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Highly Sensitive Covalently Functionalised Integrated Silicon Nanowire Biosensor Devices for Detection of Cancer Risk Biomarker

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Abstract

In this article we present ultra-sensitive, silicon nanowire (SiNW)-based biosensor devices for the detection of disease biomarkers. An electrochemically induced functionalisation method has been employed to graft antibodies targeted against the prostate cancer risk biomarker 8-hydroxydeoxyguanosine (8-OHdG) to SiNW surfaces. The antibody-functionalised SiNW sensor has been used to detect binding of the 8-OHdG biomarker to the SiNW surface within seconds of exposure. Detection of 8-OHdG concentrations as low as 1 ng / ml (3.5 nM) has been demonstrated. The active device has been bonded to a disposable printed circuit which can be inserted into an electronic readout system as part of an integrated Point of Care (POC) diagnostic. The speed, sensitivity and ease of detection of biomarkers using SiNW sensors render them ideal for eventual POC diagnostics.

Keywords

Silicon nanowire, Biosensor, Surface functionalization, Electron beam lithography, 8-hydroxydeoxyguanosine (8-OHdG), Electronic readout.

1. Introduction

Highly sensitive, reliable, low-cost, user friendly rapid diagnostic biosensor devices are required for a variety of biological and biomedical applications (Vu et al., 2009). Nanotechnology based biosensor devices have the potential to overcome many of the disadvantages of conventional health diagnostic and monitoring methods. For instance, electrochemical nanoscale biosensors offer the ability to measure biomedical parameters directly and rapidly, without using fluorescent labels. Nanoscale sensors also offer the potential for *in vivo* sensing.

Semiconducting silicon nanowire (SiNW) biosensor devices are capable of high sensitivity, label-free detection of biomolecular interactions at their surfaces (Ahn et al., 2010; Masood et al., 2010; Gao et al., 2011). SiNW biosensors have been developed for applications including characterisation of protein-protein interactions (Erhola, Toyokuni et al. 1997), virus detection (Zhang, Zhang et al. 2010) and detection of nucleic acids (Zhang, Huang et al. 2010) (Gao, Lu et al. 2011) and biomolecules including the prostate cancer biomarker prostate specific antigen (PSA) (Zheng, Patolsky et al. 2005; Ansoon Kim and Chang-Guen Ahn 2007). SiNW biosensors often consist of a conductive silicon channel – functionalised with a “bioreceptor” which is “gated” by the binding of a target disease biomarker to surface-attached bio-receptors. The gating effect results from changes in the surface charge density, which induce a depletion or accumulation region in the SiNW consequently modifying the electrical conductance of the functionalised SiNW sensor (Zhang, Huang et al. 2011). Electrochemical detection of even small numbers target biomarker molecules (Aoh, Chuang et al. 2013) has been reported, with detection limits as low as 1 pg / ml (Zheng, Patolsky et al. 2005) and fg / ml (Ansoon Kim and Chang-Guen Ahn 2007). The detection limit is highly dependent on the diameter of the SiNW devices and to a lesser extent by the SiNW doping.

Investigations of different diameter SiNWs (Elfström, Karlström et al. 2008; Wu, Ko et al. 2009) concluded that the greater sensitivity of smaller diameter nanowires is related to their higher surface to volume ratio. Smaller SiNWs are more influenced by surface charges which induce a depletion or accumulation region in the SiNW, resulting in a greater effect on the conductance / resistance of the SiNW sensor device.

Consequently, many SiNW sensor fabrication processes used a tetramethylammonium hydroxide (TMAH) wet etchant nanowire thinning method to reduce the diameter of the nanowire (Vu, GhoshMoulick et al. 2010; Gao, Lu et al. 2011; Kong, Su et al. 2012).

Doping plays a relatively minor role in the sensitivity of the sensor, where the SiNW is lightly or moderately doped (Elfström, Karlström et al. 2008), and fg / ml sensitivity has also been achieved using highly n-doped ($3 \times 10^{18} \text{ cm}^{-3}$) SiNWs (Ansoon Kim and Chang-Guen Ahn 2007).

Most researchers use doping concentrations in the range 10^{13} to 10^{19} cm^{-3} and are able to achieve detection of biomarkers at concentrations down to 10^{-12} to 10^{-15} g/ml (Ansoon Kim and Chang-Guen Ahn 2007; Zhang, Chua et al. 2009; Vu, GhoshMoulick et al. 2010; Zhang, Huang et al. 2010; Zhang, Luo et al. 2010; Zhang, Zhang et al. 2010; Gao, Lu et al. 2011; Kao, Shankar et al. 2011; Zhang, Huang et al. 2011; Kong, Su et al. 2012).

Both bottom-up; (Cui, Duan et al. 2000; Valavanidis, Vlachogianni et al. 2009) and top-down (Ansoon Kim and Chang-Guen Ahn 2007; Wu, Ko et al. 2009) fabrication methods have been used to develop SiNW sensors. The “top-down” fabrication process provides a solution for manufacturing reliable biosensors on a wafer scale - because it is compatible with silicon-based complementary metal oxide semiconductor technology (Ansoon Kim and Chang-Guen Ahn 2007; Zhang, Chua et al. 2009; Vu, GhoshMoulick et al. 2010; Zhang, Huang et al. 2010; Zhang, Luo et al. 2010; Zhang, Zhang et al. 2010; Gao, Lu et al. 2011; Zhang, Huang et al. 2011; Kong, Su et al. 2012). This is in contrast to the bottom up approach which can yield a more random arrangement of nanowires, but can be used to produce very small diameter nanowires – offering advantages in terms of sensitivity.

SiNW biosensors utilise functionalization of the silicon surface with bioreceptor molecules. There are several well-known methods for covalent functionalization of SiNWs including amino termination using (3-Aminopropyl) triethoxysilane (APTES) linking chemistry, which has previously been applied to realise DNA and peptide nucleic acid (PNA) attachment to SiNW in DNA/PNA biosensors (Li, Chen et al. 2004; Zhang, Liu et al. 2004; 2005; Li, Rajendran et al. 2005; Gao, Agarwal et al. 2007; Zhang, Chua et al. 2009; Ryu, Kim et al. 2010), and photochemical grafting using alkene derivatives (Stewart 2004).

In this article we present an electrochemical method for functionalisation of SiNW surfaces (Fig. 1a) via an aryl amine linking molecule (Fig. 1(b)). Using chemical functionalisation of SiNW with nitrobenzene, via coupling with an aryl diazonium salt, and subsequent reduction of the nitro group to an amine, aniline can be attached to the SiNW. The amino group of the aniline molecule has been used to graft antibodies targeted against the oxidative stress biomarker 8-hydroxydeoxyguanosine (8-OHdG) onto the SiNW surface (Fig. 1(c)). 8-OHdG is formed through hydroxylation of the guanine base by radical oxygen species (ROS). Following oxidation, damaged DNA is repaired by cellular mechanisms, and the hydroxylated guanine is excreted in bodily fluids. Consequently, levels of 8-OHdG in the blood and urine correlate with the degree of internal DNA damage and 8-OHdG has been used as a marker for impaired metabolism, mitochondrial dysfunction (Cui, Duan et al. 2000) and disease modelling (Cui, Wei et al. 2001) with links to number of cancers (Cui, Zhong et al. 2003).

Attachment of surface bound quantum-dot labelled anti-8-OHdG antibodies to SiNW channels has been verified using Laser Scanning Confocal Microscopy (LSCM). Interaction of the 8-OHdG target biomarker with the “bio-receptor” functionalised SiNW surface has been detected by monitoring conductance changes in response to concentrations of the target analyte as low as 0.1 ng / ml (0.35 nM), using current voltage (I-V) measurements. The generic sensor technology can be adapted to selectively and specifically detect other biomarkers – depending on the bio-receptor molecule attached to the SiNW. A hand-held, point of care (POC) system, where the SiNW chip is wire-bonded to a “bio-smartcard” and subsequently slotted in to an electronic readout device, has been developed. The SiNW sensor chip and electronic readout device have been used to detect binding of the 8-hydroxydeoxyguanosine (8-OHdG) biomarker with the SiNW surface within seconds of exposure of the sensor to 8-OHdG. The speed, sensitivity and ease of detection of biomarkers using SiNW sensors render them ideal for eventual POC diagnostics and monitoring devices.

2. Material and methods

2.1. Fabrication of SiNW biosensor array

SiNW arrays were fabricated on 10 mm² silicon-on-insulator (SOI) substrates. The substrates have boron doped top Si layers with thicknesses of 88 nm and a measured resistivity of 9 - 15 Ω .cm, implying an approximate doping concentration of 10¹⁸ cm⁻³. Lower doped silicon layers have also been investigated, but these yielded unreliable contacts – often Schottky in nature. In order to obtain a reliable Ohmic contact to the SiNW, with consistent and repeatable I-V characteristics, a higher doping concentration is desirable. In practice, we achieved low resistance, reliable Ohmic contacts using the 10¹⁸ cm⁻³ doping. Beneath the Si layer, there is a buried oxide layer with a thickness of 1400 - 1500 Å, which is supported by an 800 μ m thick silicon substrate. The SOI samples were first cleaned using a standard RCA cleaning procedure consisting of solvent, acid and alkali cleaning steps, and incorporating a 10 second hydrofluoric acid (HF) immersion step after both the acid and alkali cleans. The samples were then rinsed thoroughly in DI water. SiNWs were fabricated using a combination of electron beam lithography (EBL) (Raith E-Line Instrument, Raith) and optical lithography (Mask Aligner, MA/BA8 Gen 3 from SUSS MicroTec). PMMA-coated SOI substrates were spin-coated with PMMA (950K PMMA: Chlorobenzene = 1:3) using a spin speed of 4000 rpm for 40 seconds, to produce films 256 nm in thickness. The PMMA was subsequently soft-baked at 85°C for 2 minutes before exposure to an electron beam for the direct-write EBL process. The SiNW device consists of two micro sized contact pads at either end of a SiNW (Fig. 1(a)). The PMMA was patterned using EBL parameters: aperture size = 30 μ m, acceleration voltage = 10 kV and beam current = 0.20167 nA. The micro contact pads of the device were patterned using a dose area exposure of 100 μ A/cm², and the SiNW channel was patterned using a line exposure dose of 500 pAs/cm². Then, the sample was developed in PMMA developer (isopropyl alcohol (IPA): methyl isobutyl ketone (MIBK) = 3:1) for 1 minute, followed by soaking in IPA for 30 seconds and finally rinsing with DI water.

A lift-off process, using a 100 nm Al coating (deposited using a KJ Lesker PVD 75 Sputtering System) on top of the nanoscale PMMA pattern, was used to define an Al hard mask on top of the SOI substrate. The Al mask was used to selectively protect areas of Si during the

Reactive Ion Etching, using 10:30 sccm SF₆ / O₂ etch gas at a pressure of 200 mTorr and an RF power of 200 mW for 60 seconds in an Oxford Instruments PlasmaLAB 80plus. Following etch removal of the exposed areas of the 200 nm silicon layer that were not protected by the Al mask, crystalline silicon nanowires are revealed. A second 1 μm thick Al deposition step was then performed to fabricate the metal contact pads at either end of the SiNW. Contact pads were defined using photolithography (using a SUSS MicroTec MA/BA8 Gen 3 Mask Aligner and AZ ECI3027 positive-tone resist from AZ Materials) with metal etch-back. The sample was then thermally annealed using Rapid Thermal Annealing (RTA Anneal Sys) under a 50 sccm nitrogen (N₂) flow at 400 °C for 5 minutes in order to form an ohmic contact. The fabricated SiNW was then treated in a CHF₃ / argon (Ar) plasma RIE process for 20 seconds in order to hydrogen terminate the silicon surface in readiness for subsequent chemical functionalisation of the SiNW.

2.2. Surface Functionalisation

Cyclic Voltammetry (CV) was used to electrochemically functionalise the surfaces of SiNWs with nitrobenzene. CV measurements were carried out at room temperature, with a potentiostat in a three-electrode configuration. All experiments used the SiNW chip array as the working electrode, a platinum (Pt) auxiliary electrode and an Ag/AgCl electrode as the reference electrode (purchased from BASi Company, USA).

Initial attachment of nitro-phenyl groups to the SiNWs was performed using CV to induce an electrochemical reaction of 4-nitrobenzene diazonium tetrafluoroborate (2 mM) (Sigma-Aldrich) in a non-aqueous acetonitrile (Fisher) / 0.1 M tetrabutylammonium tetrafluoroborate (NBu₄BF₄) (Fisher) electrolyte with the surface of the SiNW electrode, forming a thin film of covalently attached nitro phenyl (PhNO₂) groups (illustrated in Fig. 1(b)). CV was performed using a scan rate of 100mV/S over a potential sweep range of +0.2V to -0.9 V. The CV process was repeated for more than 20 cycles (up to 100 cycles) to ensure the grafting reduction reaction is completed. The optimal conditions use 20 cycles. The duration of each cycle was 22 seconds using a scan rate of 100mV.

After PhNO₂ functionalisation, the SiNW sample was cleaned with acetonitrile then rinsed with dichloromethane in order to remove any physisorbed organic residues on the SiNW surface.

The second step of the functionalization process is the reduction of the grafted nitrobenzene (PhNO₂) groups to aniline molecules (PhNH₂).

The potential for reduction of the PhNO₂ to an amine (PhNH₂) was identified using CV by sweeping through a potential range of 0V to -1V in an 0.1M KCl (H₂O: EtOH, 90:10) electrolyte with scan rate 100mV/S. CV measurements indicated that the reduction reaction occurs at -0.9V. Therefore the reduction of the PhNO₂ to PhNH₂ was completed using chronoamperometry with a constant voltage (-0.9 V) for 10 minutes using Ag/AgCl as the reference electrode and Pt as an auxiliary electrode.

To assess the progression of the reduction reaction towards completion, further CV measurements were performed at specific time intervals after chronoamperometry, in order to assess the progress of the reduction reaction toward completion. If the reaction was incomplete, chronoamperometry was performed for an additional 5 minutes using a subsequent CV scan to reassess the progress of the reaction. This process was repeated iteratively until the end point of the reduction is reached. This resulted in an aniline (PhNH₂) functionalised SiNW surface.

The surface-bound amino group can subsequently be used to covalently bind to virtually any biomolecule containing a carboxyl group - forming an amide link.

2.3. Biofunctionalisation

Following chemical functionalisation with the aniline linking molecule, the SiNW chip was biofunctionalised using an antibody bioreceptor, targeted against the oxidative stress biomarker, 8-OHdG (Fig. 1(c)). The primary antibody, mouse monoclonal anti-8 Hydroxyguanosine antibody (anti-8-OHdG, purchased from Abcam, UK), was diluted in phosphate buffered saline (PBS) pH 7.4 to a concentration of 2 µg/ml and applied to the SiNW channels and incubated at 4 °C for 4 hours before rinsing 4 times in deionised water and drying under a nitrogen gas flow.

To confirm successful and specific binding of the primary antibodies to the SiNW channels, a secondary quantum dot labelled antibody, Qdot 655 goat F(ab')₂ anti-mouse IgG

conjugate (Life Technologies Ltd, UK), was diluted to 20 nM in PBS pH 7.4 and applied to the SiNW channels for 12 hours at 4 °C. The SiNW devices were then rinsed 5 times in deionised water to remove any excess unbound secondary antibodies, before drying. Fluorescence microscopy (LSCM) was used to verify successful antibody attachment to the SiNW surface via fluorescent emission from the conjugated secondary antibody. The excitation and emission wavelength of the Q-dot labelled secondary antibody was 530 nm and 651 – 658 nm respectively. Remaining free aniline groups on the SiNW surface were blocked using 5 % bovine serum albumin (BSA) in PBS for 15 minutes at room temperature, to prevent nonspecific binding of the secondary antibody to any free surface amine groups. Fluorescence should then only be detected from the SiNW where the labelled secondary antibody has bound to the primary antibody. Substrates were subsequently rinsed 3 times in deionised water and dried.

2.4. Microscopy and Spectroscopy

Scanning electron microscopy (SEM) (Ultra-High Resolution FE-SEM S-4800, Hitachi) was carried out at 10kV acceleration voltage and a 9.8 μ A emission current. The magnification was 2200 and working distance was 29.9 mm. The SEM scan resolution was typically 640 x 480 pixels.

Atomic force microscopy (AFM) was carried out using a JPK NanoWizard® 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 $2 \times 2 \mu\text{m}^2$. TESPA Veeco cantilever tips with dimensions of $T = 3.5 - 4.5 \mu\text{m}$, $L = 110 - 140 \mu\text{m}$, $W = 25 - 35 \mu\text{m}$ and drive frequency (F_0) = 304 – 338 KHz respectively, were used in this work.

Contact Angle (C.A) measurements have been used to monitor changes in the surface chemistry of silicon surfaces during surface treatment and surface functionalisation steps. The investigation was carried out using Fibro DAT 1100 Dynamic Contact Angle and Absorption Testers (FIBRO System AB, Sweden).

X-ray photoelectron spectroscopy (XPS) was used to analyse the surface chemistry of samples. Samples were scanned under ultra-high vacuum (UHV) conditions with a base pressure of 4×10^{-10} mbar using an ESCALAB system (VG ESCALAB MKII). A chromatic Al x-ray

source of 350 W with photon energy 1253.6eV with analytical 90° take off angle and pass energies of 50 eV for the full range and 10 eV for the peak areas were used.

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 μ s. 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).

2.5. Electrical Measurements

Characterisation of SiNW channel device was performed using a Hewlet Packard 4142B DC parametric analyser running ITC characterization software in conjunction with a Karl Suss MP4 probe station. Current-voltage (I-V) measurements were performed using a voltage sweep of -1.2 V to +1.2 V between the two metal contacts of the SiNW device.

2.6. Electronic readout device and “bio-smartcard”.

A portable electronic readout system incorporating SiNW biosensor arrays has been developed that contains an interface to a “bio-smartcard”, and “card readout” device which incorporates a data acquisition circuit and microcontroller. The electronic readout system is designed with a disposable “bio-smartcard” to allow the sensor measurement to operate in a liquid environment and using clinical samples.

The “bio-smartcard” was fabricated on a one-sided copper board, identical in size to a credit-card with a thickness of 0.8 mm. The fabrication process follows a standard etching procedure that is used in the making of PCBs. The “bio-smartcard” has two sets of contact array areas. The first contact array is used to connect the SiNW sensor to the “bio-smartcard”. The second contact array is used in a pressure contact, connecting the “bio-smartcard” to a spring connector inside the card-reader. The “bio-smartcard” is inserted into the readout device, whereupon a calibration I-V measurement is performed on the SiNW, before the SiNW is exposed to a test solution containing the target biomarker. The concept of the “bio-smartcard” is such that the SiNW biosensor chip and the “bio-smartcard” are both disposable after a single-use test.

A wedge wire bonder (K&S 4523, Kulicke and Soffa Ltd.) was used to wire bond electrical connections from the SiNW chip to the “bio-smartcard” using Al-Si 1%, 25 μm bonding wire)). The “bio-smartcard” is then immersed in the electrolyte solution where the electrochemical functionalisation of the SiNW device is performed. To prevent the electrolyte solution reacting with the copper tracks of the PCB, a D2020823D2 Polymer Dielectric (Gwent Group Ltd.) was used to encapsulate the copper tracks on the “bio-smartcard”.

The “card readout” device contains an LCD screen that is used to display instructions to the user on how to perform the diagnostic test. After the completion of the testing process the results, (I-V characteristics of the nanowire after 8-OHdG exposure, compared to the initial calibration scan), are displayed on the LCD.

To perform the I-V measurement and generate the graphics for the display, a microcontroller PIC18F26K22 (Microchip Technology Inc.) was used. Software was written for the PIC microcontroller using an Integrated Development Environment (IDE) (Microchip Technology Inc.) using a ‘C’ language toolset for compilation and assembly.

The electronic readout device performs measurements by use of a potential divider with the SiNW biosensor used as the impedance branch of the divider, which enables improved reliability and rapid detection of target biomarkers (results in less than 30 seconds).

For performing the I-V measurements, the microcontroller is equipped with an internal 10-bit Analog-to-Digital Converter (ADC) giving an accuracy of 1.2 mV per step. The ADC was configured such that the acquisition time was 1 μs . To ensure consistent accuracy of the acquisition of the I-V characteristics over time, before each test the battery voltage is measured to ensure that the voltage exceeds a threshold of 0.5 volts above the minimum voltage of the voltage regulator supplying the SiNW measurement element and the voltage reference.

3. Results and discussion

3.1. Device fabrication and surface functionalisation

P-type SiNW arrays were fabricated using a top-down combined EBL and photolithography process and thus are fully compatible with CMOS technology. Each device consists of three nanowires each 410nm in diameter, which act as conductive channels between two metal contact pads. The SiNWs are 50 μm in length with 10 μm spacing between each wire. Each 8mm by 8mm square silicon chip is patterned with of arrays of the 3-nanowire devices. Twenty arrays per chip enable reliability testing of the sensors to be performed.

Each SiNW device must then be functionalised with a bioreceptor antibody in order to covert the SiNW into a biosensor. The antibody is attached to the SiNW via a PhNH_2 linking group. The PhNH_2 linking group is introduced by using cyclic voltammetry (CV) to covalently bind nitrobenzene (PhNO_2) to the SiNW surface, via reaction of 4-nitrobenzene diazonium tetrafluoroborate in acetonitrile / 0.1 M NBu_4BF_4 with a hydrogen-terminated SiNW electrode, and by subsequently reduction of the PhNO_2 group to PhNH_2 . Fig. 2(a) shows the CV curves obtained during the initial functionalisation process (attachment of PhNO_2), performed using a voltage sweep from -0.9 V to +0.2 V using a scan rate of 100mV/s.

In a control experiment, CV sweeps were conducted in the absence of the diazonium reagent (Fig. 2(a), orange curve) to show that no redox reaction occurs in the absence of diazonium. The next stage, (Fig. 2(a), blue curve), was performed in the presence of the 4-nitrobenzene diazonium tetrafluoroborate.

The early stages of the electrochemical grafting reaction (cycle 1) after the application of the diazonium salt (2mM in ACN) (Fig. 2(a), blue curve) results in a broad reduction peak in the CV plot, related to the irreversible reaction of 4-nitrobenzenediazonium tetrafluoroborate and with the SiNW surface. The decomposition of the diazonium molecule, releases nitrogen to form a nitro-phenyl radical which reacts with hydrogen terminated silicon, grafting nitrophenyl groups on to the SiNWs electrode surface. The latter stages of this reduction reaction (Fig. 2(a), yellow curve) show the attachment reaction is virtually complete after around 20 CV cycles, and the surface is saturated with nitro-phenyl groups. After attachment of the nitro-phenyl group, CV was also used to monitor the progress of the attachment reaction. The broad peak in the blue

curve of Fig. 2(a) indicates that the aryl reduction reaction occurs in the range +0.2V to -0.9V, rather than at a specific voltage.

Initial CV experiments used a potential sweep from 1V to -1V. However, at voltages greater than 0.2V, no further reduction, or indeed any useful electrochemical reactions occurred. Therefore, further CV experiments were performed using the optimal voltage sweep range of +0.2V to -0.9V.

As the aryl reduction reaction proceeds, the reduction and oxidation curves in the CV measurement move closer together. The end point of the reduction reaction, represented by the superposed yellow curves, indicates that no further oxidation or reduction processes are occurring i.e. the surface is saturated with nitrobenzene – and no additional grafting of aryl radicals takes place.

The advantage of using the CV method over chronoamperometry is that it provides a broader voltage range over which to perform the reduction reaction – thus giving a higher probability of a successful reduction. In addition, using CV, we can simultaneously monitor the progress of the reaction.

The derivitisation takes only 10 minutes and therefore represents a significant process time saving over alternative silicon functionalization methods using 3-aminopropyltriethoxysilane (APTES) which reportedly take several hours to complete (Kong, Su et al. 2012). The electrochemical grafting process is also far more controllable than photochemical grafting to silicon using alkene derivatives.

After the PhNO₂ functionalisation, the SiNW sample was cleaned with acetonitrile then rinsed with dichloromethane in order to remove any physisorbed organic residues on the SiNW surface.

The second step of the functionalization process is the reduction of the grafted nitrobenzene groups to aniline molecules.

The potential for reduction of the PhNO₂ to an amine (PhNH₂) was identified using CV as -0.9 V. This is a higher potential than that used for the initial nitrobenzene grafting step. Chronoamperometric reduction was performed at a constant voltage (-0.9 V) for 10 minutes using Ag/AgCl as the reference electrode and Pt as an auxiliary electrode.

To assess the progression of the reduction reaction towards completion, further CV measurements were performed at specific time intervals after chronoamperometry. This process

may be repeated iteratively until the end point of the reduction is reached. As the reduction reaction proceeds, the reduction and oxidation curves in the CV measurement move closer together. The end point of the reduction reaction, represented by the CV curves being superposed, indicates that no further oxidation or reduction processes are occurring i.e. the nitrobenzene has been completely reduced to aniline or NHOH. Incomplete reduction, indicated by non-superposed CV curves, instigated additional chronoamperometry for periods of up to 5 minutes. Subsequent CV scans were performed to reassess the progress of the reaction.

The reduction of NO_2 to NH_2 is also evidenced by XPS data (see Section 3.2), which shows the increase of the NH_2 and NHOH peaks and simultaneous decrease in the intensity of the NO_2 peak.

3.2. X-ray photoelectron spectroscopy for chemical surface analysis

Surface functionalisation with PhNO_2 and subsequent reduction to PhNH_2 was verified using X-ray photoelectron spectroscopy analysis. Following the surface functionalisation process, a N_{1s} core peak at 405.2 eV can be seen which was not observed prior to functionalization (Fig 2(b)). This signifies successful attachment of PhNO_2 groups to the silicon surface. Reduction of the PhNO_2 groups was performed by applying a constant voltage of -0.9 V, to yield a partially terminated PhNH_2 surface as demonstrated by the peak at 398.75 eV, (Fig. 2(b)). Examination of the N_{1s} core level peak, and curve fitting of the spectra, showed that the N_{1s} peak actually consisted of 3 different peaks. These peaks can be attributed to the different states of hybridisation of the nitrogen atom: the highest in energy (Fig. 2(b) (in yellow)) is assigned as a nitrogen atom in a nitrobenzene group; the lowest energy atom may be attributed to an amino-phenyl group (Fig. 2(b) (in light-blue)), while the intermediate peak is attributed to a partially reduced state of the nitrobenzene, i.e., an H-N-OH termination (Fig. 2(b) (in purple)). Complete conversion of PhNO_2 to PhNH_2 does not occur, this is reflected by the three peaks observed in XPS results, even after electrochemical reduction.

3.3. AFM and contact angle surface analysis

The effect of surface functionalisation of silicon with amine linking group has been monitored using AFM. The surface roughness of SOI substrates before and after chemical modification of silicon surfaces (Fig. 3) shows clear changes in surface topography related to the attachment of PhNH₂ to the silicon surface. The H-terminated silicon surface is observed to be atomically flat and uniform with typical RMS (Root Mean Square) roughness of 0.13 nm before functionalisation (Fig. 3(a)). Attachment of PhNH₂ compounds to the H-terminated silicon resulted in a marked change in the surface topography; modified surfaces had an RMS roughness of 0.625 nm (Fig. 3(b)). The surface RMS roughness is increased to 0.946 nm following surface-attachment of the antibody (Fig. 3(c)). The effect of surface functionalisation on the hydrophobicity of the surface has been monitored using Contact Angle (C.A) measurements. The contact angles, θ , for H-terminated silicon (70°), PhNO₂ functionalised silicon (43°) and PhNH₂ functionalised silicon (37.5°) show that the surface becomes increasingly hydrophilic after PhNO₂ functionalisation and subsequent reduction of nitrobenzene to aniline (phenyl amine). This is expected, as the NH₂ group is polar, relative to the H-terminated silicon surface, and is able to form hydrogen bonds to water molecules.

3.4. Fluorescence verification of binding of Q-Dot labelled second antibodies on the SiNW

Functionalisation of the SiNW surface with PhNH₂ was employed as an intermediate step for bio-attachment of antibodies to SiNW surfaces, via the formation of an amide bond between the PhNH₂ group and the antibody. Confirmation of successful primary antibody attachment to the SiNW surface was achieved using fluorescent QD-labelled secondary antibody conjugates, which bind selectively to the surface attached primary antibodies. Subsequent detection of the QD-labelled antibody, attached to silicon surfaces, using fluorescence microscopy techniques enabled the success of the functionalisation process to be evaluated.

The SiNW device before functionalisation can be seen SEM (Fig. 4(a)). The same SiNW devices after functionalization are shown in Fig. 4(b), which represents a typical LSCM image of SiNWs functionalised with red emitting QD labelled antibodies. Strong, localised fluorescent signals can be seen from the SiNWs, with little non-specific labelling of the surrounding (non-

functionalised) regions adjacent to the nanowires. In addition, a number of control experiments were undertaken (data not shown): Firstly, control samples were prepared where attachment of antibodies to surfaces that had not been functionalised with PhNH₂ was attempted. Secondly, control samples were prepared where PhNH₂ functionalisation of the SiNW had been performed, followed by exposure of this surface to the labelled secondary antibody, but without attaching the primary antibody. In both control experiments, no fluorescence was detected from the surfaces during LSCM investigations.

The localised fluorescence signals shown in Fig. 4(b), together with the control experiments undertaken, demonstrate that the QDs have indeed selectively bound to the primary antibodies which are attached to the silicon surface. Furthermore, these results demonstrate that the primary antibodies have successfully attached to the SiNW surface only.

3.5. Electrical characterisation and electronic readout detection of biomolecules

Electronic biosensing using SiNWs is based on detecting changes in resistance of the SiNWs that occur during functionalisation and target-receptor binding events. The sensing mechanism can be understood in terms of the change in charge density at the SiNW surface upon receptor-target binding, the so-called “field effect”. Each SiNW is contacted by independent metal contacts, and the SiNW resistance can thus be individually measured. Resistances of each SiNWs were measured by probing the source (S) and drain (D) terminals electrodes using an $I-V$ probe station. Upon functionalisation, a change in charge density at the SiNW surface results from attachment of the PhNH₂ groups to the SiNW. This leads to a decrease in conductance or an increase in resistance of the SiNW. To verify this resistance change, the SiNW resistance was measured at each stage of the functionalisation process: (1) before functionalisation, (2) after attachment of PhNH₂ and subsequent reduction to PhNH₂ (3) after binding of the antibody to the PhNH₂ terminated SiNW, (4) after applying PBS as a control solution to the antibody-functionalised SiNW and (5) after binding of target (8-OHdG) to the surface-attached antibody.

Fig. 5(a) shows the $I-V$ characteristics of SiNW biosensors device at each stage of the functionalisation process. The orange line in Fig. 5(a) represents the $I-V$ characteristic prior to any modification to the SiNW. The initial electrochemical attachment and reduction of amino group (PhNH₂) results in an increase in the resistance of the SiNW (Fig. 5(a)) (light-blue line))

due to depletion of charge carriers when the lone pair electrons (from PhNH₂) donate electron density to the p-type SiNW. It is suggested that this results in a depletion of charge carriers in the conductance SiNW channel, causing an increase in the SiNW channel resistance.

It has been also been reported that amino functionalization (via APTES attachment) of silicon surfaces yields a positive charging effect at the SiNW surface (Pui, Agarwal et al. 2009). This would be expected to yield an increase in the depletion width of the SiNW and hence an increase resistance. This would also agree with the observed increase in resistance shown in Fig. 5(a).

The next step; attachment of the anti-8-OHdG bioreceptor antibody to the amino group of the PhNH₂ functionalised SiNW device (Fig. 5(a) (red line)) yielded a decrease in resistance of 2.23 K Ω with respect to the PhNH₂ functionalised SiNW surface. This resistance change may be attributed to negatively charged antibodies binding to the amine terminated SiNW surface and reducing the depletion width, resulting in an enhanced hole current density in the p-type SiNW (Fig. 5(a) (green line)) increasing the current transported through the SiNW. No significant change in resistance was observed when a target biomarker 8-OHdG was applied to devices that were not functionalised with the antibody receptors, which indicates that non-specific adsorption of 8-OHdG on the SiNW surface is negligible (Fig 5(a)).

Crucially, modified SiNWs also have no response in control experiments when the sensor is exposed to the buffer (PBS) with no 8-OHdG present (Fig. 5(a) (green line)). Excellent stability of SiNW biosensors toward non-specific molecules was also observed by T. Kong et al (Kong, Su et al. 2012) in their experiments on the response of SiNW based FET biosensors to a control PBS solution. Kong's SiNW FET sensors were used in the detection of cardiac marker troponin I for acute myocardial infarction diagnosis.

The final change in the I-V characteristics is related to the interaction of the surface-bound antibody bioreceptor with the target 8-OHdG biomarker (Fig. 5(a) (purple line)). This receptor-target binding event results in an increase in the resistance of the SiNW device with increasing 8-OHdG concentrations. It is unclear at this stage, the origin of the resistance change effected by 8-OHdG binding.

The functionalized SiNW biosensor thus produces a specific current response on interaction with the target biomolecule (8-OHdG) and is only influenced by specific binding of the target to the antibody.

Incomplete PhNH₂ functionalisation of SiNW surface results in insufficient antibody attachment and lower sensitivity. However, optimisation of the electrochemical attachment of PhNH₂ to the SiNW surface allowed successful antibody attachment and subsequent detection of 8-OHdG at nM concentrations. This indicates that direct covalent attachment of PhNH₂ groups to SiNWs, using diazonium chemistry allowed successful attachment of antibodies to the SiNW surface and subsequent high sensitivity detection of the DNA oxidative stress adduct, 8-OHdG.

Quantitative detection of 8-OHdG was performed on 3 μm diameter wire with 50 μm length (developed using a photolithography process) using a range of 8-OHdG concentrations. Sensitivity and detection limits of the sensor devices have been determined by measuring the resistance changes of our antibody-functionalised sensor on exposure to 8-OHdG solutions with concentrations of the target biomarker 8-OHdG of 0.01 ng/ml (35 pM), 0.1 ng/ml, 1 ng/ml, 5 ng/ml, 10 ng/ml, 20 ng/ml, 40 ng/ml and 80 ng/ml (280 nM) respectively. A plot of the measured sensor resistance in response to varying 8-OHdG concentrations is given in Fig. 5(b). Reliable 8-OHdG detection in a linear response range was observed between 1 ng / ml (3.5 nM) to 40 ng / ml (141 nM). Below 0.1 ng / ml (0.35 nM), the data is unreliable, while above 40 ng / ml, the sensor appears to saturate.

Of course, the sensitivity and detection limit of our sensors could be improved by using a smaller diameter SiNWs. However, our current sensors are capable of detecting 8-OHdG in the clinically relevant concentration range for urinary 8-OHdG (1 nM to 100 nM), and can be fabricated using a photolithography process i.e. without the need for a more expensive electron beam lithography process step. This implies a simple low-cost fabrication process.

The SiNW sensor has been integrated with an electronic readout device (Fig. 6), which performs measurements by use of a potential divider with the SiNW biosensor used as the impedance branch of the divider (Fig. 6(a)). This method for electrical characterisation can be used to establish the drain-source current changes across an array of up to seven SiNWs which enables improved reliability and rapid detection of target biomarkers (results in less than 30 seconds).

For performing the I-V measurements, the microcontroller is equipped with an internal 10-bit Analog-to-Digital Converter (ADC) giving an accuracy of 1.2 mV per step. The ADC was configured such that the acquisition time was 1 μs. To ensure consistent accuracy of the acquisition of the I-V characteristics over time, before each test the battery voltage is measured

to ensure that the voltage exceeds a threshold of 0.5 volts above the minimum voltage of the voltage regulator supplying the SiNW measurement element and the voltage reference.

The “card readout” device was equipped with a presence detect switch to facilitate recognition of insertion of the “bio-smartcard” (Fig. 6(b)). Once insertion was detected, an initial calibration sequence was performed by running a minimum of 18 successive captures of the measured voltage (V_M) by using the internal 10-bit ADC of the microcontroller. Each capture involves a number of steps, firstly, the voltage output to the potential divider is measured and stored, then the voltage across the $680\ \Omega$ sense resistor R_C is measured. By using ohms law ($V=IR$) the current is calculated flowing through the potential divider. The resistance of the SiNW is then calculated by obtaining the difference between the voltage supplied to the potential divider and the voltage across the sense resistor R_C . During the calibration sequence the deviation of the result from a moving average of all of the calibration samples is monitored. If the deviation of the latest sample is within 1% of the average and the calculated resistance of the SiNW under test does not exceed $400\ K\Omega$, the calibration is seen to be complete confirming that a good contact is established to the “bio-smartcard”. The resistance of the SiNW is then stored internally in the microcontroller for later calculations.

After a successful calibration, instructions are then presented to the user via the LCD instructing the user to inject a test solution onto the active area of the “bio-smartcard” (Fig. 6(b)). The device waits for the user to indicate that the solution has been applied via a tactile button. Upon sensing the user has pressed the button, the device performs another sequence of acquisitions of the potential difference across the SiNW. As in the calibration sequence, a series of acquisitions is sought to ensure that the SiNW resistance has stabilised.

A second I-V measurement is then performed. A comparison between the calibration acquisitions obtained after exposure to the test solution is then performed and the program displays an appropriate graphic to indicate the result to the user – if the biomarker is present or otherwise. The user is then advised to seek further medical attention (if the biomarker is detected) or else is given the all clear.

4. Conclusion

SiNWs have been successfully fabricated via a top-down fabrication approach using a combination of electron beam lithography and photolithography. A key part of any biosensor technology is surface functionalisation. Functionalisation of SiNW was achieved in a matter of minutes, using an electrochemical diazotization method for nitro phenyl attachment to silicon surface and a subsequent reduction process, converting the nitro group to an amine, under mild conditions. This represents a far more effective and production friendly process than any other reported diazotization. The advantage of this functionalisation method over previously reported techniques (Allongue et al., 1997) lies in its simplicity, avoidance of harsh oxidation chemistry and speed of reaction. Characterisation of functionalised surfaces using XPS, Contact Angle measurements and AFM have been used to confirm attachment of nitro phenyl and aniline groups to the silicon surface. AFM showed that surface roughness of SiNWs increased after surface modification with nitro phenyl or aniline groups. Contact Angle measurements showed that the surface becomes more hydrophilic after chemical modification. Fluorescence microscopy (LSCM) has been used to verify subsequent biofunctionalisation of the SiNW channels, using fluorescently labelled antibodies.

Selectively functionalised SiNWs have been used in the development of a sensor for the detection of the oxidative stress biomarker 8-OHdG, which has been related to prostate cancer risk. Changes in conductivity of the channel devices were observed at each stage of the functionalisation process. The critical detection step; binding of the target 8-OHdG biomarker to the SiNW-bound “bioreceptor” antibody, yielded a detectable decrease in the SiNW channel resistance. Reliable detection of the prostate cancer risk biomarker 8-OHdG at concentrations as low as 1 ng / ml has been achieved, with a linear quantitative response to 8-OHdG over the 1 ng / ml to 40 ng / ml concentration range. SiNW biosensors offer high sensitivity and selective detection of disease biomarkers at a potentially low cost.

Moreover, the generic functionalisation technology developed could be used to attach a wide range of bio-receptor molecules to the SiNW surface and thus be used in sensors for early diagnosis and monitoring for a variety of diseases.

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