were studied on the device performance, i.e., the FET devices with lower resistances were selected for further sensing experiments.

C. Assembly of NTFET Devices

A droplet of SWCNT dispersion was placed over the microfabricated FET surface; after a delay of 5 minutes, the

droplet was blown off using a nitrogen flow to form an SWCNT network bridging the FET electrodes.

Electrical measurements were made with a parameter analyzer (HP4155C, Japan), using the Desktop EasyExpert software for the acquisition of data in real-time, in air, and at room temperature. The source, gate and drain of FET devices were connected through connections cables in a closed test fixture (Agilent 16442A, Japan) where each device was positioned for measurements.

Each FET device was characterized from the output data recorded by measuring the drain current (I_D) obtained with the applied drain voltage (V_D, between 0 and +2V), as well as the measurement of I_D values against back-gate voltage (V_G, between -5 and +5V) at a fixed drain voltage (V_D = +1V) to obtain transfer characteristics.

D. Sensing Experiments

As the immunoreaction was used for the signal transduction of NTFET devices, a droplet of anti-CRP solution was pippeted on NTFET surface and stored overnight at 4ºC.

Diluted solutions of CRP, in the 10^{-3} to 10^{2} µg/mL range, were prepared by serial dilutions from a 10^{2} µg/mL stock solution. About 1 µL of each CRP solution were successively dispensed on the NTFET surface; after 15 minutes of incubation, the NTFET surface was washed with distilled water, and then the drain current (I_D) was measured three times (at V_D = +1V, and V_G = +1V), and on three individual devices.

The analytical signal was considered as the change of current for each CRP concentration after their interaction with specific antibodies.

IV. Highlights of the Results

The dispersed SWCNT were characterized by Raman spectroscopy and UV-Vis spectrometry. Typical Raman spectra of the dispersed SWCNT, taken with 1064 nm laser excitation and averaged from 1500 scans, is shown in Fig. 2a. The radial breathing modes (RBM), observed between 100 and 300 cm^{-1}, indicate that the nanotubes have diameters (d) in the range of 0.9-1.8 nm (d= 234 $cm^{-1}nm/\omega_{RBM}$-10). The G-bands, shown between 1550 and 1605 cm^{-1}, indicate the presence of semiconducting nanotubes (around 1590 cm^{-1}), and metallic nanotubes (around 1570 cm^{-1}). Fig. 2b shows an absorbance spectrum where the typical interband electronic transitions were observed between 400-600 nm (assigned to metallic SWCNT), and between 600-800 nm (assigned to the semiconducting SWCNT).

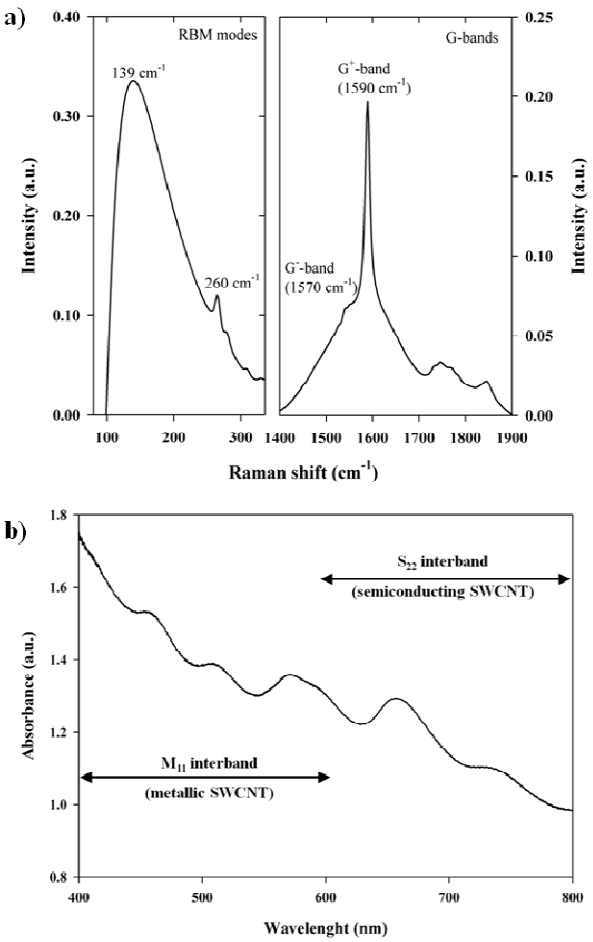

Fig. 2 a) Raman spectrum of dispersed SWCNT showing the characteristic RBM modes and G-bands; and b) absorbance spectrum of dispersed SWCNT in SC

SWCNT were then deposited onto FET surface to provide the assembly of NTFET devices. The developed NTFET devices were electrically characterized and a hole transport conduction was observed as the conduction process.

Schematic diagrams of the successive steps from the deposition of SWCNT onto FET surface to the application of NTFET devices to CRP standard solutions were shown in Fig. 3, as well as the respective electrical response. For each step, the output signal was the drain current (I_D) values measured at V_D=+1V and V_G=+1V.

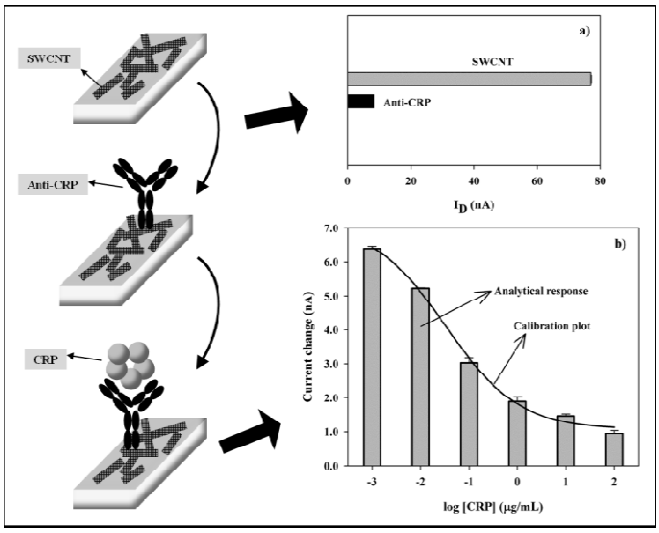

Fig. 3 Successive steps for the detection of CRP through interaction with their specific antibodies (anti-CRP) which were previously immobilized in NTFET surface; (a) Change in current before and after immobilization of anti-CRP; and (b) analytical response from the detection of CRP by NTFET devices and corresponding calibration model

Fig. 3a shows the change in I_D values of a typical NTFET device, before and after the modification of sensing surface with specific antibodies for CRP (anti-CRP). It was observed that the I_D values have decreased after the immobilization of anti-CRP on NTFET surface, suggesting the adsorption of such charged biomolecules on the SWCNT surface.

In turn, Fig. 3b shows the analytical response (in a logarithmic scale) obtained from the detection of CRP standard solutions at different concentrations (from 10^{-3} and 10^{2} µg/mL), as well as the better calibration model obtained for such analytical response.

The analytical response of NTFET devices, which was considered as the change in I_D values after the interaction of CRP with their specific antibodies, decreases with the CRP concentration, suggesting that a reducing of holes mobility exists; it can be due to the antigen-antibody interaction which causes geometrical deformations on nanotubes, which consequently increases the scattering centres among them.

The analytical parameters for the calibration model obtained from the analytical response of the NTFET devices to CRP (Fig. 3b) were summarized in Table I.

TABLE I
ANALYTICAL PARAMETERS OF CALIBRATION MODEL FROM ANALYTICAL RESPONSE OF NTFET DEVICES TO CRP

Calibration model	$y = D + [(A - D) / (1 + 10^{(x - logC)*B}]$	
Coefficients	A (µg/mL)	7.1380 ± 0.5382
	B (nA.(µg/mL)$^{-1}$)	0.5764 ± 0.1112
	logC (µg/mL)	-1.4771 ± 0.1725
	D (µg/mL)	1.0694 ± 0.1834
R^2	0.9809	
p value	< 0.0001	

From Table I, a high correlation between the analytical response and the concentration of CRP was found (R^2 of 0.9809, $p < 0.0001$).

In terms of reproducibility, an adequate variability was observed from the application of NTFET devices to CRP detection, i.e., an average coefficient of variation of 8.5±4.8% was obtained (calculated from the average and standard deviation obtained on three individual NTFET devices for all concentration of CRP tested). Furthermore, it was found that there is not a statistically significant difference ($p = 0.9428$) between various NTFET devices for the same CRP concentration, when an ANOVA analysis was applied to the experimental data.

V. CONCLUSION

The major scientific impact of the CARDIOSENSOR project is to provide suitable and disposable biosensing devices which can improve the diagnosis of the risk of cardiovascular diseases in subjects, through a non-invasive methodology. The tasks concerning the fabrication of FET devices, the assembly of NTFET devices through the deposition of SWCNT, and the application of NTFET devices to CRP standard solutions were completed.

At this time, the collection of real samples and questionnaires about the medical story and diary habits of volunteers subjects have been made for the further application of developed NTFET devices to such real samples, i.e., blood by invasive sampling, and saliva by non-invasive sampling. As next task, comparison between the results obtained by NTFET devices and those of standard ELISA assays will be performed to provide the validation of developed biosensing devices. Furthermore, results from the detection of CRP on blood and saliva will be compared in order to know if a correlation exists between the levels of CRP on these two biological fluids; this correlation can then be used to develop a prototype of NTFET devices for the screening of CRP by non-invasive methodology.

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