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Electrochemical Biofunctionalization of Highly Oriented Pyrolytic Graphite for Immunosensor Applications

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The present research demonstrates a procedure for surface modification of Highly Oriented Pyrolytic Graphite (HOPG) electrodes intended for use as immunosensors. The HOPG surface is linked to the molecule 8-hydroxydeoxyguanosine (8-OHdG), an oxidative stress biomarker for DNA damage, though the aniline mediator covalently bonded to electrode and biomarker. An electrochemical procedure to graft the mediator is described and the presence of biomarker at surface is demonstrated by using a fluorescence-labeled immune-reagent. An electrochemical functionalization process has been employed for attachment of functional aminie (NH_2) linking groups to graphitic surfaces, which consists of two stages: (i) a reaction with a diazonium salt to covalently bond nitrobenzene groups to the surface and (ii) electrochemical reduction of the nitro group ($-NO_2$) to an amine group ($-NH_2$). The shape of the CV curve indicates that the redox reactions are taking place at the HOPG electrode surface. The amine group can subsequently be used to covalently link to an antibody biorecptor. The presence of 8-OHdG, indicative of DNA damage, has been linked to increased cancer risk. Detection of this oxidative stress biomarker is an important tool for the early diagnosis of disease. [DOI: 10.1380/ejssnt.2016.193]

Keywords: Biofunctionalization; Hydroxydeoxyguanosine; Highly Oriented Pyrolytic Graphite

I. INTRODUCTION

A biosensor is a self-contained integrated device that is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element, which is in direct spatial contact with a transduction element. The transductant in this study is an antibody that enables specific detection of a biomarker, 8-hydroxydeoxyguanosine (8-OHdG), the presence of which results in a change registered in the biosensor [1]. The investigation of biosensor devices has been prompted by a demand for high sensitivity diagnostic sensors for biomarkers for a range of diseases. Highly Oriented Pyrolytic Graphite (HOPG) sensors offer the potential for biosensing applications which is related to its electronic properties. HOPG is chemically similar to graphene but is used as a low cost development model for surface functionalization research [2–4]. The mechanical, electrical and chemical properties of chemically modified graphene (CMG) are intrinsically linked to its structure [5].

The modification of the surface chemistry of semiconductors via chemical functionalization can be used for introducing new properties to the material. A property of graphene is that it has an intrinsic zero band-gap energy. functionalization modifies the carrier concentrations and other electronic properties of the graphene substrate [6]. In order to use semiconductors as a sensor, the band-gap has to be opened in order to allow it to be used as a biosensor [7]. By altering the electronic structure, structural imperfections this can alter the chemical properties and reactions of semiconductors.

functionalization of semiconductors can take place at several different sites such as: dangling bonds, defects, edges and the basal plane itself. functionalization of graphene may also be influenced by methods of graphene

production, chemical reactivity and solubility. Differences in electron transfer rates may be important for selecting and manipulating graphitic materials on-chip [8]. HOPG surfaces can be functionalized and can be utilized specifically for electrochemical biosensors [9]. It has been reported that the surfaces, electronic and structural properties of graphene-based films fabricated by thermal reduction of exfoliated graphite oxide are very similar to those of HOPG [10], and HOPG has thus been used extensively in this work as a model for graphene.

II. RESEARCH METHODOLOGY

A. Functionalization of the HOPG Surface

Cyclic Voltammetry (CV) has been used to electrochemically functionalize the surfaces of HOPG with nitro groups ($-NO_2$) and subsequent chronoamberommetry used to convert it to an amine group ($-NH_2$). CV measurements were carried out with a potentiostat in a three-electrode configuration, in which the HOPG arrays were used as the working electrodes. A platinum (Pt) auxiliary electrode and an Ag/AgCl or Ag/Ag+ reference electrode were used. The attachment of the nitro phenyl groups to the surface was performed in the presence of non-aqueous 4-nitrobenzene diazonium tetrafluoroborate (2mM Sigma-Aldrich) in acetonitrile (ACN) and tetrabutylammonium tetrafluoroborate (NBu_4BF_4) 0.1 M (Fisher). After the chemical functionalization reaction to attach nitrobenzene to HOPG, the samples were cleaned with acetonitrile (CH_3CN) then rinsed with dichloromethane (CH_2Cl_2) in order to remove any physisorbed organic residues on the sample surfaces.

In order to fabricate biosensor devices, the semiconductor is functionalised with a "receptor" which interacts with a target molecule to give an electrical signal response. The attachment of a "bioreceptor" to the semiconductor surface required chemical functionalization of the semiconductor surfaces with "linking" groups or molecules, which can then be used to bind to biorecep-

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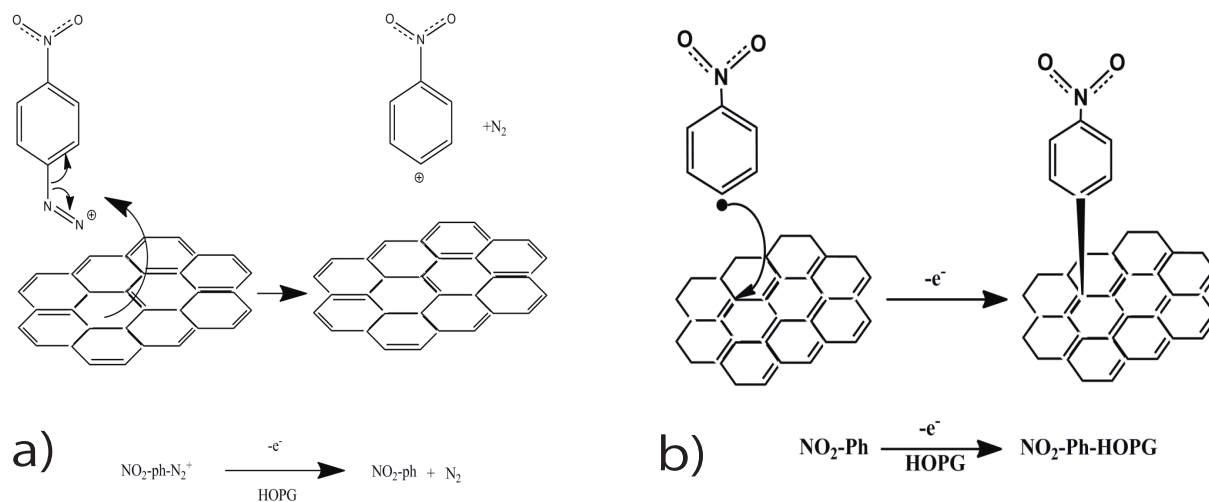
FIG. 1. (a) Electron transfer from an HOPG electrode to diazonium cation and subsequent loss of N_2 to form an aryl radical (b) Nitrophenyl radical and an electron from the π bond of surface HOPG generates covalent bond and releases an electron.
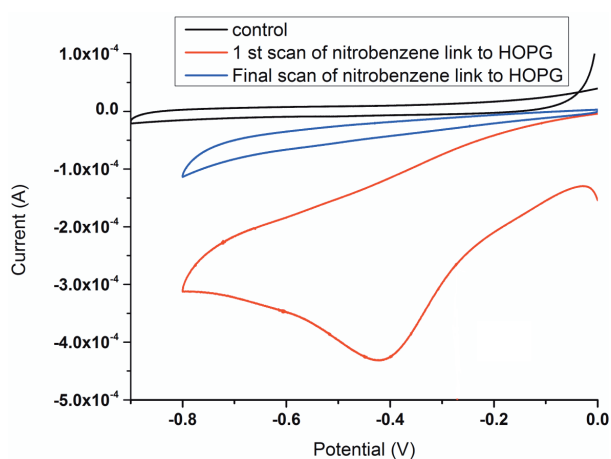

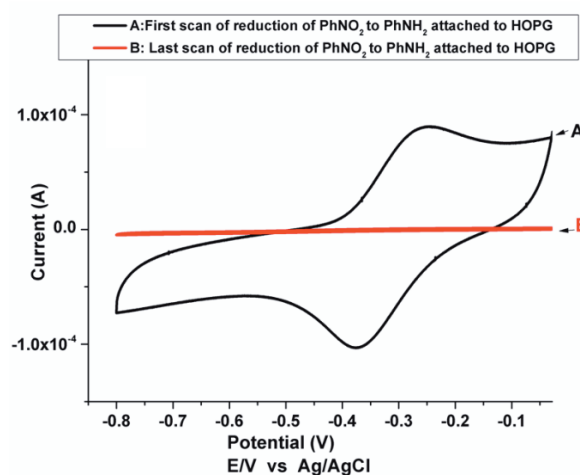FIG. 2. Cyclic voltammogram of a functionalized HOPG surface.

FIG. 3. Cyclic voltammograms of the reduction of surface-grafted 4-nitro-phenyl on (1) the HOPG electrode; (2) the HOPG electrode in 0.1 M KCl in ethanol/water (1:9), at 100 mV s^{-1}.

tor biomolecules. Cyclic Voltammetry (CV) was used to electrochemically functionalise the surfaces of HOPG with nitrobenzene. CV measurements were carried out with a potentiostat in a three-electrode configuration.

All experiments used the Highly Oriented Pyrolytic Graphite (HOPG) surface as the working electrode, a platinum (Pt) auxiliary electrode and an Ag/AgCl electrode as the reference electrode (purchased from BASi Company, USA). The first functionalization step used an electrochemical reaction of 4-nitrobenzene diazonium tetrafluoroborate (2 mM) (Sigma-Aldrich) in an acetonitrile (Fisher) / 0.1 M tetrabutylammonium tetrafluoroborate (NBu_4BF_4) (Fisher) electrolyte with the surface of the HOPG electrode, to form a thin film of covalently attached nitro phenyl ($PhNO_2$) groups on the HOPG surface. After the $PhNO_2$ functionalization, the HOPG sample was cleaned with acetonitrile then rinsed with dichloromethane in order to remove any physisorbed organic residues on the HOPG surface. The second stage of the functionalization process reduced the $PhNO_2$ to an amine (NH_2) in an electrolyte, 0.1M KCl (H_2O: EtOH,

90:10). This resulted in an aniline ($PhNH_2$) functionalised HOPG surface. The surface-bound amino group can covalently bind to virtually any biomolecule containing a carboxyl group forming an amide link.

B. Biofunctionalization Processes

Following the chemical functionalization process with the aniline linking molecule, the SiNW chip was biofunctionalized using an antibody bioreceptor, targeted against the oxidative stress biomarker, 8-OHdG. The primary antibody was that of a mouse monoclonal anti-8 hydroxyguanosine antibody which was diluted in PBS to a concentration of 2mg/ml and applied to the SiNW and incubated at 4°C for 4 hours before rinsing in deionized water. In order to confirm the successful primary antibody attachment to the SiNW surface, this was achieved
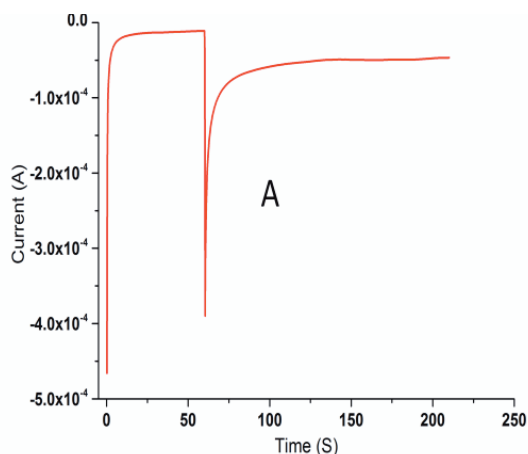
FIG. 4. (c) Chronoamperometry of HOPG for the second stage of reduction. Chronoamperometry was measured in 0.1 M KCl in a solution of ethanol/water (1:9). Voltage applied was −0.8 V.

using fluorescent Quantum Dot (QD)-labeled secondary antibody, which binds selectively to the surface of the primary antibodies.

C. Characterization and spectroscopy

Scanning electron microscopy (SEM; Ultra-High Resolution FE-SEM S-4800, Hitachi) was carried out at 10 kV acceleration voltage and a 9.8 mA emission current. The magnification was 2200 and working distance was 29.9 mm. The SEM scan resolution was typically 640×480 pixels. Atomic force microscopy (AFM) was carried out using a JPK NanoWizards II

AFM mounted on an inverted epifluorescence microscope (Zeiss Axiovert 200). Topographic images of SiNW were acquired in tapping mode in air, collected at a scan rate of 1.5 Hz over a scan area of 2 mm^2.

Laser scanning confocal microscopy (LSCM) was undertaken using an LSM 710 confocal microscope (Carl Zeiss Microscopy, Cambridge, UK). The LSCM scan resolution was typically 512×512 pixels with a pixel dwell time of 3.15 ms. The laser excitation wavelength and optical light path filters were set appropriately for the fluorescent QD under examination (405 nm and 600–700 nm respectively).

III. RESULTS

A. Functionalization of HOPG

In order to fabricate biosensor devices, HOPG electrodes must be functionalized with a receptor which interacts with a target biomolecule to give an electrical signal response. The attachment of a bioreceptor to the HOPG surface requires chemical functionalization of the semiconductor surfaces with linking groups or molecules, which can then be used to bind to bioreceptor biomolecules. HOPG is chemically similar to graphene and has been

used as a model for future graphene functionalization research.

The functionalization of HOPG with nitro-phenyl groups involves a reaction of HOPG with a diazonium cation as shown in Fig. 1. The first stage of the reaction involves the transfer of an electron from the HOPG electrode substrate to the aryl diazonium cation and formation of an aryl radical through the release of a nitrogen molecule. There is a subsequent reaction of this aryl radical with the substrate, yielding a nitrobenzene functionalized surface.

B. Cyclic Voltammogram Analysis

Figure 2 shows the CV curves obtained for the reactions between the HOPG working electrode and the diazonium electrolyte. The black curve is the blank or control CV scan, which is performed without the active diazonium compound present in the electrolytic solution. This flat curve indicates that no redox processes are occurring during the control CV experiment – as there is no diazonium for the HOPG to react with. The red curve shows the first CV scan, which is performed in the presence of the diazonium salt. The first part of the scan ramps the voltage from 0 V to −0.85 V and the changes observed indicate the start of the functionalization process – initial transfer of an electron from the HOPG electrode substrate to the aryl diazonium cation and formation of an aryl radical through the release of a nitrogen molecule and subsequent reaction of this aryl radical with the HOPG substrate.

The attachment of the nitro-phenyl group to the HOPG electron constitutes an overall reductive process. Because of the dissociation of the unstable diazonium cation, to the aryl radical and nitrogen, this reduction reaction is an irreversible process. After the first reduction cycle, half a mono-layer of nitro-phenyl groups are attached to the HOPG surface in which 65% of all electrons are exchanged within this first cycle. The shape of the CV curve indicates that the redox reactions are taking place at the HOPG electrode surface. As the reaction proceeds, the set of redox CV curves become closer and closer together. This indicates that the reaction is nearing completion. The blue curves shows the end point of the reaction, at which point no more oxidation or reduction processes are occurring, indicating that the reaction is complete.

The nitro phenyl is bound to HOPG and the nitro is reduced to amine. However, the p-nitro phenyl is not the only isomer can be obtained. By voltammograms showed in Figs. 2 and 3, the CV reduction can be assigned to the reaction $PhNO_2 + 4H^+ + 4e \rightarrow Ph\text{-}NHOH + H_2O$ and the reversible couple may be the nitroso/hydroxylamine pair: $Ph\text{-}NHOH \leftrightarrow Ph\text{-}NO + 2H^+ + 2e$. This assignment is also consistent with the currents showed in voltammograms.

In the second stage of the functionalization process, the surface nitrophenyls were converted to the aminophenyls by electrochemical reduction. The electrode is subjected to two different electrochemical reduction strategies including Chronoamperometry at a constant potential and cyclic scanning between 0 and −1 V versus Ag/AgCl to find the optimum reduction conditions. To identify the potential at which the NO_2 to NH_2 reduction occurs as
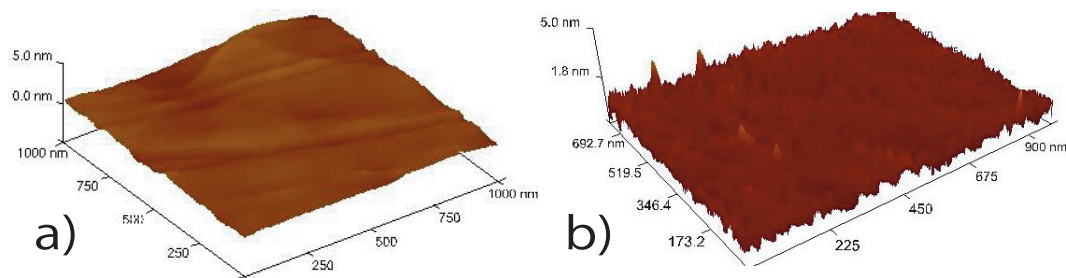
FIG. 5. a) AFM scan of blank HOPG $R_a = 0.14$ nm and b) AFM scan of HOPG with nitrophenyl $R_a = 0.33$ nm
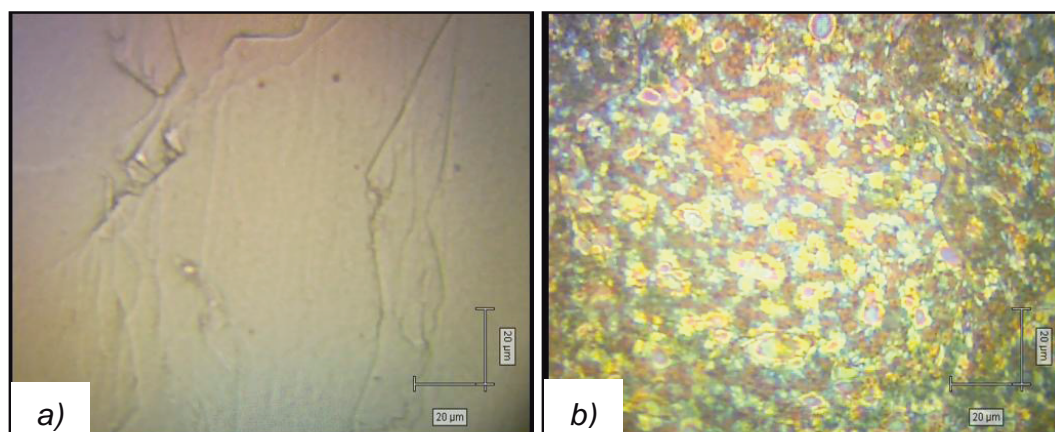

FIG. 6. a) Micrograph of blank HOPG and b) Micrograph of HOPG with nitrophenyl.

shown in Fig. 2, a CV measurement is used. The cathodic sweep of the cyclic voltammogram of the 4-nitrophenyl modified HOPG electrodes are similar to each other, with a reversible reduction peak at around $(-0.4$ V) and in the final scan the pair of reversible redox peaks disappears, as shown in line b. This reversible electrochemistry is attributed to the formation of the hydroxylamine intermediate (hydroxyl amino-phenyl).

C.   Chronoamperometry

Once the reduction voltage has been identified, Chronoamperometry is used to complete the reduction reaction as shown in Fig. 4. To assess the progression of the reduction reaction towards completion, further CV measurements are performed at specific time intervals. As the reduction reaction proceeds, the reduction and oxidation curves in the CV measurement move closer together. When the curves are superimposed on one another, this indicates the end point of the reduction reaction and functionalization of the HOPG surface.

D.   Characterization and HOPG Surfaces

AFM measurements performed on HOPG before and after functionalization, were used to assess the surface roughness and surface topography after functionalization as shown in Fig. 5a and 5b. The surface roughness of HOPG increases by 0.19 nm after surface functionaliza-

tion when amino-phenyl groups are attached to the surface. Optical micrographs of HOPG before and after functionalization show the differences after functionalization, where iridescence observed after functionalization is indicative of a thin layer of nitrophenyl present on the surface as shown when comparing Figs. 6a and 6b. Attaching aniline to HOPG, via coupling with an aryl diazonium salt, the amino group of the aniline molecule has been used to graft 8-OHdG - onto the HOPG surface. Using SEM, it is possible to observe and compare the surface of the device at different stages of biofunctionalization. Difference between the surface characteristics of HOPG as observed with AFM, micrographs and SEM, comparing surfaces before and after functionalization and biofunctionalization with the antibody targeted against 8-OHdG. After attachment of the antibody to the surface shows further features as shown in Figs. 7a and 7b.

E.   Laser Scanning Confocal Microscopy

The functionalized HOPG surface as shown in Fig. 8 shows fluorescence (red) from the secondary labeled antibody, bound to the primary antibody, targeted against 8-OHdG, applied on the surface of HOPG. Chemical analysis (X-ray photoelectron spectroscopy (XPS)) of the functionalized surface shows the NH_2 peak changed, rather than the NO_2. This is expected due to the antibody attachment to the NH_2 group rather that the NO_2 group.

As shown in Fig. 8 the anti-8-OHdG antibody was specifically and selectively attached to the surface. This
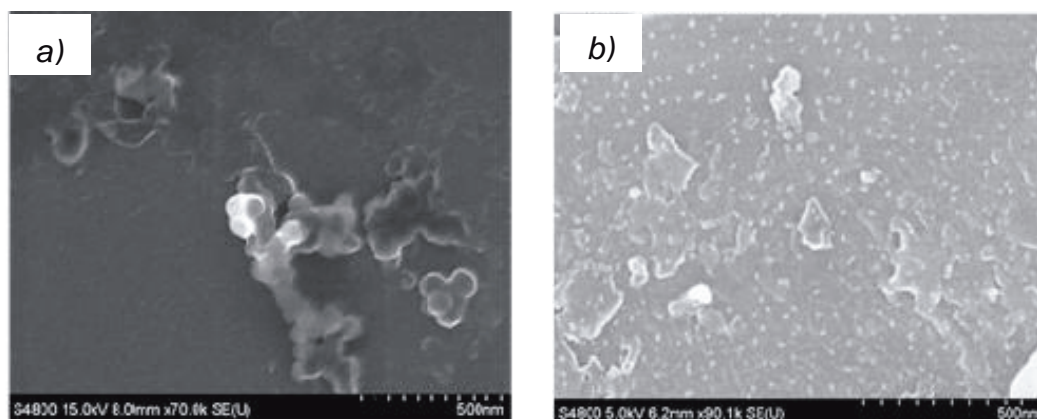
FIG. 7. Difference between the surface characteristics of HOPG a) After functionalization and b) 8-OHdG antibody bonded onto the functionalized surface.
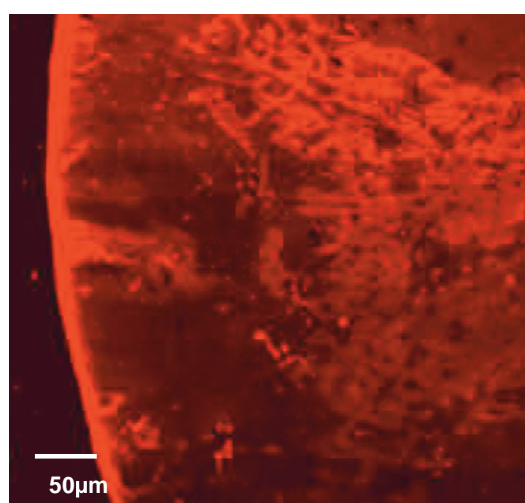

FIG. 8. Laser scanning confocal micrograph of HOPG, selectively functionalized with an antibody targeted against 8-OHdG and bound to a 2nd antibody labeled QD655.

occurs as the surface is functionalized with amino-phenyl groups – which are able to bind to the antibody. This sensor technology developed uses an antibody functionalized semiconductor channel to detect specific disease biomarkers. The antibodies are used as bioreceptors in these sensors, capable of binding with the target disease biomarkers. Biomarkers have been covalently attached to HOPG in which the surface attachment has been verified using laser scanning confocal fluorescence microscopy.

IV. CONCLUSION

During this research a critical component in the development of a reliable biosensor; the surface functionalization of a HOPG surface with a bioreceptor molecules, has been achieved. In order to attach bioreceptor molecules, an electrochemical functionalization process for attaching aniline linking groups to HOPG surfaces was developed. This involved electrochemical diazotization for grafting of nitro phenyl to HOPG using CV. Subsequent reduction of the grafted nitro-phenyl groups on the working electrode surface to amino-phenyl groups was performed using Chronoamperometry by applying a constant current to the working electrode. CV was used to monitor the progress and extent of the functionalization and reduction reactions. The surfaces of HOPG when functionalised via an NH_2 terminated linking molecule can be attached to a primary antibody, and thus can be used as bioreceptors for detecting their corresponding disease biomarker protein antigen.

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