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Protein immobilization on 3C-SiC (100) as a substrate for detecting the onset of acute myocardial infarction (AMI)

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Abstract— **Silicon Carbide (SiC), has been shown to be a bio- and hema-compatible substrate that could potentially be used in biosensor applications. The development of a viable biorecognition interface using SiC as the substrate material for bio-detection is described. Surface modification with 3-aminopropyltriethoxysilane (APTES) and immobilization via covalent conjugation of anti-myoglobin (anti-Myo) on the modified surfaces is achieved, which are initial steps for immunosensing based devices. Successful formation of APTES layers and antibody immobilization were identified with surface water contact angle (SWCA), X-ray photoelectron spectroscopy (XPS) and atomic force microscopy (AFM).**

I. Introduction

The early and accurate diagnosis of disease using key biomolecular biomarkers is of major importance for the medical community. Enabling the continuous monitoring of physiological parameters that provide a physician with the knowledge to determine a proper therapy for their patient is slowly becoming a major concern in the biomedical research [1].

Rapid diagnostics using post-operatory devices for short and long term monitoring of patients, especially following coronary surgeries, presents an opportunity for the development of biosensors [2]. The use of biomarkers, in addition to other techniques, can produce more effective and targeted diagnoses [3]. On the other hand, the availability of materials that can be implanted long term is one of the main components in such a system.

Individuals with silent cardiac syndrome develop myocardial infarctions (MI) that go unnoticed and require periodic examinations [4]. Around 80-100% of patients who have suffered MI manifest silent ischemic episodes [5] and the main tool for diagnostic is the registration of non-specific ST changes in the patient's electrocardiogram (ECG). Unfortunately, there may be 10 sec following the occurrence of a MI before electrocardiographic changes can be detected [4].

The continual monitoring of Troponin I, Creatine kinase (CK1) and Myoglobin (Myo) can provide highly specific diagnostic data even before the clinical manifestations of a MI becomes apparent. In particular, Myo concentrations rise quickly after a MI event and is thus considered a biomarker for early detection of MI [6].

Silicon Carbide (SiC), more specifically 3C-SiC, has been shown to be a bio and hema-compatible semiconductor material [7–9], extensively used and studied as a heart stent coating [10], [11] that can be used in long-term biological applications. This material offers a high degree of chemical and mechanical stability and current silicon device (CMOS) process technology can be used for device fabrication. The fact that 3C-SiC can be grown by chemical vapor deposition on Si substrates has made it an attractive material for MEMs and Bio-MEMs applications [9], [12].

In this paper we describe the characterization of 3C-SiC surfaces following activation, after surface modification with 3-aminopropyltriethoxysilane (APTES) and subsequent bio immobilization with anti-myoglobin (anti-Myo). This is the preliminary step to building a 3C-SiC impedance-based device to determine Myo concentration. Several characterization techniques, including surface water contact angle (SWCA), X-ray photoelectron spectroscopy (XPS) and atomic force microscopy (AFM), were used as means to confirm the suitability of SiC as a biosensor substrate.

II. Experimental

A. Surface cleaning and preparation

3C-SiC was grown in a horizontal, low pressure, CVD reactor on (100) silicon (Si) substrates at 1385°C [13]. The 3C-SiC samples were prepared by solvent cleaning, first in acetone then in isopropanol for 5 min each, followed by piranha cleaning (H_2SO_4:H_2O_2) (1:1) for 10 min and then immersed in HCl: H_2O (1:2) for 2 min and finally dipped in 5% aqueous hydrofluoric acid (HF). Surface hydroxylation (-OH termination) was achieved with a final HF treatment done immediately prior to the functionalization reaction.

B. Surface functionalization

Surface functionalization of 3C-SiC with APTES (Sigma-Aldrich) was done by immersion in a 1% APTES/toluene solution for 90 min. The samples were then rinsed by ultrasonication with toluene, toluene/isopropanol (1:1) and isopropanol for 20 min and 10 min for the last two steps.

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This was followed by a baking step at 100°C for 1 hr in an oven. Static water contact angle (SWCA) measurements were acquired using a KSV CAM 101 system in which 3 ml DI water droplets were deposited on the hydroxylated 3C-SiC and the APTES modified 3C-SiC. In addition, XPS was used (SPECS XR-50 Mg-Anodex-ray source, EKα = 1253:6 eV and photoelectron detection with a SPECS Phoibos 100 hemispherical analyzer and a MCD-5 detector) to measure the elemental composition and the chemical state of the elements on the SiC surface. The comparison of the survey and core level spectra of the hydroxylated SiC and the functionalized surfaces are used to confirm the successful formation of APTES layers on SiC. In addition, a XEI 100 scanning probe atomic force microscope (Park Systems) was used to analyze the surface topography before and after functionalization.

C. Anti-myoglobin immobilization

The immobilization of anti-myoglobin (Fitzgerald Industries) on the APTES modified SiC surface was performed via covalent conjugation at a concentration of 100 ug/ml.. Covalent coupling was achieved by activating carboxylic acid groups of the anti-myoglobin with 2 mM 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) (Proteochem™). Since EDC produces a reactive unstable intermediate, 5 mM *N*-hydroxy-sulfosuccinimide (Sulfo-NHS) (Proteochem™) are also added in MES buffer (2-(*N*-morpholino)ethanesulfonic acid) at pH 5. The EDC, sulfo-NHS and antibodies are deposited directly on the APTES|SiC surface and incubated overnight at 4°C, this process is illustrated in **Fig. 1**. After incubation of the antibodies, subsequent washes with PBS and 0.05% Tween 20 were performed followed by a blocking step consisting of washing with 1% Bovine Serum Albumin (BSA) in Phosphate Buffered Saline (PBS).

The surfaces used as controls for this experiment included, i) physical adsorption of the antibody on the surface, which was studied by omitting the EDC-Sulfo NHS covalent conjugation step, and ii) omission of the BSA blocking step. The former control reports differences between covalent and non-covalent immobilization of anti-myoglobin. The latter control investigated blocking by the adsorption of BSA as a means to prevent non-specific adsorption of Myo. AFM was employed to monitor the surface topography after antibody immobilization and to check the uniformity of the surface.

III. RESULTS

A. Surface functionalization

SWCA following surface cleaning and hydroxylation of the 3C-SiC substrates was found to be 16±3° and after APTES surface modification it was 62±1°, which shows an increase in surface hydrophobicity similar to the findings of Schoell *et al.* for 3C-SiC functionalization with aminopropyldiethoxymethylsilane [14], Bierbaum *et al.* of aminopropyltrimethoxysilane on Si (111) [15] and Baur *et al.* of APTES on GaN and AlN [16]. AFM analysis of the surface topography of the 3C-SiC and chemically modified surfaces demonstrated that no surface aggregates or new features were produced, which is evident in the small change of the value of the surface roughness. For the bare surface the RMS roughness was ~0.9 nm and for the APTES-modified surfaces the RMS roughness value was 1.1 nm. These observations suggest formation of a smooth uniform organic layer (**Fig. 2a and 2b).**

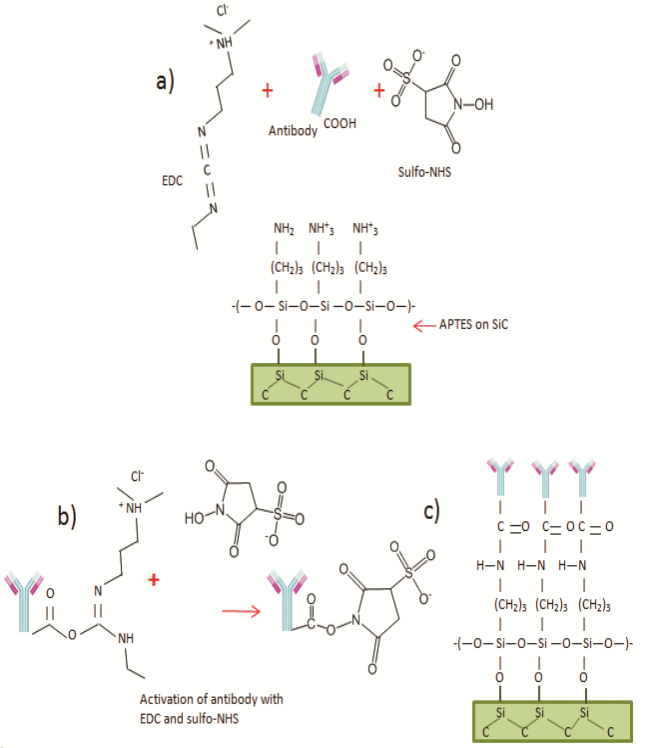
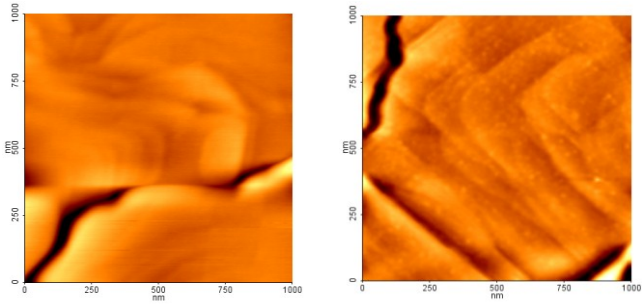


Figure 1. Process followed for anti-myoglobin immobilization. a) EDC-Sulfo NHS solution and anti-myoglobin are deposited on APTES|SiC samples, b) Activation of the antibody carboxylic group with EDC, sulfo-NHS produces a semi-stable amine-reactive ester and c) Antibodies coupled to the surface.



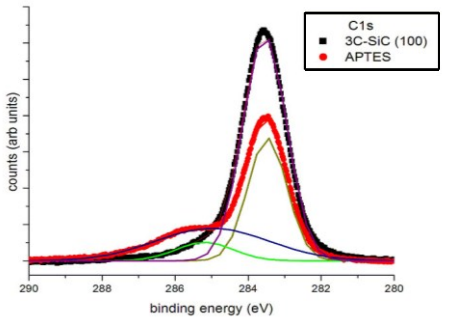
a) b)

Figure 2. AFM micrographs (1000 X 1000 nm^2, z scale 3 nm) of a) hydroxylated 3C-SiC. b) Corresponding APTES modified surfaces. Images taken in non-contact mode.

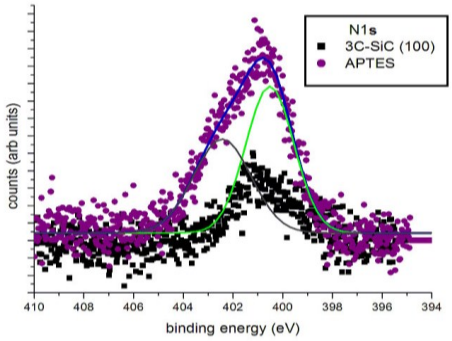
XPS analysis revealed that the 3C-SiC was successfully modified by the identification of new carbonaceous and amino species on the modified surfaces. The deconvolution of the C1s spectra resulted in two components one at 283.5 eV that can be attributed to the C of the SiC substrate and

another one at 285.5 eV (**Fig. 3a**) due to the APTES-modified samples, which can be attributed to hydrocarbons at the surface. These peaks have also been identified by Schoell *et al.* and Rosso *et al.* in previous reports on 6H-SiC and 3C-SiC surface functionalization [14], [17], [18]. Following surface modification with APTES, an attenuation of the SiC substrate peaks for the C1s core level was observed, as seen in **Fig. 3a**), due to the presence of overlying organic layers.

For the N1s core level spectra, two components were resolved at 400.5 eV and 404.2 eV (**Fig 3b**), which arise from terminal -NH_2 and -NH_3^+ functional groups [14], [15]. This result demonstrates the effective formation of thin organic layers with a high concentration of reactive NH_2 groups that would allow for successful attachment of the anti-myoglobin on the surface.



a)



b)

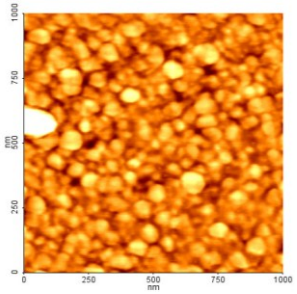
Figure. 3. a) C1s core level spectra and. b) N1s core level spectra of the 3C-SiC after HF dip and APTES surface functionalization. The solid lines give the fitted spectral components of the deconvoluted data.

B. Anti-myoglobin immobilization

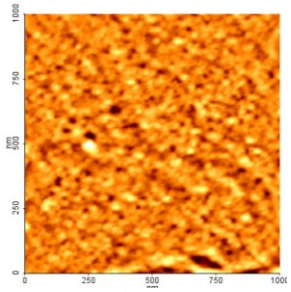
The qualitative evaluation of the anti-myoglobin immobilization was also done with AFM. Three types of surfaces were evaluated: Surface A, covalent conjugation of anti-myoglobin to the surface followed by BSA blocking, Surface B, non-covalent linkage of anti-myoglobin to the surface followed by BSA blocking, and Surface C, with no BSA blocking step following the EDC/Sulfo-NHS covalent coupling of anti-Myo to the surface.

Surface A shows a uniform and densely packed antibody film with some isolated proteins. Some of the images present particles with diameter ~20 nm while for others the length was close to 50 nm (**Fig. 4a**). We also observed that the particles have a globular shape due to its protein nature, as it was reported by several authors[19–21]. On the other hand, Surface B presents smaller particles packed in molecular arrays where the antibodies appear to adsorb in clusters. However, there are certain regions where no antibody adsorption occurred as seen in **Fig. 4b**). This surface presents non-specific adsorption of anti-Myo because of the absence of covalent conjugation with EDC/Sulfo NHS. In addition, there a lower amount of anti-Myo on Surface B compared to Surfaces A and C. A blocking agent was used to fill up those gaps where covalent immobilization of anti-Myo has not occurred and to prevent cross-reactivity and/or non-specific adsorption of other proteins.

For Surface C, where the BSA blocking step was omitted, similar images to **Fig 4a**) were obtained; however, there were fewer clusters and less protein accumulation, which can be seen in some of the darker spots in **Fig. 4c**).The larger aggregates on Surface A are likely due to the presence of BSA, a well know sticky protein, that readily adsorbs and forms aggregates. Surface C reflects the absence of the blocking protein, as the size of the particles have been reduced to an average of 30 nm. If the blocking step with BSA is omitted, the antibody immobilization process is not affected. However, it may impact the successful quantification of the antibody-antigen interaction because non-specific binding of the Myo (antigen) could possibly take place on the surface, which leads to poor results.



a)



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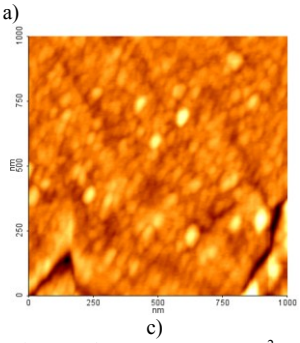

c)

Figure. 4. AFM micrograph (1000 X 1000 nm^2, z scale 5 nm, non-contact mode) after myoglobin immobilization a) surface A (covalent immobilization and BSA blocking), b) surface B (non-covalent adsorption) and c) surface C (covalent immobilization and no BSA blocking.

IV. DISCUSSION

In this article an efficient means for the attachment of biomolecules to SiC has been described. We have demonstrated and successfully achieved immobilization of anti-myoglobin on 3C-SiC. Surface functionalization of the 3C-SiC surface with APTES served as the starting point to link antibodies to the substrate. The fact that the APTES layer was uniform, as seen with AFM, and showed a change in surface wettability after chemical treatment of 3C-SiC, allowed the antibodies to attach to the functional moiety of the self-assembled monolayer. The use of covalent conjugation agents like EDC/Sulfo-NHS on Surface A compared to Surface B (where no EDC/Sulfo-NHS was used) yielded appreciable differences in the molecule conformation on the surface, i.e. homogeneous and uniform for Surface A while Surface B had areas where no proteins have been attached. We have also seen ovoid protrusions with diameters from 45 to 60 nm similar to the work done by Ehrhart *et al.* for hepatitis detection on polystyrene [19]. This could be due to the superimposition of two Y shaped immunoglobulins (IgG) that were randomly distributed on the surface. We would not expect the molecules to exhibit a Y shape because of the conformational flexibility of IGgs and interactions with the substrate. The results presented in this article suggest that 3C-SiC is a suitable substrate material for immunosensing based devices as was shown in Section III. Further investigation to optimize the immobilized antibody concentration as well as the blocking step (influence on myoglobin detection) needs to be performed with fluorescence tagged proteins immobilized to the anti-myoglobin surface using the appropriate controls.

V. CONCLUSION

This study shows successful formation of APTES layers that produce $-NH_2/-NH_3^+$ free functional groups for later covalent immobilization of bio-molecules. We have studied the viability of using 3C-SiC as a biosensing platform implementing an immunosensor based approach. In this case, we are focused on diagnostic myocardial devices and, for this reason we performed covalent immobilization of anti-myoglobin using EDC-Sulfo NHS chemistry. The XPS and AFM results presented show evidence of homogeneous APTES formation as well as uniform coverage of antibodies on the surface as an optimal starting point for a biomarker detection device. Nevertheless further investigation into a lower antibody concentration and adequate blocking step need to be performed to guarantee the specificity of myoglobin detection.

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