Ultra-sensitive electrochemical nanobiosensor for the determination of 17beta-estradiol in municipal wastewater (ENDOTEK)

Report to the
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by

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EXECUTIVE SUMMARY

BACKGROUND

Advances in wastewater treatment strategies ensure that only very low levels of estrogenous endocrine disrupting chemicals (e-EDCs) and other organic pollutants remain in the effluent. Current treatment methodologies can eliminate up to 99% of 17β-estradiol (E2) and 78% 17α-ethynylestradiol (EE) in the wastewater effluent (Stalter *et al.* 2010b, Ternes *et al.* 1999). In general significant amount of estrogens still remains in wastewater effluent with the result that new techniques, such as the activated carbon and ozonolysis technologies, are being incorporated in wastewater treatment plants for total elimination of estrogens in effluents (Stalter *et al.* 2010a; Hecker *et al.* 2011). However, the amount of estrogens that continue to enter the aquatic environment through wastewater effluents is still capable of causing harmful health effects. The ENDOTEK project focused on the development of biosensor methodology for real-time determination of low levels of estrogens in municipal wastewater effluents (which is mainly the state that interacts with aquatic environment), with particular emphasis on 17α-estradiol as a model e-EDC.

RATIONALE

Estrogenous endocrine disrupting chemicals pollute municipal wastewater through the discharge of human and agricultural wastes and may have serious health implications if they enter the food chain in large quantities. In South Africa, the extent of this class of pollution is not yet thoroughly studied. In addition to e-EDCs determination in, or decontamination from, polluted wastewater streams, one way of assisting environmental policy makers in South Africa will be the introduction of cost-effective, reliable and easy-to-use technologies which are suitable for (i) accurate and real time determination of estrogens in wastewater stream, (ii) collecting data for setting environmental standards, and (iii) deployment as alarm devices for monitoring and ensuring compliance to set standards. The ENDOTEK project involved the construction and testing of aptamer biosensor for 17β-estradiol determination in wastewater. Since aptamers are known for their ultra-sensitivity and excellent selectivity for target analyte (Nonaka *et al.* 2013; Savory *et al.* 2014), biosensors containing aptamers are one of the most reliable methods of determining estrogenic EDCs. This report deals with the construction of Au|G2PPT-co-PEDOT| electrode system and its application in the development of aptameric biosensor by incorporating an E2-specific 76-mer biotinylated ssDNA aptamer.

OBJECTIVES AND AIMS

AIM 1: Synthesis of DNA-aptamer which have corresponding selectivity for 17β-estradiol by Systematic Evolution of Ligands by Exponential Enrichment (SELEX).

AIM 2: Fabrication and characterisation of screen-printed carbon and gold microsensor chips.

AIM 3: Construction of estrogen nanobiosensors with dendritic star polymer and DNA-aptamers on disk electrodes and microsensor chips.

AIM 4: Electrochemical and instrumental characterisation of the biosensors.

AIM 5: Optimisation of the amperometric and impedimetric response parameters of the nanobiosensor and application to the analysis of wastewater samples.

AIM 6: Comparative studies by high pressure liquid chromatography (HPLC) and enzyme-linked immunosorbent assay (ELISA) techniques.
AIM 7: Modelling of the aptamer biosensor's amperometric and impedimetric responses.

METHODOLOGY

The first step in the development of the DNA-aptamer based biosensor for E2 was the preparation of the component materials of the biosensor sensitive layer. That involved the synthesis of the 17 β-estradiol-selective 76-mer-ssDNA aptamer by SELEX and the electrosynthesis of G2PPT-co-PEDOT. The two materials were used in the construction of the aptameric biosensor designated as Au|G2PPT-co-PEDOT|76-mer-ssDNA-Aptamer. The sensor parameters were optimized to achieve high sensitivity for E2. Part of the goal of the study was to perform comparative studies of estrogen analysis by chromatographic (HPLC) and enzyme-linked immunosorbent assay (ELISA) techniques.

RESULTS AND DISCUSSION

Single stranded DNA aptamer and a generation-2 dendritic star co-polymer, G2PPT-co-PEDOT, were the biomaterial and electroactive polymeric materials used for the construction of sensor systems. The biosensor preparation parameters were optimised and their characteristic electrochemical, electronic, spectroscopic and microscopic properties were determined. The biosensor was calibrated with standard E2 solutions and applied in real sample analysis. Comparative studies of the aptameric biosensor responses were performed with chromatographic and biosensor methods.

Aim 1: A 76-mer biotinylated ssDNA aptamer for 17β-estradiol was synthesised by SELEX. The sequence of the 17β-estradiol aptamer is 5´-BiotinGCTT...AAGC-3´.

Aim 2: Screen-printed carbon electrode (SPCE) was found to be suitable for microscopic analysis and multichannel robotic sensor test station electroanalysis of the aptameric biosensors. Gold electrodes/microsensor chips were suitable for aptameric sensors containing dendritic star co-polymer (G2PPT-co-PEDOT).

Aim 3: The DNA-aptamer biosensor for 17β-estradiol was developed with the 76-mer biotinylated ssDNA aptamer immobilised on a gold disk electrode (AuDE). The coupling of the aptamer onto the electrode required the functionalisation of AuDE with G2PPT-co-PEDOT dendritic star co-polymer.

Aim 4: The electro-microscopy and the electro-spectroscopy of the Au| PPT--co-PEDOT|76-mer-ssDNA-Aptamer bioelectrode were studied by cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS), Fourier transform infrared spectroscopy (FTIR), and scanning electron microscopy (SEM). The structural bases of the reactivity as well as chronoamperometric response characteristics of the sensor device were ascertained.

Aim 5: The conditions for optimal performance and the response parameters {dynamic linear range of application (DLR) limit of detection (LOD) reproducibility, sensitivity and selectivity} of the aptameric sensor were determined. For real sample applications, wastewater effluents supplied by Scientific Services of the City of Cape Town were used. All sample preparation and storage were in accordance to the specifications of the U.S.A. Environmental Protection Agency (2005) standard.

Aim 6: Chromatographic experiments were performed to validate the results obtained with the aptasensor. Agilent 2D HPLC-GPC chromatographic instrument operating on HPLC mode was used in the experiment. Treated wastewater was the sample of choice as that

was the main type that enters into the environmental water system. For the ELISA validation studies, the E2 content of water samples were analysed using 17β-estradiol ELISA kit for environmental water (ALPCO Diagnostics ELISA kit).

AIM 7: The amperometric responses of the voltammetric experiments were modelled to the hyperbolic Langmuir isotherm which enabled the determination of the analytical dynamic linear range (DLR). The DLR is the region of the hyperbolic sensor calibration curve which directly gives a linear signal-to-concentration relationship without the necessity of additional curve linearization mathematical procedures. The EIS data were fitted to appropriate electrical circuits that best represent the interfacial reactions occurring on the electrode surface. The resultant charge transfer resistance values were profiled to ascertain whether the interaction between the aptasensor and E2 was a simple Langmuir isotherm case (that gives a hyperbolic signal/concentration plot) or it is an allosteric interaction (that gives a sigmoidal plot). The results indicated that the aptasensor response was typical of what was expected for a biochemical binding processes following a Langmuir model.

GENERAL

The ENDOTEK project involved the use of Au|G2PPT-co-PEDOT|76-mer-ssDNA-Aptamer biosensor to determine 17β-estradiol in water samples. Sensor validation tests including cross reactivity, precision, recovery and reproducibility tests were performed in accordance to the EPA Standard Operating Procedures (U.S. EPA 2005). The nanosensor's analytical parameters were validated with chromatographic studies as well as ELISA. The ENDOTEK sensor methods had better sensitivity than chromatography performed on conventional C-18 column.

CONCLUSIONS

The ENDOTEK sensor analytical parameters were DLR = 0.05-307 pg/mL and LOD = 0.043 pg/mL 17β-estradiol. This makes it more sensitive than recorded experimental and commercial immunoassay and chromatographic methods. The DLR values predicates the sensor suitability for E2 determination in the effluent of wastewater treatment plants, as well as for the determination of very low levels of E2 that may be present in drinking water or food products. The ENDOTEK sensor will also be suitable for measuring deviation from acceptable daily intake (ADI) of E2 through all sources. The ADI limit for E2 is 0.05 μg/kg body weight as determined by the Food and Agriculture Organisation of the United Nations (FAO). Also the aptameric sensor technology can be adapted to develop sensor systems for other types of water pollutants including cyanotoxins.

RECOMMENDATIONS FOR FUTURE RESEARCH

In its present form, the project and its findings represent a proof of concept. Further work is required for actual development of device prototype. Also further work is required to develop new aptameric biosensor for other e-EDCs including 17α-ethynylestradiol, estriol and estrone, as well as combinatorial aptamer biosensor that will be used for the determination of the total e-EDC content of a water sample. Considering that only 78% of 17α-ethynylestradiol (EE), the major component of birth control pill, is removed by water treatment plants, it is very urgent to develop aptasensors for monitoring EE level in water for domestic usage. Another important research to undertake is the development of electrochemical ELISA library for the major estrogenous endocrine disrupting chemicals. Commercial ELISA systems are mainly based on UV-Vis measurements.

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LIST OF ABBREVIATIONS

ADI	Acceptable daily intake
Aptasensor	Aptamer biosensor
ATP	Adenosine triphosphate
COMPRENDO	Comparative research on endocrine disruptors
CREDO	Cluster for research on endocrine disruption
CV	Cyclic voltammetry or cyclic voltammogram
DCM	Dichloromethane
DELFIA	Dissociation enhanced lanthanide fluorescence immunoassay
DLR	Dynamic linear range
DNA	Deoxyribonucleic acid
DPA	3, 3'-Dithiodipropionic acid
EDAC	Ethylenediamine acetic acid
EDCs	Endocrine disrupting chemicals
EDEN	Endocrine disruptors: exploring novel endpoints, exposure, low-dose and mixture effects in humans, aquatic wildlife and laboratory animals.
EDOT	3,4-ethtylenedioxythiophene
EE	17α-ethynylestradiol
EEQ	17β-estradiol estrogenic equivalent
EIS	Electrochemical impedance spectroscopy
e-ELISA	Electrochemical enzyme-linked immunosorbent assay
ELISA	Enzyme-linked immunosorbent assay
ENDOTEK	Endocrine disrupting chemicals sensor technology
EPA	USA Environmental Protection Agency
ER-α	estrogen receptor α-recombinant protein
ER-CALUX	Estrogen receptor-mediated chemically activated luciferase gene expression assay
EU	European Union
EURISKED	Multi-organ risk assessment of selected endocrine disruptors
E2	17β-estradiol
FIRE	Risk assessment of brominated flame retardants as suspected endocrine disruptors for human and wildlife health
FAO	Food and Agriculture Organisation of the United Nations
FTIR	Fourier transform infrared spectroscopy
GC-MS	Gas chromatography-Mass spectrometry combination
GPC	Gel permeation chromatography
G2PPI	Generation-2-poly(propylene imine) dendrimer
G2PPT	Generation-2-poly(propylene thiophenoimine)
G2PPT-co-PEDOT	Generation-2-poly(propylene thiophenoimine)-co-poly(3,4-ethylenedioxythiopene) dendritic star co-polymer
HFBA	Heptafluorobutyric anhydride
^{1}HNMR	Proton nuclear magnetic resonance
HPLC	High-performance liquid chromatography
HPLC/UV	High-performance liquid chromatography with UV detector

HRP	Horseradish peroxidase
IgG	Immunoglobulin G
LOD	Limit of detection
NHS	N-Hydroxysuccinic acid
OECD	The Organisation for Economic Co-operation and Development
OPD	o-Phenylenediamine dihydrochloride
PAMAM	Polyamidoamine dendrimer
PBS	Phosphate buffer saline
SA-HRP	Horseradish peroxidase-labelled streptavidin
SELEX	Systematic evolution of ligands by exponential enrichment
SEM	Scanning electron microscopy
SPEs	Screen-printed electrodes
ssDNA	Single stranded deoxyribonucleic acid
SWV	Square wave voltammetry (SWV)
UV/Vis	Ultraviolet-Visible spectroscopy
WHO	World Health Organisation
YES	Yeast estrogen screen method
2D HPLC-GPC	Two dimensional high pressure liquid chromatography – gel permeation chromatography combination
76-mer-ssDNA	76-mer biotinylated single stranded deoxyribonucleic acid

1 INTRODUCTION AND OBJECTIVES

There is a current concern in South Africa that water resources are heavily contaminated with pollutants generally classified as endocrine disruptors or endocrine disrupting chemicals (EDCs). The focus of this study was on endocrine disruptors that are natural or synthetic estrogenic hormones such as estrone (E1), 17β-estradiol (E2), estriol (E3) and 17α-ethinylestradiol (EE). Estrogenic hormones have disrupting potencies that are several thousand times higher than those of other chemicals (Bolong *et al*. 2003, Nghiem *et al*. 2004). This implies that natural and synthetic estrogens can be biologically reactive even at low nanogram per litre levels (Purdom *et al*. 1994). Consequently, the detection of these trace contaminants in municipal water resources and their elimination are very important areas of current research interest. Due to the fact that in the urban areas estrogens enter surface water through human urine, the actual concentration of estrogenic compounds in municipal wastewater depends on treatment/reclamation plants and also on the population of the catchment area that contribute to the wastewater (Eckstein and Sherk 2011, Gadd *et al.* 2003). Results from other countries show that municipal wastewater estrogenic endocrine disrupting chemicals (e-EDCs) vary for studies performed in UK, Germany and Japan (ng/L): UK – estrone 15-220, estradiol 7-88; Germany – estradiol 0.9, ethinylestradiol 1.4; and Japan – estradiol 2.7-48 (Bolong *et al*. 2003, Desbrow *et al*. 1998). In USA oral contraceptives such as 17-ethynylestradiol (100 ng/L median value) occur in wastewater more than natural hormones (Kolpin *et al*. 2002). The level of contamination of municipal wastewater in South Africa by individual synthetic and natural estrogens is not known, and there is no available technology for their real time determination. The main methods for the determination of e-EDCs have been through vitellogen (a biomarker for EDCs) enzyme-linked immunosorbent assay (ELISA) on fish samples or by high-performance liquid chromatography (HPLC) of wastewater. They are very technical methods requiring extensive sample pre-treatment and highly qualified personnel. Thus the development of rapid, simple and low-cost procedure for detection of e-EDCs in wastewater samples is of utmost importance. The ENDOTEK research project dealt with the development of electrochemical DNA aptamer biosensor for detecting and quantifying 17β-estradiol (a model e-EDC) in wastewater samples down to the femto- or atto-molar range using multichannel sensor system.

1.1 Introduction

An endocrine disrupting chemical may be a pharmaceutical substance or other substances that are potentially capable of altering the endocrinology of organisms in which these compounds accumulate in small amount. Endocrine disruptors interfere with normal physiological actions of hormones (Masikini *et al.* 2011) leading to (i) breast, testicular and prostate cancers, (ii) malformation of male reproductive tract, (iii) low sperm count, (iv) endometriosis and (v) extensive sexual disruption in aquatic and terrestrial wildlife. Being several thousand times more disrupting than their phenolic counterparts, natural and synthetic estrogens can be biologically reactive even at nanogram per litre levels. This means that their content in municipal wastewater if unchecked and uncontrolled may have deleterious effects on the general quality of human health, increase in endocrine related health conditions, and decrease in the rate of population growth which may eventually affect the overall level of productivity of a community. The ENDOTEK sensor system that contains SELEX-produced estrogen-specific aptamer was conceptualised as a point-of-need a

deployable devices for determining and monitoring levels E2 of in a wastewater sample. The implication is that this easy-to-use and real-time sensor system will enable the control of the contaminants from affecting humans through aquatic and agricultural food chains and other possible use of water that is not specifically treated for estrogens.

There has been concerted effort world-wide to develop reliable and cost effective technologies and procedures for detecting EDCs at very low levels and in real time. For example, the European Union's 'CREDO cluster' consisted of four EU-funded EDCs research programmes (COMPRENDO, EDEN, EURISKED and FIRE) involving 63 laboratories (European Commission Environmental Research Themes, 2006). Available instrumental methods (chromatographic, spectroscopic, atomic absorption and mass spectrometric) for detecting these compounds are expensive, involve extensive sample pre-treatment and require high technical skills (Jiang *et al*. 2009). In contrast, it is known that biosensor devices based on electrochemical principles can offer analytical solutions with low cost, simplicity and fast sample through-puts (Iwuoha *et al*. 2007), while being suitable for on-the-spot testing of environmental samples. This study involved the development of DNA aptamer sensor technology for rapid, highly specific and simultaneous determination of EDC in municipal waste water.

1.2 Estrogenicity of EDCs

Endocrine disrupting chemicals impact negatively on the endocrine system of humans and wildlife (Amaral-Mendes 2002). Due to their interference with and mimicking of the biochemistry of natural hormones, it is of utmost importance to continuously measure the amounts of EDCs in domestic wastewater and river water. Estrone (E1), 17β-estradiol (E2) and estriol (E3) are the most stable of the 20 common steroidal hormonal compounds classified as estrogens. Generally all the steroidal estrogens metabolise very rapidly into the highly stable E1, E2 and E3, making the three the most dominant and persistent in the environment. 17β-estradiol (an 18-carbon steroid – see Fig. 1) is the most potent natural estrogen as well as an EDC which enters the water systems through domestic wastewater containing human wastes, as well as through agricultural wastewater from life stock farms as shown in Fig. 2. High concentrations of E2 in water or food chain disrupts the physiology of the endocrine system of various animal species, leading to feminisation in fish and stimulates the proliferation of cancer cells in humans (Safe 2004, 2005, Schilirò 2009). E2 is a very potent EDC and is used as an index for reporting the endocrine disrupting potential of chemicals: EDC’s concentrations are reported in terms of their pg/mL or ng/L 17β-estradiol equivalent (EEQ).

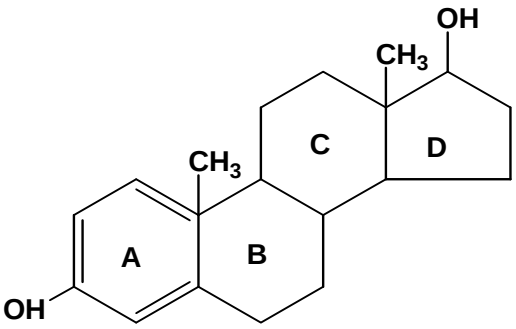


Figure 1. Chemical structure of 17β-estradiol (E2).

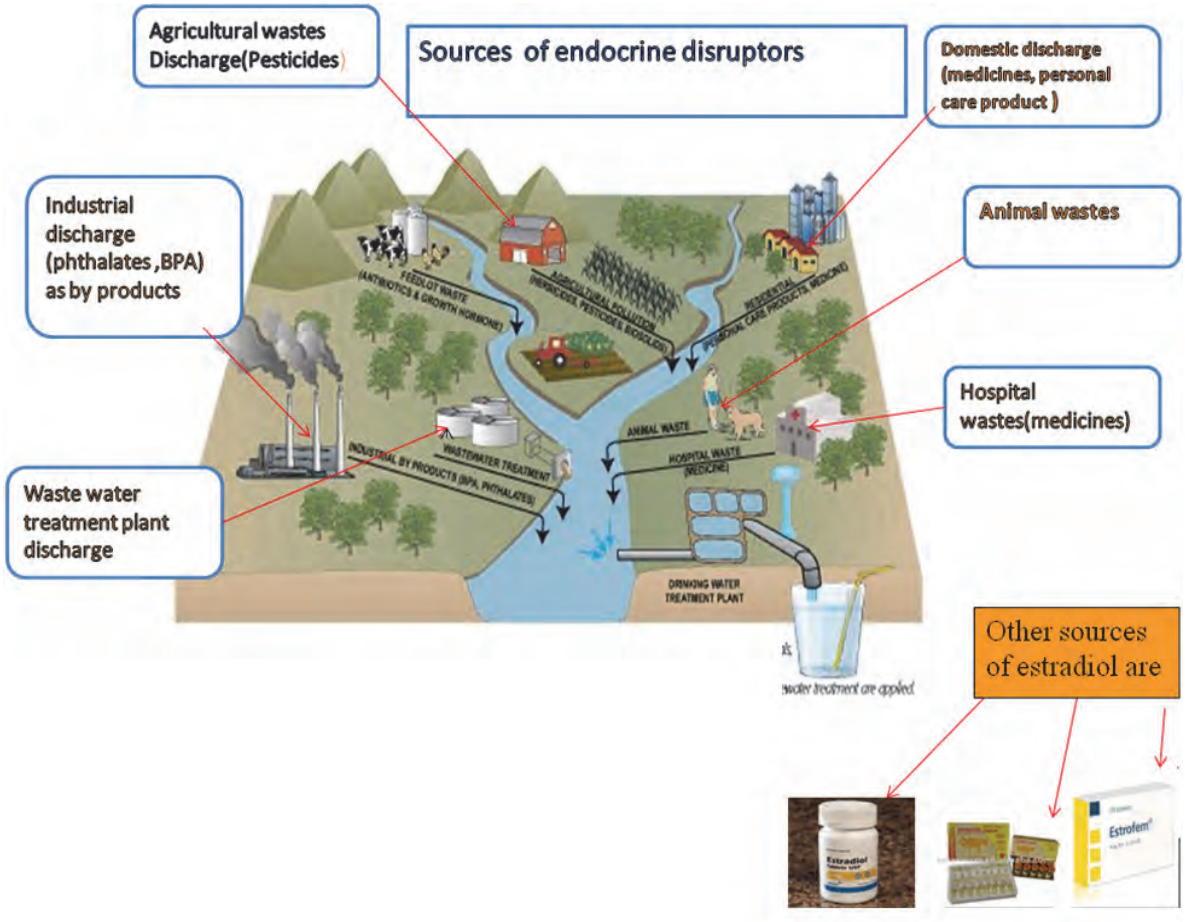


Figure 2. Sources of endocrine disrupting chemicals.

1.3 Aptameric biosensor

Aptamers are short, single stranded and artificial receptors with a special three-dimensional conformation of primary structures of RNA or DNA that can bind to their targets with high affinity and selectivity (Zhao and Yang 2010). These targets include low molecular weight organic and inorganic compound, as well as macromolecules such as drugs, proteins and even whole cells (Zhao and Yang 2010). Due to these properties aptamers are widely explored as biological recognition elements for specific sensing of a variety of analytes.

Selectivity of aptamers for the specific target is based on *in vitro* selection from random sequence nucleic acids combinatorial libraries by Systematic Evolution of Ligands by Exponential Enrichment (SELEX) (Tombelli 2005). Aptamers possess many advantages over the traditional recognition molecules, such as antibodies and enzymes, due to their chemical simplicity, non toxicity, specific binding ability, ease of synthesis, lack of immunogenicity, good stability during long-term storage and ease of functionalization for immobilization procedures (Zhao and Yang 2010, Tombelli 2005). Smith and his co-workers (Smith *et al.* 2007) used aptamer-conjugated magnetic nanoparticle for the collection of cancer cells, while target cells labelled with aptamer-modified fluorescence nanoparticles was required for the optical detection. An aptamer based electrochemical sensor for label-free recognition and detection of cancer cell with a detection limit of 6×10^3 cells/mL has been reported (Pan *et al.* 2009). Electrochemical aptamer biosensors are of immense current interest due to the specificity of aptamers in the recognition of their target analytes (Zhao and Yang 2010, Tombelli 2005, Liu *et al*. 2009). Recently, a highly sensitive, reusable aptasensor for adenosine, which has a detection limit of 1.65×10^{-8} M was reported (Liu *et al*. 2009). Also a cocaine aptasensor prepared with gold nanoparticles was reported by Xiaoxia and co-workers (2008). In order to amplify signals, aptamers have been labelled with electroactive materials such as ferrocene and methylene blue (Fabien *et al*. 2006). Also Liu and co-workers (Liu *et al*. 2009) reported an ultrasensitive aptasensor (with a limit of detection of 75 fmol) based on an aptamer functionalised with a ferrocene-bearing cationic polythiophene. Yan and his co-workers developed a multifunctional label-free electrochemical aptamer-based biosensor for the detection of adenosine triphosphate (ATP) (Yan *et al.* 2008). This report contains the development of electrochemical aptameric sensor based on label-free aptamer system incorporated onto a dendrimeric co-polymer platform.

Dendrimers are a class of three-dimensional macromolecules with a well-defined and highly branched tree-like morphologies (Zhang *et al.* 2009). The unique properties of dendrimers such as biocompatibility, controlled composition, adequate functional groups for chemical fixation and structural homogeneity make them suitable for a wide range of biosensing applications (Yan *et al.* 2008, Zhang *et al.* 2009). The application of dendrimers in biosensor development has been reported for various signal transduction modes. Zhang *et al.* (2009) constructed an impedimetric aptasensor based on polyamidoamine (PAMAM) dendrimer-modified gold electrode for the determination of thrombin. Another dendrimeric biosensor based on PAMAM modified with gold nanoparticle exhibited very low detection limit (1.4×10^{-14} M) (Li *et al.* 2009). One of the advances of dendrimer-based sensor systems is the possibility of using hybrid dendrimers containing encapsulated metal nanoparticles or dendritic co-polymers, both of which make them very useful for application in catalysis and electrocatalysis (Deng *et al*. 2005). The formation of dendritic co-polymers with conducting polymers such as polyaniline, polythiophene and polypyrole should increase the conductivity and give rise to nanostructurisation of the co-polymer, due to the elongation of the conjugation chain and an unhindered π stacking of the polymer molecules by the dendrimer (Deng *et al*. 2005, Malenfant *et al.* 2000). This report contains the preparation of G2PPT-co-PEDOT for application as biocompatible platform for the fabrication of aptameric biosensor for the determination of 17β-estradiol (an estrogenous EDC that mimics the endocrine activities in humans and wild life and produce deleterious consequences including cancer and reproductive abnormalities) (Amaral Mendes 2002).

Estrogens pollute municipal waste water through the discharge of human and agricultural wastes and may have serious health implications if they enter the food chain in large quantities. In South Africa, the extent of this class of pollution is not yet thoroughly studied. Biosensor technology provides a rapid response and easy-to-use approach for point of need assessment of EDC content of waste water, which is more cost effective than other traditional analytical methods (Koester *et al*. 2002). Electrochemical determination of E2 has been performed on electrodes modified with poly(L-serine) (Song *et al.* 2008) or with carbon nanotube and ionic liquid (Tao *et al.* 2009) . Since aptamers are known for their ultra-sensitivity and excellent selectivity for target analyte, biosensors containing aptamers will be one of the most reliable methods of determining estrogenic EDCs. This report deals with the construction of Au|G2PPT-co-PEDOT star co-polymer electrode system and its application in the development of aptameric biosensor for E2, by incorporating an E2-specific 76-mer biotinylated ssDNA aptamer into the polymer.

2 EXPERIMENTAL

The experimental procedure for the construction of the G2PPT-co-PEDOT aptasensor involved: (i) the synthesis of generation 2 poly(propylene thiophenoimine)-co-poly(3,4-ethylenedioxythiophene) dendritic star co-polymer (G2PPT-co-PEDOT) on gold electrode and (ii) the electrostatic integration of ssDNA aptamer produced by the systematic evolution of ligands by exponential enrichment (SELEX) procedure. The preparation of the dendritic star co-polymer and its application in the construction of the aptamer biosensor are described. Also presented in this section are the methods used in the characterisation of dendritic co-polymer systems and the biosensor. The morphological properties of the dendrimeric sensor platform were interrogated by scanning emission microscopy (SEM) and atomic force microscopy (AFM), while their spectroscopic characteristics were studied by Fourier transform infra-red spectroscopy (FTIR) and fluorescence spectroscopy. The electrochemical behaviour of the aptasensor and its constituent materials were studied by electrochemical impedance spectroscopy (EIS), cyclic voltammetry (CV) and square wave voltammetry (SWV).

2.1. Synthesis of the G2PPT

The synthesis of generation two (G2) poly(propylenethiophenoimine) dendrimer (G2PPT), which is 2-thiopene aldehyde-functionalized G2 poly(propylene imine) dendrimer (G2PPI), was carried out by the condensation reaction of PPI with 2-thiophene aldehyde. A reaction mixture of 1.65 g or 5.2 mmol G2PPI and 2.34 g or 20.85 mmol 2-thiophene aldehyde in a 50 mL dry methanol was stirred magnetically under a positive pressure of nitrogen gas for 2 days in a 100 mL three-necked round-bottom flask. The removal of the methanol from the reaction mixture was done by rotatory evaporation and the residual oil was dissolved in 50 mL dichloromethane (DCM). The organic phase formed was then washed 6 times with 50 mL of water to remove unreacted monomer. Then DCM was removed by rotary evaporation and a yellow G2PPT oil was obtained. The method employed for this synthesis is a slight modification of that reported Smith and his co-worker (Smith *et al.* 2003) and Salmon and Jutzi (2001). The yield was 1.55 g, 65% (Scheme 1), ^{1}HNMR ($CDCl_3$ 200 MHz, ppm): 1.34 (s,br,4H,H-1), 1.74(t8H,H-2), 2.42(m,br,12H,H-2&3), 3.51(t,8H), 6.90(t,8H,H-8), 7.01(s4H,H-7), 7.23(s,4H,H-6), 7.8(SC_4H_3S). The G2PPT PPT gave a new ^{1}HNMR chemical shift at 8.3 ppm and a strong FTIR peak at 1622 cm^{-1} due to the formation of N=C bond in the dendrimer moiety, while out-of-plane vibration for thiophene ring was obtained at 789 cm^{-1}, which in agreement with the report of other workers (Deng *et al*. 2005).

2.2 Electrosynthesis of G2PPT-co-PEDOT

The star co-polymer, G2PPT-co-PEDOT, was electrochemically synthesised on a 1.6 mm gold disk electrode (Au). The gold electrode was first polished to a mirror-like surface with alumina powder of sizes 1.0, 0.3 and.05 µm, respectively. The electrode was immersed in piranha solutions (a 3:1 mixture of concentrated sulphuric acid and 30% hydrogen peroxide) for 10 min for effective cleaning followed by sonication of the electrode in ethanol and water, consecutively, for 5 min. The electrode was further cleaned electrochemically in sulphuric acid by cycling between the potential of -200 mV to +1500 mV *vs*. Ag/AgCl reference electrode until a reproducible cyclic voltammogram was obtained. Subsequently, the gold electrode was rinsed with copious amount of water and absolute ethanol. 6 µL of 10 mg/mL G2PPT oil was drop-coated on the surface on the gold electrode and left for 12 h for effective self-assembly of the G2PPT film. The modified Au electrode was immersed into an

aqueous solution of 0.1 M lithium perchlorate ($LiClO_4$) containing 0.1 M 3,4-ethylenedioxylthiopene (EDOT) and 0.1 M sodium dodecyl sulphate (SDS) and the electrode potential was cycled from -0.1 to +0.1 mV at a scan rate of 50 mV/s for ten cycles to obtain generation 2 poly(propylenethiophenoimine)-co-poly(3,4-ethylenedioxythiopene) film on gold electrode (Au|G2PPT-co-PEDOT). The reaction scheme for the formation of G2PPT-co-PEDOT is shown in Fig. 3.

For the preparation of G2PPT-co-PEDOT on screen-printed electrodes, the same procedure was used except that there was no electrode pre-treatment, as the electrodes were used as supplied.

2.3 Fabrication of the aptasensor

A self-assembled monolayer of 3,3'-dithiodipropionic acid (DPA) on the G2PPT-co-PEDOT film-modified Au was formed by the incubation of Au|G2PPT-co-PEDOT in an ethanolic solution of 50 mM DPA for 30 min. The unbound DPA was removed by washing the electrode system in absolute ethanol followed by Millipore water.

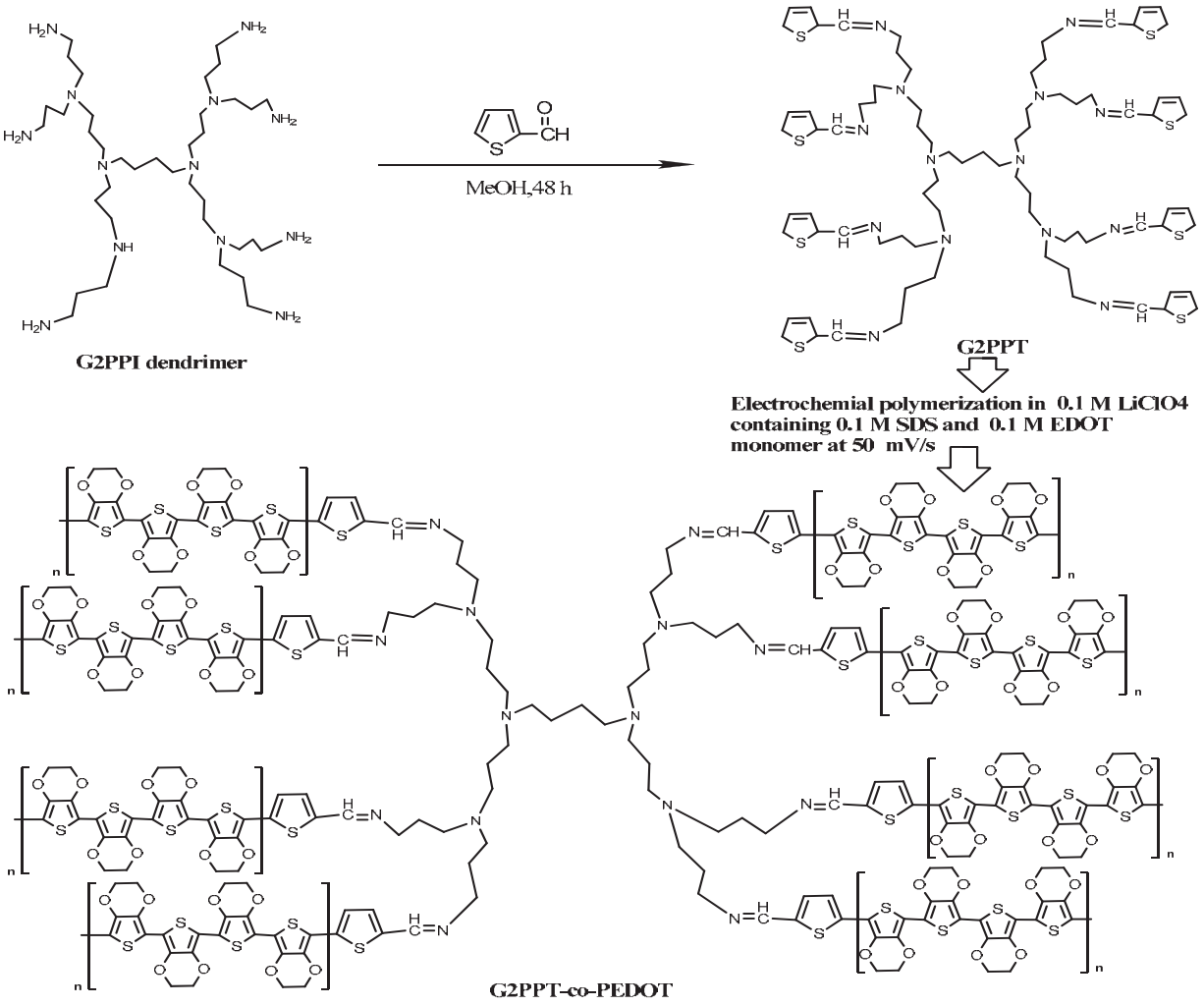


Figure 3. Reaction scheme for the preparation of G2PPT-co-PEDOT.

The DPA film on the Au|G2PPT-co-PEDOT electrode was activated by immersing the electrode in a 1:1 mixture of 1 mM ethylenediamine acetic acid (EDAC) and 1 mM N-hydroxysuccinic acid (NHS) for 1 h. (This procedure activated the carboxylic group of DPA for easy bonding with the amine group of streptavidin). The electrode was then functionalized with streptavidin by incubating it in a 0.1 M phosphate buffer (pH 7.5)/0.1 M KCl saline solution (PBS) containing 10 µg/mL streptavidin for 60 min at 25 °C and was later rinsed in PBS. 10 nM biotinylated 76-mer-ssDNA-Aptamer was placed on the surface of the streptavidin-modified electrode for 120 min at 25 °C to form Au|G2PPT-co-PEDOT|76-mer-ssDNA-Aptamer biosensor via biotin-streptavidin interaction. The procedure for the aptasensor formation and its detection of 17β-estradiol is schematically represented in Fig. 4.

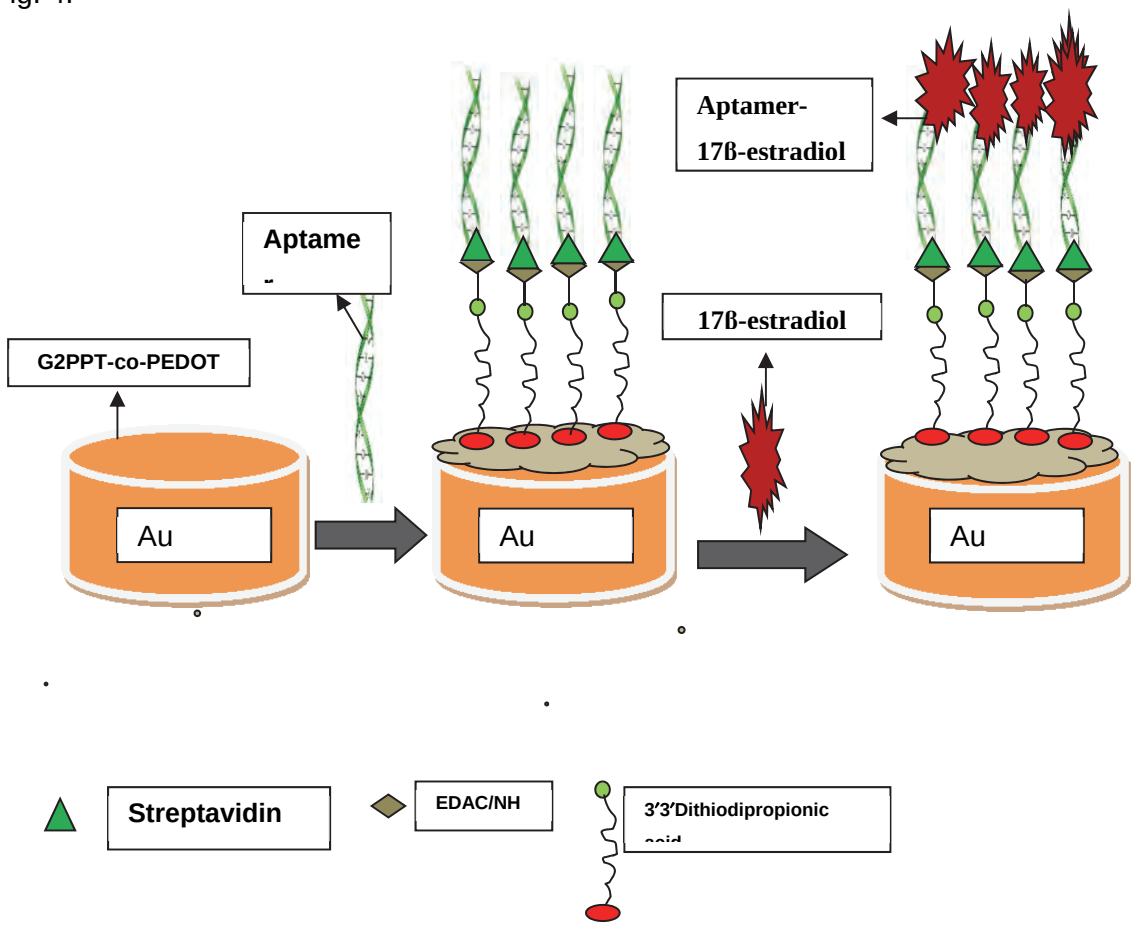


Figure 4. Schematic representation of the construction of Au|G2PPT-co-PEDOT|76-mer-ssDNA-Aptamer biosensor.

2.4 Electrochemical measurement

A three-electrode cell system was used to perform all electrochemical experiments. Gold disk electrode with a diameter of 1.6 mm was used as the working electrode (or the electrode for biosensor preparation), platinum wire as the counter electrode, and Ag/AgCl (3 M Cl^-) as the reference electrode. For experiment with screen-printed sensors, a 1.6 mm diameter gold working electrode, a gold counter electrode and a silver reference electrode were printed on the same strip and used with cell solutions of 50 µL. All electrochemical (voltammetric and electrochemical impedance spectroscopy (EIS)) experiments were

recorded with a Zahnner IM6 electrochemical workstation (MeBtechnik) at room temperature and in solutions de-aerated by purging with argon for 20 min. Square wave voltammetry (SWV) measurements were performed at a SWV amplitude of 25 mV and frequency of 15 Hz.

2.5 Instrumental analysis

UV/Vis spectroscopy measurements were recorded with a Nicolette Evolution 100 Spectrometer (Thermo Electron Corporation, UK). Fourier transform infrared spectroscopy (FTIR) measurements were done with a Perkin-Elmer Spectrum 100-FTIR spectrophotometer. The morphology of the samples was studied with a SEM Gemini LEO 1525 microscope. Fluorescence spectrophotometer (Horiba Nanolog^{TM} 3-22-TRIAX) with double grating excitation and emission monochromators and a slit width of 3.2 nm was used for fluorescence experiments. Proton NMR (^{1}H NMR) measurements were performed with a Varian GerminiXR200 (200 MHz) spectrometer using $CDCl_3$ as solvent and tetramethylsilane as an internal standard.

3 EXPERIMENTAL PROCEDURES

The ENDOTEK project focussed on the construction, optimisation, characterisation and application of a dendritic aptamer biosensor for E2 developed with G2PPT-co-PEDOT star co-polymer and 76-mer-ssDNA-Aptamer. The methodology for the characterisation, optimisation and calibration of the aptasensor and its application in real sample are presented.

3.1 Water samples handling

Standard solutions of 17β-estradiol and treated wastewater samples (effluent of water treatment plant) were used. The Environmental Protection Agency of USA recommended methods for water and wastewater sampling were adopted as specified in the EPA-600/4-82-0129, Handbook for Sampling and Sample Preservation in Various Waters and Wastewaters (U.S. EPA 1982). Accordingly, freshly collected effluents of treated wastewater samples were used for the experiments reported in this study. Samples were stored in small aliquots at -30 °C when not used freshly. Thawed samples were not refrozen. All measurements were in triplicates.

The USA Environmental Protection Agency's Standard Operating Procedure for the analysis of steroid hormones in aqueous samples (U.S. EPA 2005) was adopted in the analysis of real samples, i.e. treated wastewater collected from Scientific Services Laboratory of the City of Cape Town. (Scientific Services Laboratory is responsible for the treatment of sewage and wastewater for the City of Cape Town). The analysis of the wastewater samples involved the implementation of the following steps:

(i) The determination of response to17β-estradiol in water sample.
(ii) The determination of 17β-estradiol in spiked wastewater sample.
(iii) The calculation of the amount of 17β-estradiol recovered.
(iv) The performance of precision and reproducibility tests.

3.2 Multichannel electrochemical aptamer genosensor

The multichannel genosensing was performed with calibrated aptamer biosensors to determine 17β-estradiol in wastewater samples. The wastewater samples used were the treated domestic wastewater (wastewater effluent), since that is the form of wastewater that finally enters the various water systems. The aptamer biosensor chips used in this study consisted of G2PPT-co-PEDOT|76-mer-ssDNA-Aptamer system deposited on 12 channel screen-printed Au electrodes. Each sensor chip was constructed with G2PPT-co-PEDOT and 5´-BiotinGCTT...AAGC-3´ (76-mer biotinylated aptamer) incorporated onto the gold surface of the screen-printed electrode via biotin-avidin interaction. The biosensor was calibrated with standard 17β-estradiol solution. Cyclic voltammetry and square wave voltammetry measurements were performed with a multichannel robotic sensor test system (see Fig. 5) which was used to test several samples simultaneously and the resultant voltammograms were recorded.

Figure 5. Uniscan TST-RM-12 robotic multichannel electrochemical analyser.

With the multichannel robotic sensor test system, aspects of the biosensor preparation and the determination of the analyte were carried out with 12 individually controlled electrodes of the microsensor chips programmed for reactions in a 96-well electrochemical cells system containing reagents or samples. A typical flowchart for the robotic electrochemical analyser experiment is presented in Fig. 6 for Au|G2PPT-co-PEDOT|76-mer-ssDNA-Aptamer biosensor preparation and 17β-estradiol determination.

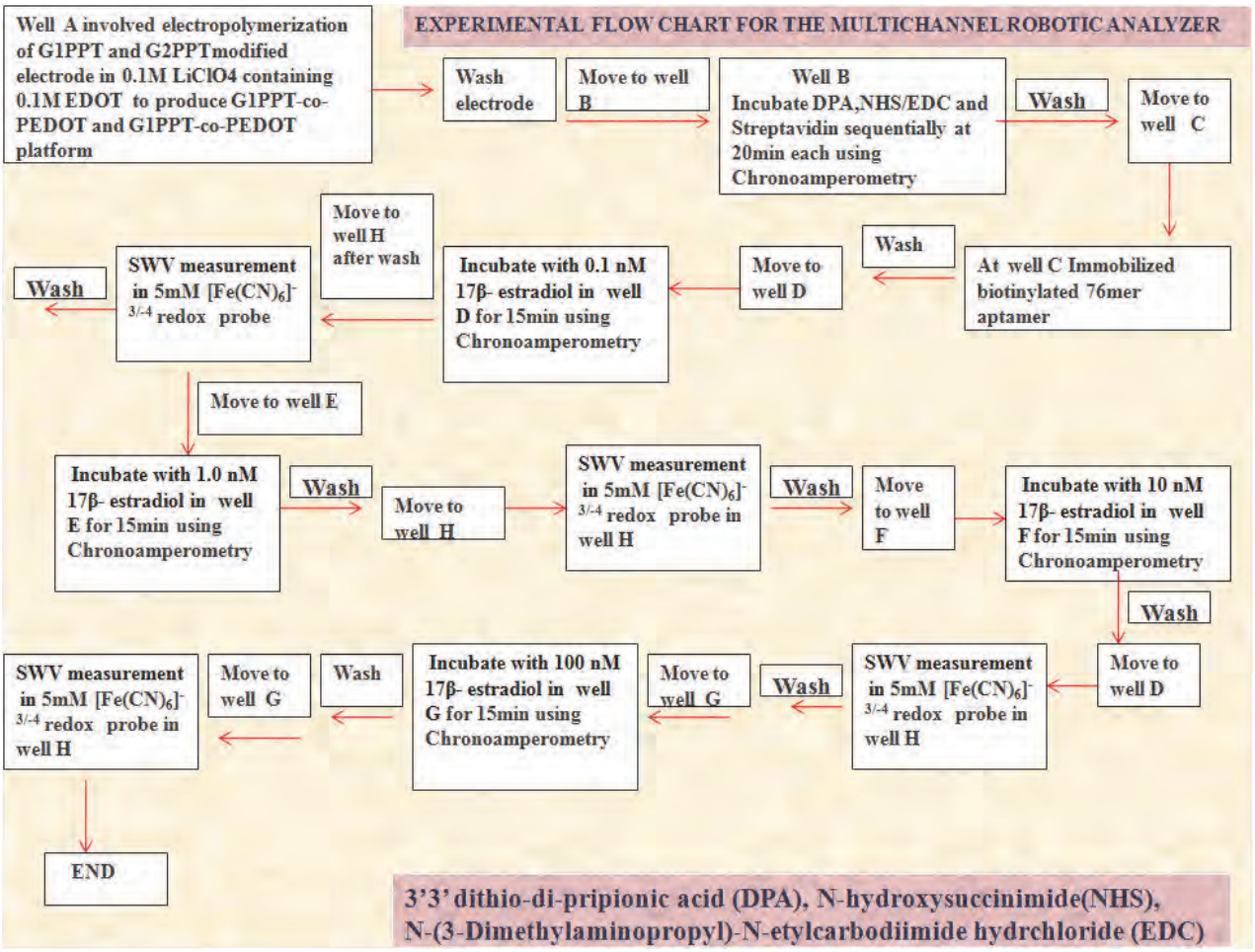


Figure 6. Flow chart for a typical experiment using the Multichannel Robotic Sensor Test Station.

3.3 17β-estradiol ELISA experiments

The 17β-estradiol ELISA methodology is based on competitive immunoassay. This ELISA kit for the determination of 17β-estradiol in environmental water is based on a competitive enzyme immunoassay that uses anti-17β-estradiol antibody and biotin-avidin affinity system. The 96-wells of the ELISA plate were coated with goat anti-rabbit immunoglobin G (IgG). Biotinylated 17β-estradiol, 17β-estradiol standard or samples and rabbit anti-17β-estradiol are added to the wells for competitive immunoreaction and incubated for 1 h at 20 °C. After incubation and plate washing, HRP-labelled streptavidin (SA-HRP) was added to form HRP-labelled streptavidin-biotinylated 17β-estradiol-antibody complex on the surface of the wells. Finally, HRP enzyme activity is determined by o-phenylenediamine dihydrochloride (OPD) and the concentration of 17β-estradiol is calculated. The calculation of 17β-estradiol is based on the fact that the amount of bound peroxidase conjugate is inversely proportional to the concentration of 17β-estradiol in the sample. The enzyme activity determination using OPD was performed by measuring the optical absorbance at 429 nm. The data obtained from wells containing standard 17β-estradiol solution was used to construct ELISA calibration curve. The concentration of 17β-estradiol in the water samples were determined with the calibration curve by reading the corresponding concentration to the absorbance values obtained.

3.4 Chromatographic determinations

Chromatographic determination of 17beta-estradiol in wastewater effluent was performed by HPLC/UV with qualitative confirmatory analyses using GC-MS.

3.4.1 HPLC analysis of wastewater sample

An Agilent 2D HPLC chromatographic instrument was used in the experiment. HPLC determination of 17beta-estradiol in wastewater was performed with a C18 bonded reversed-phase column. The mobile phase was acetonitrile-water (50:50, v/v).

3.4.2 GC-MS analysis of wastewater

The GC-MS experiments were performed with an Agilent 6890N GC coupled with an Agilent 5975 MS using Rtx®-5MS (30 m, 0.25 mm ID, 0.25 µm film thickness) column. The chemical compounds in the water samples were extracted by solid phase extraction (SPE). The retained compounds were then eluted with methanol/dichloromethane/acetone (40:40:20) v/v mixture. The eluents were evaporated to dryness with nitrogen followed by being reconstituted in acetonitrile. The samples were derivatized with heptafluorobutyric anhydride (HFBA) in acetonitrile for 1 h at 80 °C. The derivatives were again evaporated to dryness before reconstituted in hexane followed by injecting onto the GS-MS column. The stock standard solution was first dissolved first in acetone and then in methanol. The calibration standards were all dissolved in deionised water. Both the sample and standards were extracted in the same way.

4 RESULTS AND DISCUSSION

This section reports the preparation, characterisation and application of the materials that were used in aptasensor fabrication. However, this will not include the details of the synthesis of the 76-mer-ssDNA-Aptamer by the SELEX procedure. The discussion of the results will cover spectroscopic, microscopic and electrochemical methods used to analyse the chemical systems and changes in their properties within the sensor system. The spectroscopic analysis included proton nuclear magnetic resonance spectroscopy (^{1}HNMR), Fourier transfer infrared spectroscopy (FTIR) and electrochemical subtractively normalised *in situ* FTIR (e-SNIFTIR). The aptasensor response parameters and application in the analysis of real samples are also discussed.

4.1 Electrosynthesis of the G2PPT-co-PEDOT star co-polymer

Au|G2PPT-co-PEDOT| composite electrode was fabricated by the electropolymerisation of 3,4-ethtylenedioxythiophene or EDOT in the presence of G2PPT drop-coated onto an Au electrode. The experiment was performed in 0.1 M $LiClO_4$ containing 0.1 M SDS with cyclic voltammetry technique in the potential range of -1000 mV to +1000 mV at a potential scan rate of 50 mV/s. The cyclic voltammograms of EDOT with and without G2PPT were compared in Fig. 7. One of the primary differences between the two polymerisation processes is the change in current response. A slight drop in the EDOT polymerisation current was observed in the presence of G2PPT compared to the electropolymerisation of current of EDOT in the absence of G2PPT. An oxidation peak associated with G2PPT is apparent at 995 mV with no increase in current as expected for film growth, which revealed the occurrence of the electrodeposition of dendrimer on the surface of the electrode as earlier reported by Arotiba *et al.* (2010). A shoulder corresponding to the oxidation potential of EDOT is present at 986 mV.

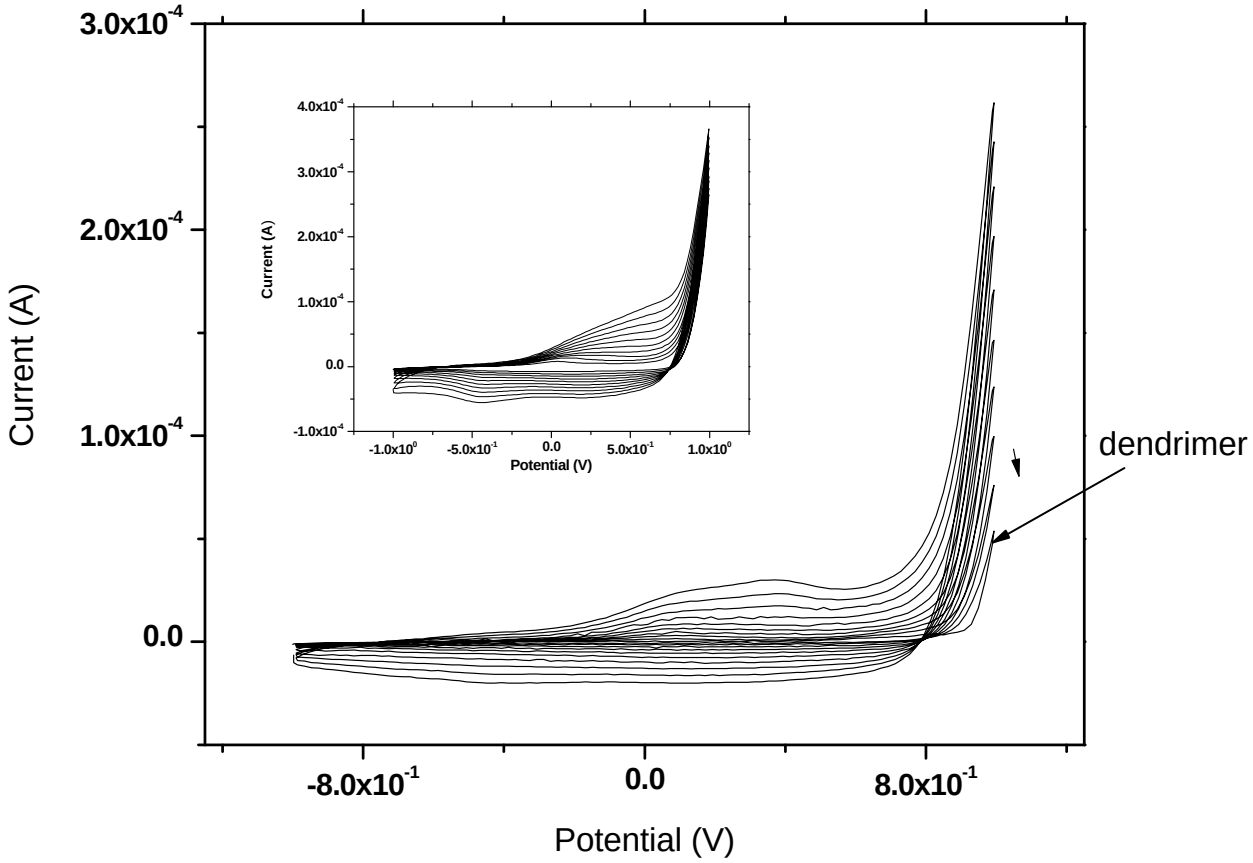


Figure 7. Electropolymerisation of G2PPT on Au electrode in 0.1 M $LiClO_4$ containing 0.1 M EDOT and 0.1 M SDS at a scan rate of 50 mV/s to form Au|G2PPT-co-PEDOT. The inset is the EDOT polymerisation voltammograms for PEDOT film formation.

The anodic and cathodic peak potentials of the co-polymer were observed at +230 mV and -490 mV respectively compared to PEDOT film which was obtained at +290 mV and -603 mV, respectively. The oxidation potential shifted to less positive values which is indicative of the formation of co-polymer (Xu *et al*. 2005). An irreversible electro-oxidation peak that occurred at +1330 mV in the voltammogram of functionalised dendrimer (G2PPT) has also been reported by Julio *et al.* (2002). The change in oxidation and reduction peaks may be attributed to the formation of the co-polymer. Co-polymers have been reported to decrease the onset of oxidation, which can successfully prevent overoxidation (Arotiba *et al.* 2010). The decrease in the onset potential for electro-oxidation can be linked to the type of dendrimer employed for this work as well as the type of electrolytes used (Xu *et al*. 2005). As would be seen from scanning electron microscope study, the film obtained for G2PPT-co-PEDOT co-polymer is quite different from the PEDOT film, which is an indication of co-polymer formation.

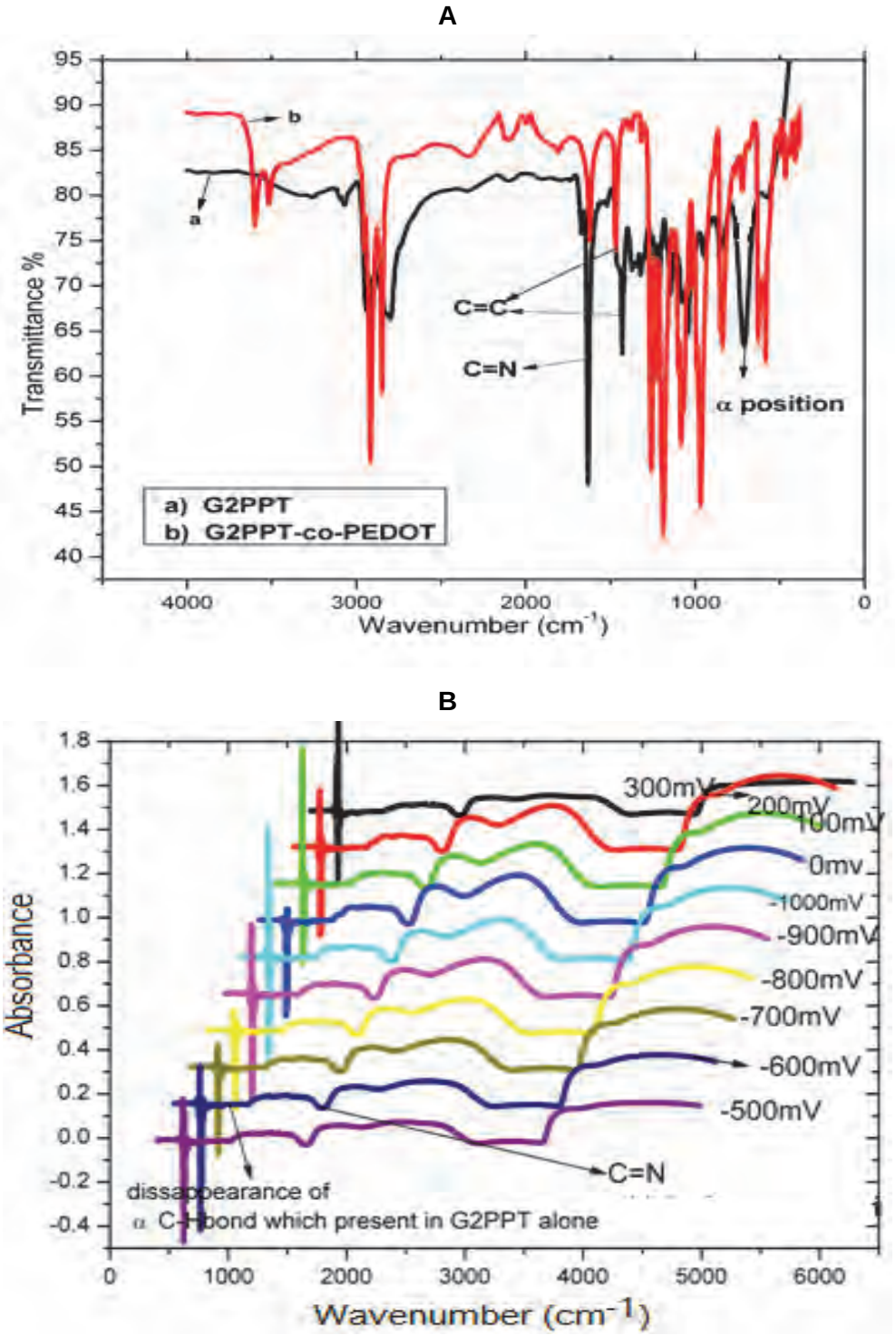


Figure 8. (A) FTIR spectra of G2PPT and G2PPT-co-PEDOT. (B) e-SNIFTIR spectra of G2PPT-co-PEDOT.

4.2 Spectroscopic analysis of G2PPT-co-PEDOT

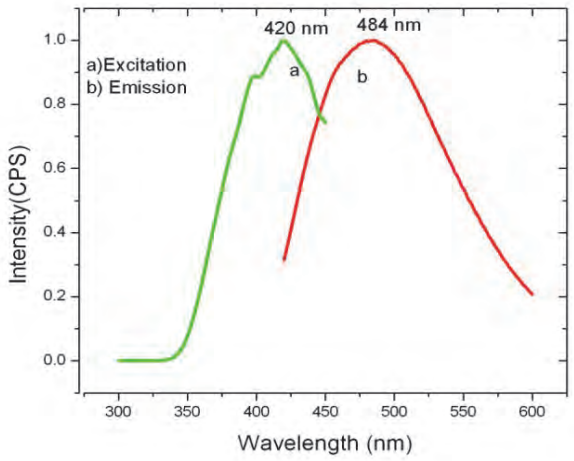
The synthesis of the G2PPT-co-PEDOT star co-polymer started with the preparation of G2PPT by the condensation of G2PPI with 2-thiophene aldehyde in dry methanol under a positive pressure of nitrogen gas for 2 days to produce a yellow oil of G2PPT (Smith *et al.,* 2003, Salmon and Jutzi 2001). This is followed by the electropolymerisation of 0.1 M EDOT in 0.1 M $LiClO_4$ solution using Au electrode on which G2PPT was drop-coated. The proton NMR (^{1}HNMR) of G2PPT gave a new chemical shift at 8.37 ppm for C=N-H which was not observed in the parent G2PPI. This was supported by a strong G2PPT FTIR band (Fig. 8) at 1622 cm^{-1} for C=N-H bond and a 788 cm^{-1} out-of-plane vibration for thiophene ring (Xu Min *et al*., 2006, Wei *et al.* 2006). The FTIR spectrum of the G2PPT showed 722 cm^{-1} out-of-plane band for C-H bending located at the α-position of the thiophene ring, which disappears after the co-polymerisation of G2PPT and EDOT to form G2PPT-co-PEDOT via α-α coupling of thiophene units.

The peaks at 1632 cm^{-1} (FTIR) and 1640 cm^{-1} (e-SNIFTIR) are C=N bond stretching vibrations present in the G2PPT dendrimer moiety. The peaks are not observed in the G2PPI-co-PEDOT spectra. Components of the G2PPT-co-PEDOT star polymer used in sensor system, including the thiophene monomer (EDOT), functionalized dendrimers (G2PPT), homopolymer (PEDOT) and the star co-polymer itself (G2PPI-co-PEDOT), were analyzed with FTIR and the spectra are drawn in Fig. 8. The spectra (not shown) of the thiophene monomer (EDOT) have several characteristic peaks at 387, 521, 631, 677, 758, 859, 933, 1054, 1099, 1135, 1183, 1271, 1468, 1583 and 2980. Stretching vibrations at 1468 and 1583 cm^{-1} originate from the stretching modes of C=C, and that at 1099 cm^{-1} from S-H of the thiophene ring. The C-H bending of thiophene can be observed at 1054 cm^{-1} and 758 cm^{-1}. In the spectrum of the functionalized dendrimers several characteristic peaks at 476, 720, 792, 862, 1012, 1258, 1213, 1417, 1633, 1672, 2120, 2917, 2962 cm^{-1} for G2PPI were observed (spectra not shown in Fig. 8). The band at 2917 and 2962 cm^{-1} for G2PPI indicate the presence of CH_2 stretching. The sharp bands at 1632 cm^{-1} is attributed to the C=N bond stretching vibration present in the dendrimer moiety. In the spectrum of the G2PPT (Fig. 8), out-of-plane bending of C-H bending located at the α-position of the thiophene ring was observed at 720 cm^{-1} (Deng *et al*. 2005). The spectra of the star co-polymer (G2PPT-co-PEDOT) (Fig. 8) and homopolymer (PEDOT) (spectra not shown in Fig. 8) prepared by polymerisation in 0.1 M $LiClO_4$ containing 0.1 M sodium dodecyl sulphate (SDS) and 0.1 M EDOT (monomer) showed several characteristics peaks at 627, 788, 837, 969, 1012, 1083, 1190, 1258, 1232, 1468, 1621, 2917, 2847, 2954 cm^{-1} and 626, 769, 850, 980, 1108, 1131, 1199, 1376, 1405, 1474, 1497 and 2920 cm^{-1}. The band at 627, 626, 1190 and 1199 cm^{-1} indicate the presence of perchlorate ion used as the dopant during polymerisation (Xu 2015). In the spectrum of the G2PPT-co-PEDOT (co-polymer) a band with a sharp peak for C=N appears at 1622 cm^{-1}, which was completely absent in the homopolymer (PEDOT) spectrum. In addition, the absorbance at 720 cm^{-1} found in G2PPT spectrum, completely disappears after the polymerisation, indicating that the G2PPT was converted to G2PPT-co-PEDOT via α-α coupling of thiophene units. The band at 789 cm^{-1} corresponds to C-H out-of-plane vibration in thiophene ring. The vibration of C-S bond in the thiopene ring is occurs at 837 cm^{-1}, which is similar to earlier reports (Deng *et al*. 2005).

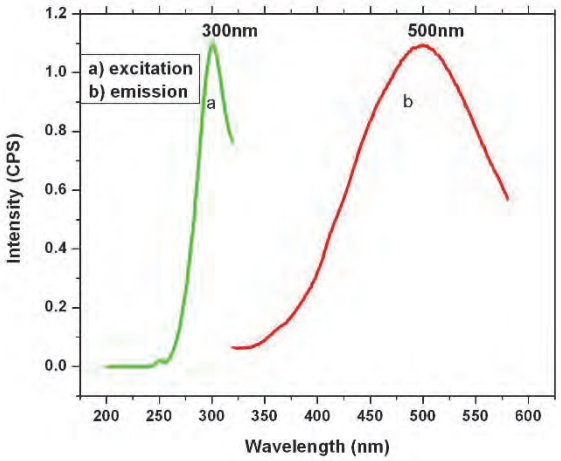
4.3 Fluorescence spectroscopy of G2PPT-co-PEDOT

The fluorescence spectra of G2PPI, G2PPT and G2PPT-co-PEDOT were investigated and recorded as shown in Fig 9. G2PPI dendrimer exhibited excitation and emission peaks at

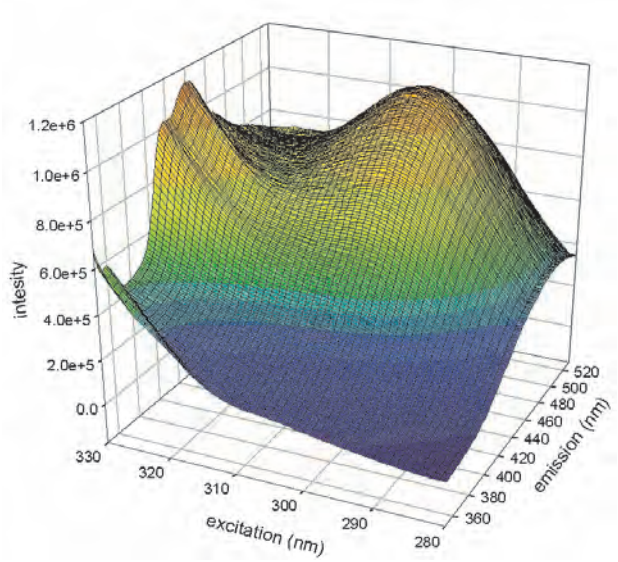
356 nm and 421 nm, respectively. The functionalization of G2PPI dendrimer with thiophene to form G2PPT, i.e. generation 2 poly(propylenethiophenoimine), increased the intensity of the emission peak at 484 nm. Similar responses have been reported for other compounds where dendrimer arms were modified with 1,8-napthalimide groups (Chen *et al.* 2006, Yang *et al*. 2007).



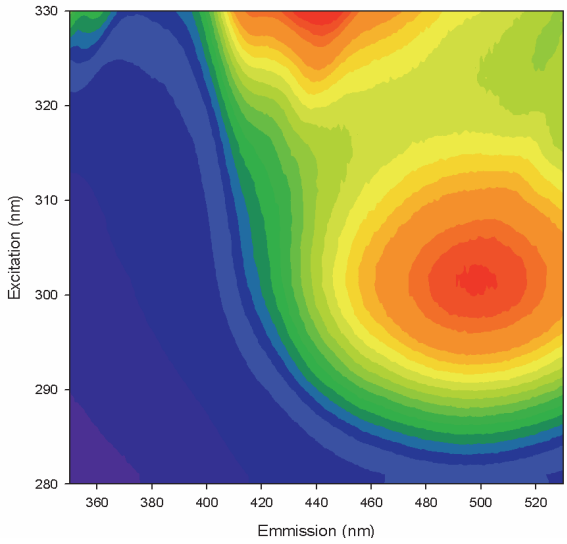
G2PPT



G2PPT-co-PEDOT



3D isograms



3D contour spectra

Figure 9. Fluorescence spectra of G2PPT and G2PPT-co-PEDOT (Top diagrams). 3D fluorescence emission isograms and contour spectra of G2PPT-co-PEDOT (Bottom diagrams).

On the other hand the formation G2PPT-co-PEDOT dendritic star co-polymer changed the chromophoric system of the parent dendrimer which gave florescence hypsochromic excitation and bathochromic emission bands at 300 nm and 500 nm, respectively, as shown in Fig 9. The strong emission band at 500 nm (in blue region) may be attributed to the

incorporation of the highly conducting PEDOT polymer at the dendrimer arm which exhibited blue coloration in its doped state due to onset of π-π* transition (Rozlosnik 2009). This bathochromic shift in emission wavelength shows that the dendritic star co-polymer may be a good blue emitter. To further confirm the formation of the star co-polymer, it was excited at the wavelength corresponding to G2PPI and G2PPT but the observed emission could not be reversed to their excitation bands upon an emission-excitation scan. This shows that an entirely new compound was synthesized.

4.4 Scanning electron microscopy (SEM)

Figure 10 is a display of the SEM images of the sensor materials used for sensor preparation. The SEM of G2PPT consists of evenly distributed globules and PEDOT showed flaky exfoliates. G2PPT-co-PEDOT revealed what could be considered an enlargement of the G2PPT dendritic globules by the incorporation of the PEDOT onto the arms of G2PPT dendrimer, which may be considered as a further proof of co-polymerisation of G2PPT and PEDOT (Olowu *et al.* 2011a, Baleg *et al.* 2014).

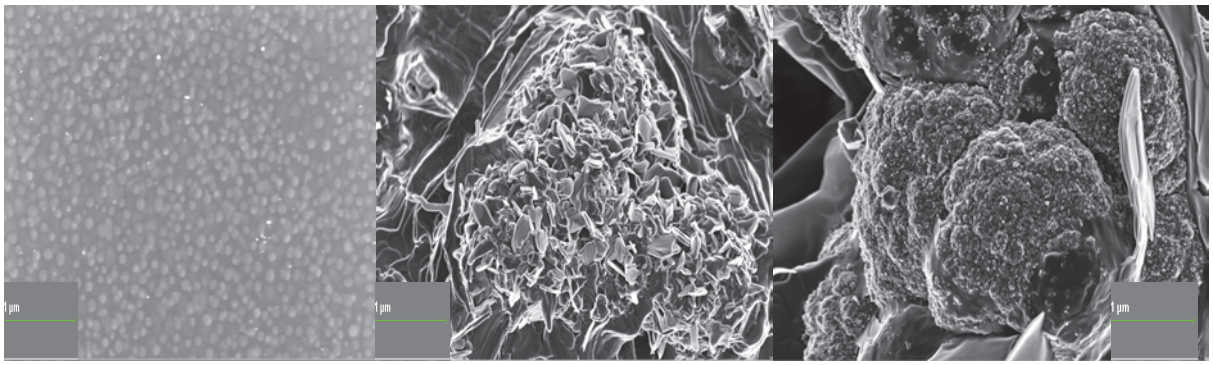


Figure 10. Scanning electron microcopy (SEM) images of dendritic star co-polymer and its constituents.

4.5 Cyclic voltammetry in ferricyanide probe

Cyclic voltammetry is an effective and convenient tool to monitor the barrier of the modified electrode, because the electron transmission between the solution species and the electrode must occur by tunnelling either through the barrier or through the defects in the barrier. In this work the electrochemical behaviour of the stepwise fabrication process was studied in PBS solution containing 5 mM $[Fe(CN)_6]^{-3/-4}$ (used as an electrochemical redox probe). The cyclic voltammogram of $[Fe\ (CN)_6]^{-3/-4}$ at different modified electrode are presented in (Fig. 11). A pair of well-defined redox peaks was observe at the bare electrode (curve a) compared to the electrode modified with functionalised dendrimer, which showed drastic decrease in peak currents (curve b). This may be attributed to the compact nature of the functionalised film coated on the electrode, which hindered the charge transfer or the electroactive redox probe from approaching the surface of the electrode. Also the S-Au linkage between the thiophene of the dendrimer and the gold electrode is negatively charged, resulting in electrostatics repulsion of the negatively charged $[Fe(CN)_6]^{-3/-4}$ redox probe (Li *et al*. 2009). An increase in peak current was observed at (curve a) on modification of the electrode with PEDOT (curve c) because it is a conducting polymer which accelerates the rate of electron transfer due to a decrease in the electron tunnelling between the probe

and the electrode; as well as a result of the electrostatic attraction between the positively charged backbone of the homopolymer and the negatively charged $[Fe(CN)_6]^{-3/-4}$ probe.

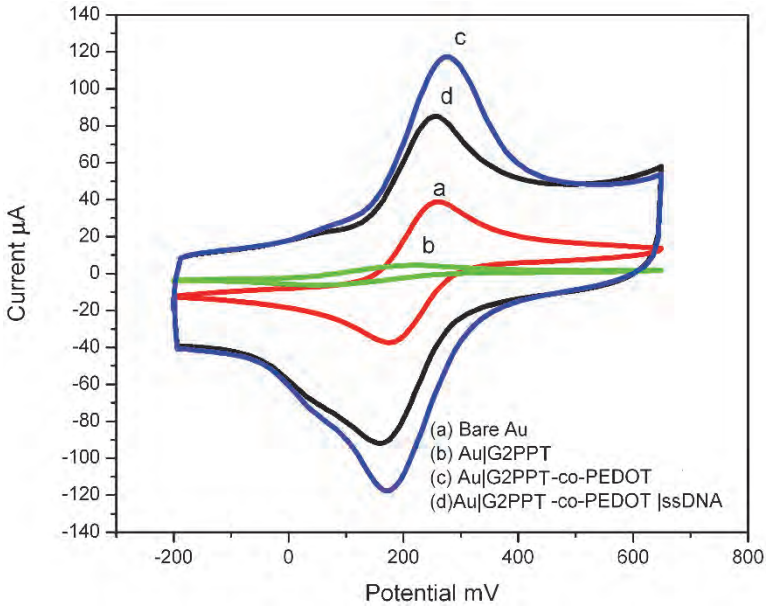


Figure 11. Cyclic voltammetry of modified electrodes in 5 mM $[Fe(CN)_6]^{-3/-4}$ PBS at (a) Au (b) Au|G2PPT| (c) Au|G2PPT-co-PEDOT, (d) Au|G2PPT-co-PEDOT|76-mer-ssDNA-Aptamer.

A significant change occurred after the G2PPT was co-polymerized with PEDOT to form a G2PPT-co-PEDOT star co-polymer on the surface of the electrode (curve c). This may be ascribed to the combination of the unique properties exhibited by the functionalised macromolecule and the conducting polymer, as well as the electrostatic attraction between the negatively charged redox probe and the positively charged back bone of G2PPT-co-PEDOT. The result is a further decrease in the electron transfer tunnelling distance, thereby increasing the rate of electron transfer at the surface of the electrode. A slight decrease in peak current of probe $[Fe\ (CN)_6]^{-3/-4}$ ion was observed for the aptamer modified electrode system (Au|G2PPT-co-PEDOT|76-mer-ssDNA-Aptamer) compared to that obtained with the co-polymer modified Au electrode (Au|G2PPT-co-PEDOT). This also reflects the occurrence of electrostatic repulsion between the negatively charged phosphate backbone of the ssDNA aptamer and the negatively charged $[Fe(CN)_6]^{-3/-4}$ probe. The CV results confirm that the 76-mer-ssDNA-Aptamer was successfully attached to G2PPT-co-PEDOT on the gold electrode surface.

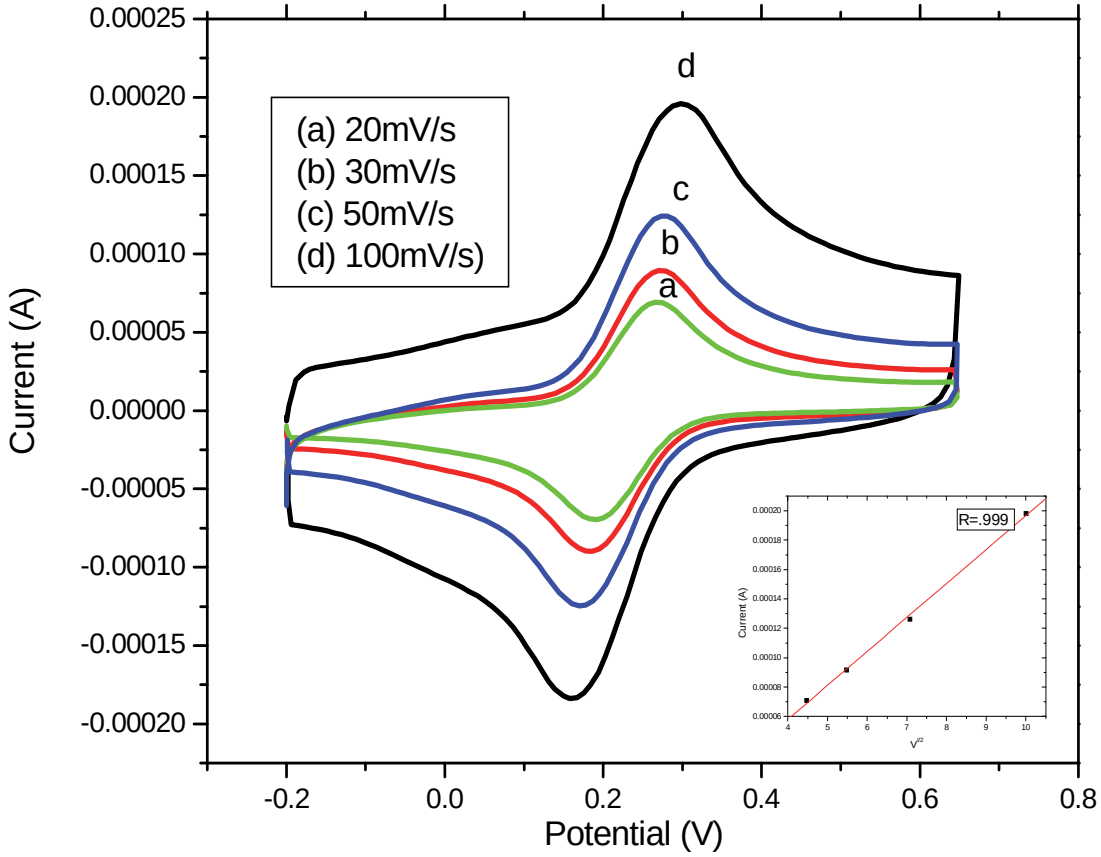


Figure 12. Cyclic voltammograms of Au|G2PPT-co-PEDOT at scan rates of (a) 20 mV/s, (b) 30 mV/s, (c) 50 mV/s and (d) 100 mV/s in 5 mM $[Fe(CN)_6]^{3/-4}$ redox probe containing 0.1 M KCl.

The scan rate dependence of the CVs of Au|G2PPT-co-PEDOT electrode in $[Fe(CN)_6]^{3-/-4}$ solution, as plotted in Fig. 12, depicts increases in the voltammetric peak currents as the scan rate increases, with no concomitant shift in the peak potential. This illustrates the presences of surface-bound thin layer of electroactive co-polymer on the electrode. A linear dependence of the anodic peak current on the scan rate was observed (Fig. 12 inset), with gave a correlation coefficient of 0.991. It can thus be deduced that the G2PPT-co-PEDOT layer behaves as a surface-bound thin film electroactive species undergoing fast electron transfer reaction at the electrode. The fact that there was no shift in potential and the I_{pa}/I_{pc} is unity also revealed the stability of the G2PPT-co-PEDOT platform in the $[Fe(CN)_6]^{3/-4}$ solution. The diffusion coefficient (D) of $[Fe(CN)_6]^{3/-4}$ to the of Au|G2PPT-co-PEDOT electrode surface was calculated to be 4.50 x 10^{-7} cm^2 s^{-1}, using the Randle Sevčik equation,

$$I_p = 2.69 \times 10^5 n^{3/2} A D^{1/2} v^{1/2} C \qquad (1)$$

where I_p = peak current, n = number of electron transfer, A = area of an electrode, D = diffusion coefficient, v = scan rate and C = concentration of $[Fe(CN)_6]^{3/-4}$ in the bulk solution.

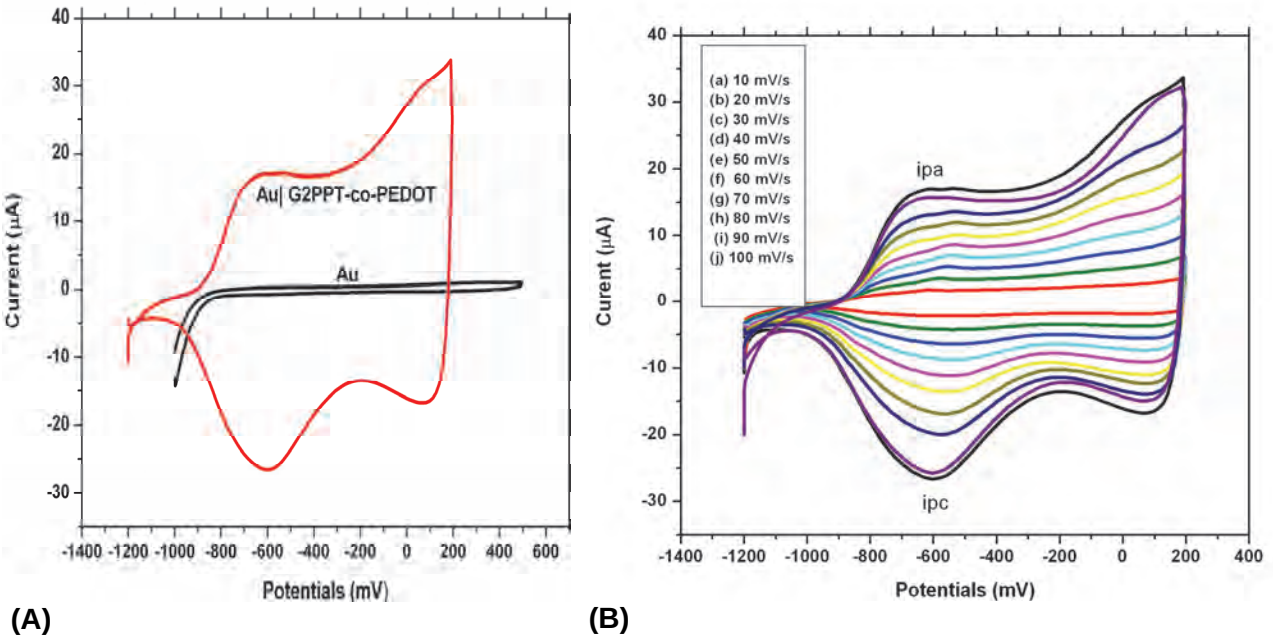


Figure 13. (A) Cyclic voltammograms of Au and Au|G2PPT-co-PEDOT in 0.1 M PBS (pH 7.5). (B) Cyclic voltammograms of Au|G2PPT-co-PEDOT at potential scan rates of 10-100 mV/s in 0.1 M PBS (pH 7.5).

The D value is high compared to 2.20 x 10^{-8} cm^2 s^{-1} obtained for $[Fe(CN)_6]^{3/-4}$ on bare Au and 6.46 x 10^{-8} cm^2 s^{-1} and 8.68 x 10^{-9} cm^2 s^{-1} reported for polyaniline (PANI)-modified electrodes (Mathebe *et al*. 2004). The high D value obtained for Au|G2PPT-co-PEDOT electrode implies a facile flow of electron due to high conductivity of the G2PPT-co-PEDOT co-polymer due to increased conjugation length, resulting in a D value that is approximately one order of magnitude higher than the values for PANI as reported by Mathebe *et al*. (2004).

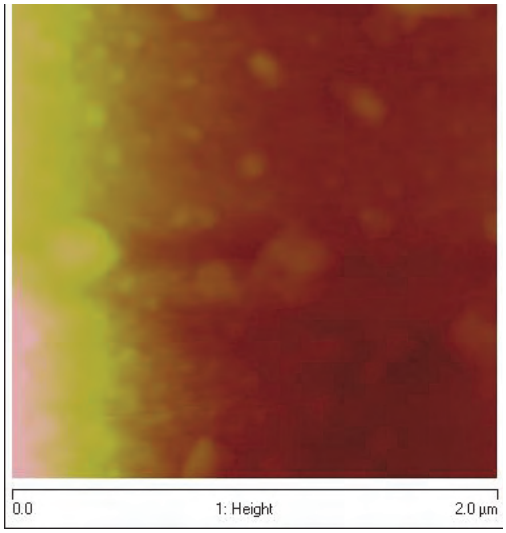
4.6. Voltammetry of G2PPT-co-PEDOT

Cyclic voltammograms of Au|G2PPT-co-PEDOT film in phosphate buffer solution are shown in Fig. 13 for which (A) compares the responses at 100 mV/s for Au and Au|G2PPT-co-PEDOT. The multi-scan rate voltammetric responses of Au|G2PPT-co-PEDOT electrode plotted in (B) indicate a surface-confined electroactive species undergoing fast reversible 2-electron redox process with a formal potential of -550 mV (Olowu *et al.* 2011a, Baleg *et al.* 2014).

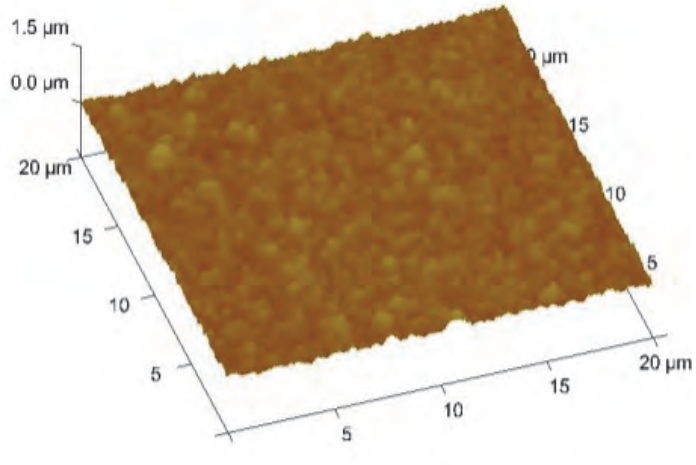
4.7 Atomic force microscopy (AFM)

The AFM images of PEDOT (Fig. 14 top images) are a display of smooth surfaces, while rough surface and globular clusters are depicted in the G2PPT-co-PEDOT images (Fig 14 bottom images). The 3D AFM image of G2PPT-co-PEDOT shows the formation of evenly-distributed dendrimer domes with small holes at the top, which differ from that of PEDOT.

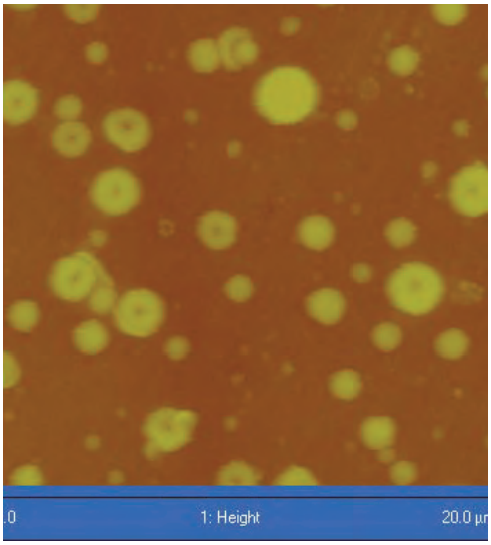
The changes in surface morphology are characteristic of the presence of G2PPT dendrimer moiety in a compound and, therefore, confirm successful electrochemical synthesis of G2PPT-co-PEDOT.



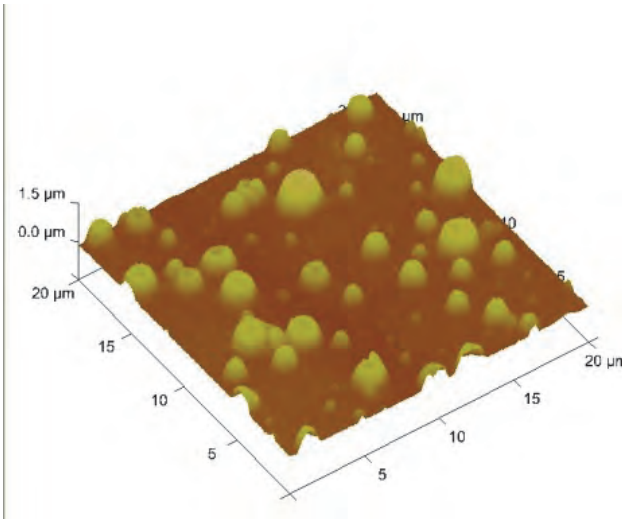
2D image of PEDOT



3D image of PEDOT



2D image of G2PPT-co-PEDOT



3D image of G2PPT-co-PEDOT

Figure 14. AFM images of PEDOT and G2PPT-co-PEDOT.

4.8 Electronics of G2PPT-co-PEDOT

The electronics of Au|G2PPT-co-PEDOT electrode was interrogated by electrochemical impedance spectroscopy (EIS) performed at 100 mHz - 100 kHz in $LiClO_4$ and phosphate buffer solutions. The EIS results indicate that the electrochemical deposition of G2PPT-co-PEDOT on gold electrode results in a decrease in the electrochemical charge transfer resistance when compared to Au and Au|G2PPT interfaces (see Fig. 15). This is attributed to an increase in the conjugation length of the polymer as result of the linking of the highly conjugated PEDOT to the dendrimer. The star co-polymer exhibited a 2-electron

electrochemistry and a surface coverage of 97.3%. The EIS responses of Au|G2PPT-co-PEDOT in aqueous phosphate buffer (pH 7.5) gave responses that were similar to those obtained in 0.1 M $LiClO_4$ which revealed that the dendritic star co-polymer can be used as a suitable platform in both electrolytes. Bode plots showed a remarkable difference in the electronics of Au, Au|G2PPT and Au|G2PPT-co-PEDOT. Bode impedimetric analysis indicates that G2PPT-co-PEDOT is a semiconductor with a phase angle shift, (|φ|, (curves with dotted lines) value of 40°, and thereby exhibits better conductivity than Au (|φ| = 35°) and Au|G2PPT (|φ| = 30°) at 100 mHz when the frequency perturbation is minimal. Also at this frequency, the total impedance (curves with full lines) of the three systems indicate a drastic drop in total impedance, Z, of the materials from $Z_{(Au|G2PPT)}$ = 18 kΩ to $Z_{(Au|G2PPT\text{-}co\text{-}PEDOT)}$ = 2.8 kΩ. The high $Z_{(Au|G2PPT)}$ value shows that G2PPT has insulating properties which is modified by co-polymerisation with PEDOT. As the frequency of the materials are scanned up to 100 kHz the materials display variations in the frequencies of maximum phase angle shift, $|\varphi|_{max}$: Au ($|\varphi|_{max}$ = 29.6° at 900 Hz), Au|G2PPT ($|\varphi|_{max}$ = 49° at 1 Hz) and Au|G2PPT-co-PEDOT ($|\varphi|_{max}$ = 11.6° at 950 Hz). The star polymer gave its $|\varphi|_{max}$ value at a frequency 900 times greater than that of AuG2PPT which confirms that Au|G2PPT-co-PEDOT is a better conductor (Arotiba *et al.* 2010, Olowu *et al.* 2011b. Rassie *et al.* 2011, Iwuoha *et al.* 2014, Tsegaye *et al.* 2014). Also the smaller Nyquist semicircle of Au|G2PPT-co-PEDOT confirms that it has lower charge transfer resistance than Au|G2PPT. The EIS parameters of the platform are collected in Table 1. Results in Table 1 indicate that the sensor platform exhibits fast electrochemistry characterised by a heterogeneous rate constant, k_{et}, exchange current, I_o, and time constant, τ, values of 3.10 x 10^{-2} cm s^{-1}, 3.01 x 10^{-4} A and 2.97 x 10^{-5} s rad^{-1}, respectively. These results clearly indicate that the platform was very suitable for the construction of aptamer biosensor.

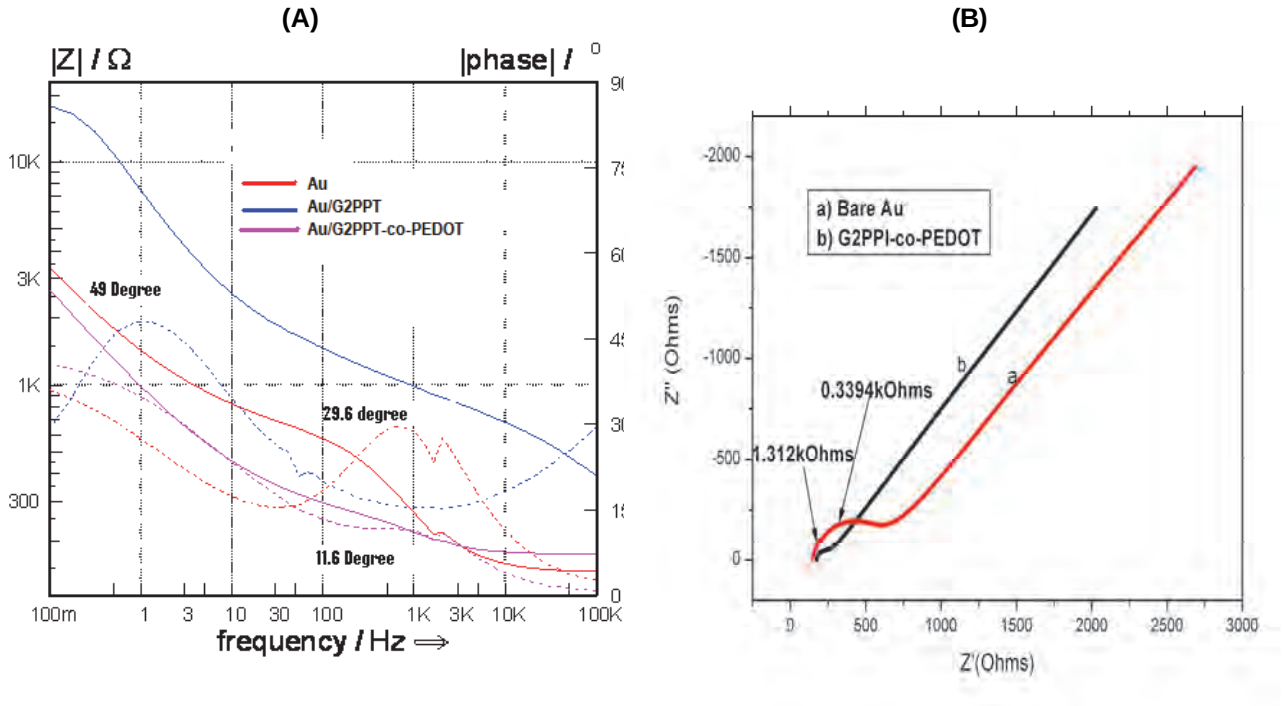


Figure 15. (A) Bode and (B) Nyquist plots of Au|, Au|G2PPT| and Au|G2PPT-co-PEDOT in $Fe(CN)_6]^{-3/-4}$ redox probe at 220 mV.

Table 1. Impedimetric parameters of aptasensor platforms.

Parameter	**Au|**	**Au|G2PPT**	**Au|G2PPT-co-PEDOT**
R_{ct} (Ω^{-1})	495.4	16210	85.31
Phase shift ($\|\varphi\|_{max}$,°)	49	29.6	11.6
ω_{max} (rad s^{-1})	1543.30	3.36	8244.6
Exchange current ($i_{o.}$ A)	5.18×10^{-5}	1.58×10^{-6}	3.01×10^{-4}
Heterogeneous rate constant (k_{et}, cm s^{-1})	5.35×10^{-3}	1.63×10^{-4}	3.10×10^{-2}
Time constant (τ, s rad^{-1})	3.21×10^{-4}	1.86×10^{-1}	2.97×10^{-5}

4.9 Optimisation of aptamer biosensor

The fabrication of the novel aptasensors was performed on G2PPT-co-PEDOT-modified gold electrode upon which 76mer-biotylated aptamer was immobilized via streptavidin-biotin to produce Au|G2PPT-co-PEDOT|76-mer-ssDNA-Aptamer for 17β-estradiol detection. The cyclic voltammetric responses of the biosensor platform and the biosensor probed via the electrochemistry of ferricyanide is shown in Fig. 16. Voltammetric and impedimetric interrogation of the sensor were performed to ascertain the conditions for optimal reactivity of the ssDNA aptamer immobilized on the Au|G2PPT-co-PEDOT| electrode. The sensor voltammetric responses were measured before and after immobilization of ssDNA aptamer on the modified electrode, and decrease in voltammetric peak currents were observed when various concentrations of ssDNA aptamer (0.1-1.0 μM) were used in the sensor construction. This may be attributed to biotinylated ssDNA aptamer interference with the electron flow (Tombelli *et al.* 2005). The decrease in current as aptamer concentration was increased may be due to the electrostatic repulsion that exist between the negatively charge phosphate backbone of the DNA aptamer and the negatively charged hydroxyl ion substituent of the target in aqueous solution, which is one of the subtle substituent for aptamer discrimination within molecule of similar structures in the presence of $[Fe(CN)_6]^{-3/-4}$ as redox probe.

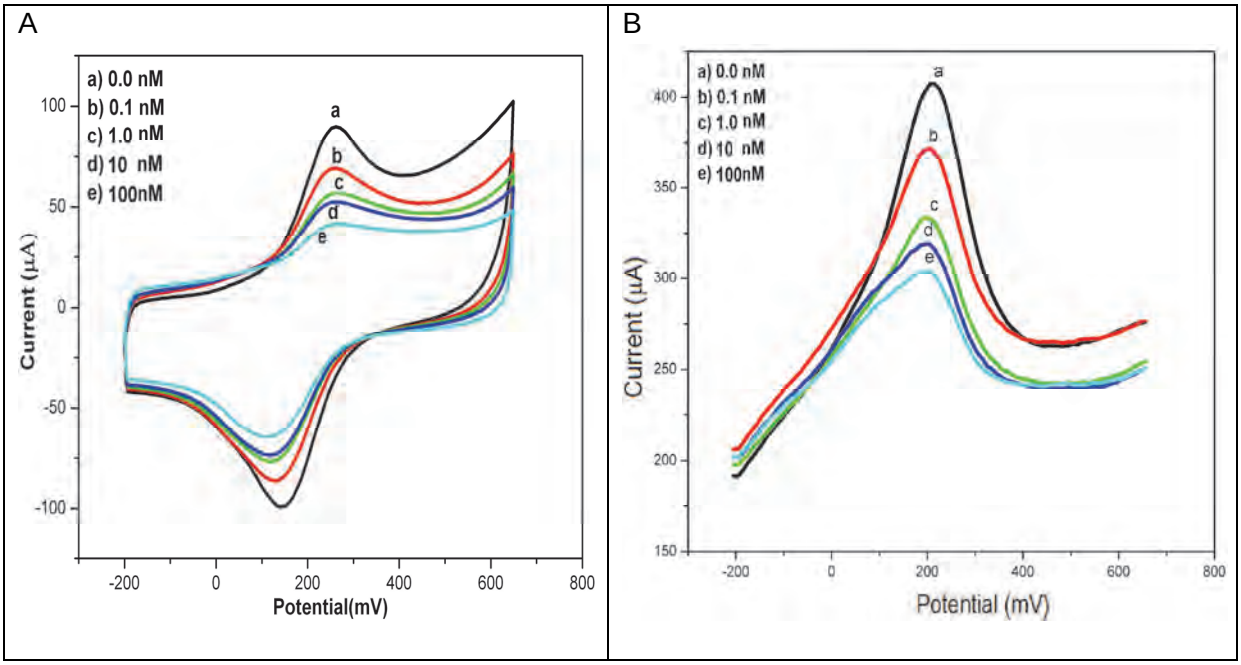


Figure 16. Cyclic voltammetric (A) and Square wave voltammetric (B) responses of the Au|G2PPT-co-PEDOT|76-mer-ssDNA-Aptamer biosensor at 100 mV/s in 0.1 M phosphate buffer (pH 7.5) containing 0.1 M KCl and 5 mM $[Fe(CN)_6]^{-3/-4}$. Results are shown for 5 concentrations of 17β-estradiol. (0.01 nM = 3 pg/mL).

4.10 Determination of 17β-estradiol

The aptasensor was made up of a biotinylated ssDNA aptamer immobilized on a gold electrode modified with G2PPT-co-PEDOT dendritic star co-polymer. The analytical performance of the aptasensor was investigated by incubating the sensor in 0.01-1000 nM 17β-estradiol solutions. Electrochemical signals were recorded by cyclic voltammetry and square wave voltammetry and results are shown in Fig. 16. The experiments were performed at 100 mV/s in 0.1 M phosphate buffer (pH 7.5) containing 0.1 M KCl and 5 mM $[Fe(CN)_6]^{-3/-4}$. The detection of 17β-estradiol was monitored through the decrease in peak current of the biosensor as the concentration of E_2 increased. This is similar to what has been reported by other workers for related systems (Kim 2009; Kim 2010) The attenuation of peak current as concentration of E_2 increases is attributed to the formation of aptamer-E_2 complex on the electrode surface which hindered the effective flow of electrons between the redox probe ($[Fe(CN)_6]^{-3/-4}$) and the electrode surface (Kim 2009). In addition aptamers are known to undergo conformational changes upon binding their targets. The detection of E_2 arises from the complexation of the aptamer and E_2 rather than through the formation of DNA aptamer duplex. This interaction of oligonucleotide with ordinary molecule rather than with complementary DNA strand increases the electron tunnelling distance between the redox probe and the electrode (Kim 2010). Zhang and his co-workers (Zhang *et al.* 2007, Tao *et al*. 2009) reported that E_2 becomes negatively charge in solution due to the formation of more hydroxyl radical ion at high pH.

4.11 Calibration of aptasensor

The biosensor was calibrated with standard solutions of E2 before it was used to test real sample. The calibration was performed with both CV and SWV voltammograms displayed in

Fig. 16 which gave the same results. The response of is Au|G2PPT-co-PEDOT|76-mer-ssDNA-Aptamer biosensor to E2 was used to construct a calibration curve for the sensor as shown in Fig. 17. The calibration curve was typical of what is expected in biochemical binding processes following Langmuir model which gives a hyperbolic response. The dynamic linear range of the Au|G2PPT-co-PEDOT|ssDNA-aptamer biosensor was 0.05-307 pg/mL E2. The detection limit was 0.043 pg/mL. This calibrated sensor gave better limits of detection than that reported by Dempsey *et al.* (2004) which had a detection limit of 4.06 μg/mL when the GCE|Tyrosinase|poly(thionine) biosensor was employed for the detection of 17β-estradiol. Ndagili *et al.* (2011) reported a detection limit of 28 pg/mL for E2 using ZnSe quantum dot-cytochrome P450 3A4 enzyme biosensor. Coille *et al*. (2002) reported a detection limit of 300 pg/mL when a solid-phase micro extraction-high performance liquid chromatography with UV and electrochemical detection method was used for 17β-estradiol detection in water samples.

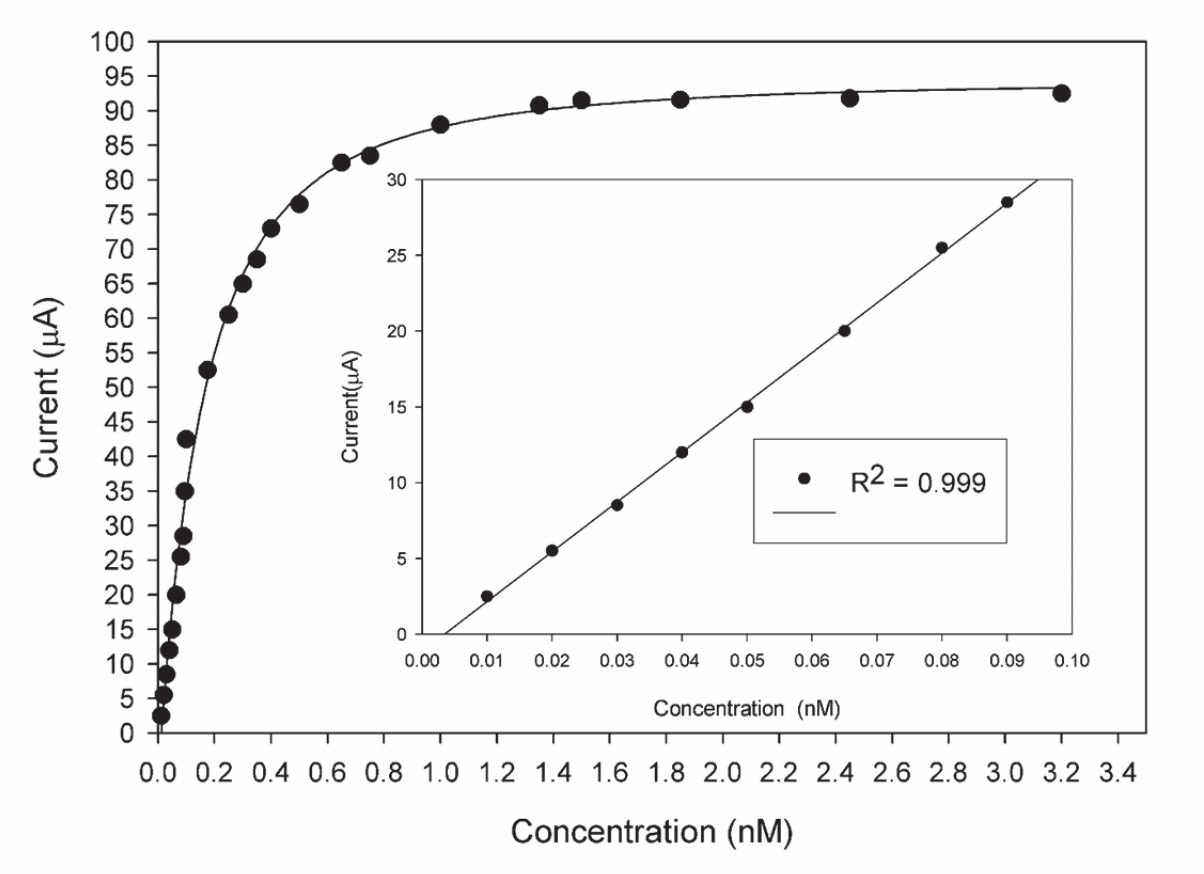


Figure 17. Calibration curve of Au|G2PPT-co-PEDOT|76-mer-ssDNA-Aptamer biosensor. Inset shows dynamic linear up to 0.1 nM (0.01 nM = 3 pg/mL).

The sensor was found to be more sensitive than most other techniques for determining E2. For example, the DL of electrochemical enzyme-linked immunosorbent assay (e-ELISA) is 20 pg/mL E2 as reported by Draisci *et al.* (2000), and the dissociation enhanced lanthanide fluorescence immunoassay (DELFIA) methodology gave a DL of 2.3 pg/mL E2 (Majima *et al.* 2002). On the other hand, the aptasensor has comparable precision (RSD%) to the immunoassay methods: aptasensor (6.8-11.5%), DELFIA (6.2-13.4%) and e-ELISA (6.7-14.3%). The analytical parameters of other techniques are compared with the aptasensor in

Table 2. The apatasensor's analytical parameters very well accommodate the maximum residue limits of 40 pg/mL permitted by Italian legislation and EU limits (Scalas *et al.* 2007).

Table 2. Comparison of analytical performance of techniques for 17β-estradiol determination.

Technique	**Detection limits (pg/mL)**	**Dynamic linear range (pg/mL)**	**Reference**
DELFIA*[1]	2.3		Majima *et al.* 2002
ABNOVA ELISA Kit	28.5.	29.3-3000	ABNOVA 17 beta-Estradiol ELISA Kit (www.abnova.com)
ALPCO Diagnostics ELISA kit	16.5	16.5-4000	ALPCO Diagnostics ELISA kit (www.alpco.com)
Electrochemical ELISA	20		Draisci *et al.* 2000
GC-MS method for analysis of beta-estradiol	150	10^3-300 x 10^3	Wu *et al*. 2009
Quantum dots labelled fluorescence immunoassay	5.42	10-10^7	Sun *et al.* 2010
*[2]Varian C-18 solid-phase extraction	0.1		Chimchirian *et al.* 2007
Au|G2PPT-co-PEDOT|ssDNA-aptamer biosensor	0.043	0.05-307	This study

*[1]Dissociation enhanced lanthanide fluorescence immunoassay.
*[2]The analytical method uses a Varian C-18 solid-phase extraction (Varian Inc., Palo Alto, California), followed by a derivatization with bis(trimethylsilyl)trifluoroacetamide.

4.12 Specificity of aptasensor

The cross reactivity of the Au|G2PPT-co-PEDOT|76-mer-ssDNA-Aptamer biosensor was studied for by checking its voltammetric response to other hormonal EDCs at a wide concentration range. The results displayed in Table 3 shows that little or no significant change was observed in the sensor responses to other compared to 17β-estradiol which may be attributed to lack of specific binding. The low cross reactivity of the sensor for related analytes demonstrates the ability of the 76-mer-ssDNA-Aptamer to retain its unique specificity for E2 even in immobilised state.

Table 3. Cross reactivity of aptasensor.

Compounds	Cross reactivity (%)
17β -Estradiol	100
d-Aldosterone	0.00
Estrone	1.03
Estriol	0.09
Ethynylestradiol	0.01
Progesterone	0.00
Testosterone	0.28
2-Methoxyestradiol	2.01

4.13 Storage stability

To characterize the reproducibility of the aptasensor five repetitive experiments involving incubating of 100 nm 17β-estradiol with the aptasensor was carried out. The binding interaction between the aptamer-target was monitored with change in peak current after five different measurements and a relative standard deviation 5 % was obtained. The stability of the fabricated aptasensor is an important issue in development and practical implementation for the detection of 17β-estradiol, therefore, aptasensor storage stability was monitored. The result showed that the response of the aptasensor to 100 nm 17β-estradiol decreased by 11.27% after storing in the refrigerator for half a month which means that the aptasensor retained 88.73% of its initial response.

4.14. Application of biosensor to real sample

Real sample used for this study was obtained from Scientific Services of the City of Cape Town. The aptasensor was applied to standard solutions of E2 treated water sample (effluent of the treatment plant). Sample volume of 100 mL was used in the study. The aptasensor was applied to standard solutions of E2, treated wastewater sample (effluent of water treatment). The Environmental Protection Agency of USA recommended methods for water and wastewater sampling were adopted as specified in the EPA-600/4-82-0129, Handbook for Sampling and Sample Preservation in Various Waters and Wastewaters (U.S. EPA 1982) as explained in Section 3.1.

Table 4. Recovery test for aptasensor.

Recovery test for treatment plant effluent			
E2 added (pg/mL)	**E2 observed (pg/mL)**	**E2 expected (pg/mL)**	**E2 recovery (%)**
0	7.2		
10	16.8	17.2	97.7
100	118.7	107.3	110.6
500	524	507	103.4

Results of the recovery experiments are shown in Table 4 for effluent water samples obtained from Scientific Services of the City of Cape Town. The precision and reproducibility tests were performed by determining the intra-assay and inter-assay covariance of the biosensor for standard solutions effluent samples and results are collected in Table 5. ELISA methods using ALPCO Diagnostics ELISA kit has intra-assay (4.6-10%) and inter-assay (2.8-13.6%) precision and reproducibility values for environmental water. The E2-specific aptasensor was used to determine the E2 levels in the municipal water samples supplied by Scientific Services of the City of Cape Town. The E2 levels were found to be 7.2 pg/mL in the effluent sample. Wide discrepancies in the concentration of estrogens are known to occur in the effluent of municipal wastewater systems. For example, for three municipal wastewater treatment plants in South-eastern Pennsylvania USA, the 17alpha-estradiol levels determined by the Varian C-18 solid-phase extraction followed by derivatisation with bis(trimethylsilyl)trifluoroacetamide ranged from 0.5-49 pg/mL (effluents) (Chimchirian *et al.* 2007). In another study on the wastewater from a sewage treatment plant in Harbin city, China the 17β-estradiol estrogenic equivalent (EEQ) values determined with the yeast estrogen screen (YES) method were 3.5-29.6 pg/mL (effluent) (Zhang *et al.* 2011).

Table 5. Precision and reproducibility test for aptasensor.

	Standard solutions	**Effluent**
Intra-assay covariance (%)	6.0-10.4	5.8-10.0
Inter-assay covariance (%)	6.8-11.5	4.9-10.7

In South Africa what is usually reported is the estrogenicity of water which determines the total EDC content of the water sample expressed in terms of its EEQ. However, since E2 is the most potent estrogenic EDC, its content in water systems can be compared with reported EEQ values or it can provide a fair estimate of what the EEQ value will be. For two drinking water sources in Molekane and Sekuruwe rural communities in Waterberg district of the Limpopo Province, Aneck-Hahn and co-workers (Aneck-Hahn *et al*., 2008) reported EEQ values of 0.63-2.48 pg/mL. Their results are in the same range as 0.16-1.92 pg/mL EEQ obtained in another study carried out on the water reservoir of the Rietvlei Nature Reserve, Gauteng sampled at the same time of the year using the same technique. The Limpopo and Gauteng EEQ values were determined with the yeast estrogen screen (YES) assay methodology. Other studies in other parts of South Africa gave EEQ values of 0.32-16.0 pg/mL for surface, ground and river water using estrogen receptor-mediated chemically activated luciferase gene expression (ER-CALUX) assay (Bornman *et al*. 2007). The ER-CALUX assay method seemed to be more sensitive than the YES assay (Schultis and Metzger 2004; Pawlowski *et al.* 2004) as it uses estrogen receptions known for their high binding affinity. In an international study, Murk *et al*. (2007) reported EEQs of wastewater treatment plants determined by ER-CALUX assay to be in the range 0.03-16.36 pg/mL for treatment plant effluent. They also determined the ER-CALUX EEQ values of water samples from four large rivers to be 0.07-0.48 pg/mL. The aptasensor reported in this study is more sensitive than the ER-CALUX assay due to the specificity and high binding affinity of the aptamer for E2, confirmed by the fact that sensor did not exhibit any significant cross reactivity.

4.15 Chromatographic Validation

Chromatographic experiments were performed to validate the results obtained with aptasensor. This is one of the methods used in the validation of the ENDOTEK sensor system. However, simple chromatographic systems may not be as sensitive as the ELISA and other affinity methods such as ER-CALUX method and YES method. However, it still serves the purpose of giving an indication of the advantages of the biosensor methodology over instrumental methods. Chromatographic determination of 17β-estradiol in wastewater effluent was performed by HPLC/UV with qualitative confirmatory analyses using GC-MS.

4.15.1 HPLC analysis of wastewater samples

The detection of 17β-estradiol occurred at 281 nm. The HPLC system gave a detection limit in the range of 3.45 pg/mL. The recovery time was greater than 90% for all water samples. It is worthy of note that chromatographic determination of E2 in wastewater effluents can give values ranging from non-detectable to 100 microgram E2 per litre of effluent depending on the source of sample and method of sample pre-treatment and storage (Karnjanapiboonwong *et al.* 2011).

4.15.2 GC-MS results

The chromatograms of the E2 analysis are shown in Figures 18-20 and the E2 results from the sample are given in Table 6. The molecular ion (664 m/z) was used to determine the presents or absence of 17β-estradiol in the samples. The following ions (mz): 237, 409 and 451 were used as qualifier ions for the compound. The results show that the GC-MS method did not detect E2 at the sensitivity level of microgram per litre. However, that no E2 is detectable at microgramme per litre of the sample agrees with the observation of Karnjanapiboonwong *et al.* (2011) that wastewater effluent samples may have non-detectable E2 levels. It has also been demonstrated by literature results described by Ingrand *et al.* (2003) that the sensitivity of chromatographic methods including GC-MS for 17β-estradiol analysis depends on the type of extraction and derivatisation procedures and the detection combination procedures developed for the study, in addition to other physical condition.

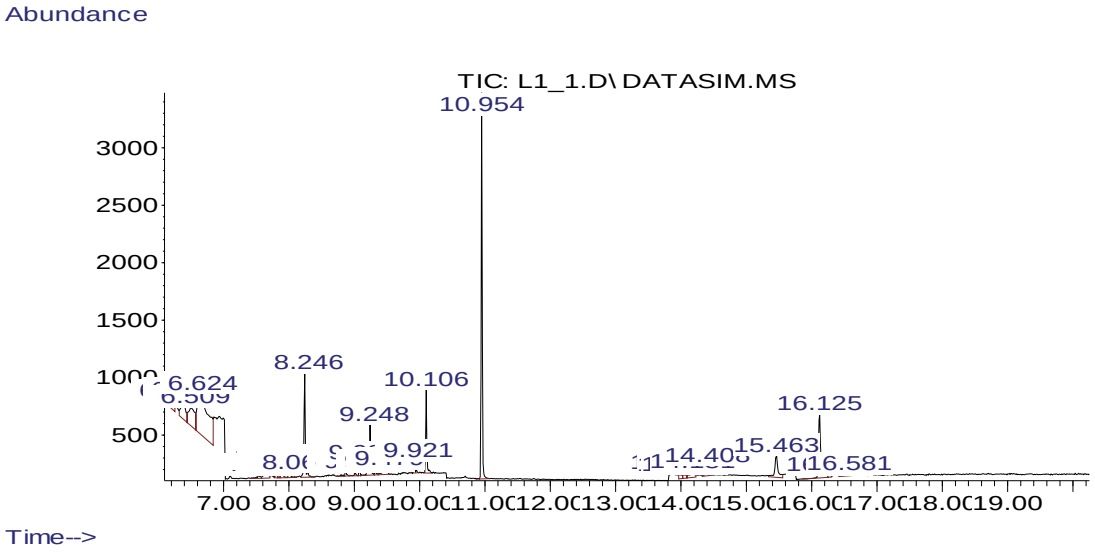


Figure 18. Total ion count chromatogram of level 1 @ 0.001 mg/L showing 17β-estradiol at 15.465 min.

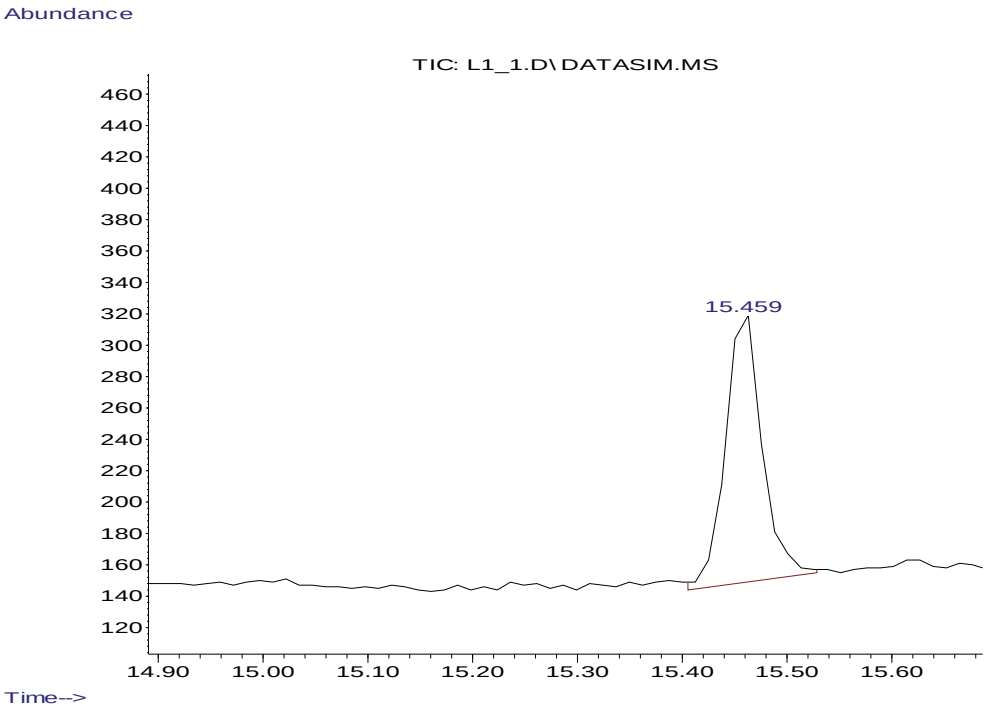


Figure 19. Single ion monitoring (SIM) Chromatogram of level 1 @ 0.001 mg/l showing 17β-estradiol at 15.465 minutes by monitoring ion (m/z) 664.

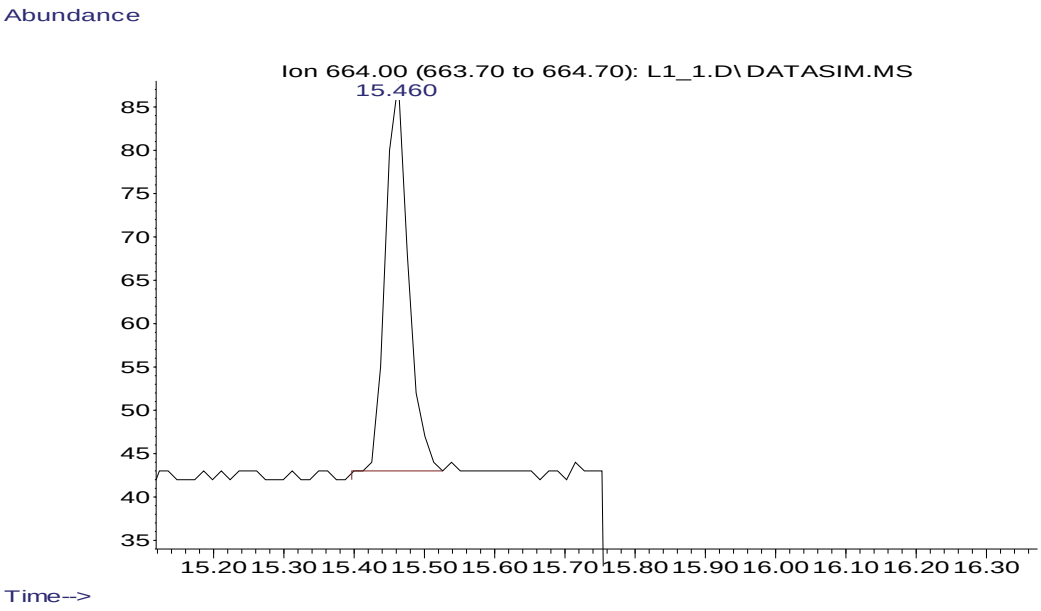


Figure 20. Single ion monitoring (SIM) Chromatogram of sample ZNDMBRFE 17β-estradiol at 15.465 minutes by monitoring ion (m/z) 664.

Table 6. The GC-MS results of 17β-estradiol in wastewater effluent samples.

Sample name	Compound Name	SIM Ion Monitored	Concentration (µg/L)
ZNDMBRFE	17Beta-Estradiol	664 (237, 409 and 451)	Not detected
HBFE	17Beta-Estradiol	664 (237, 409 and 451)	Not detected

4.16 ELISA validation

Standard solutions of 17β-estradiol and treated wastewater samples (effluent of water treatment) were used. The Environmental Protection Agency of USA-recommended methods for water and wastewater sampling were adopted as specified in the EPA-600/4-82-0129, Handbook for Sampling and Sample Preservation in Various Waters and Wastewaters (U.S. EPA 1982). Accordingly, freshly collected effluents of treated wastewater samples were used for the ELISA experiments. Samples were stored in small aliquots at -30°C when not used freshly. Thawed samples were not refrozen. All measurements were in triplicates.

4.16.1 ELISA responses for 17β-estradiol

The 17β-estradiol ELISA exhibited a sigmoidal response indicating allosteric interaction between anti-17β-estradiol-antibody and 17β-estradiol. The concentration range for the calibration is 0.3 pg/mL to 500 pg/mL. Comparatively, within the same range the aptamer nanobiosensor exhibited Michaelis-Menten binding kinetics and Langmuir isotherm which indicated a saturation of binding site that depends on the amount of aptamer contained in the biosensor. The immunoassay method involves a case where the concentration of the anti-body is high compared to those of surface-bound bio-receptor molecules, as is the case with the aptamer nanobiosensor previously reported.

4.16.2 17β-estradiol level in wastewater sample

The EPA's Standard Operating Procedure for the analysis of steroid hormones in aqueous samples (U.S. EPA 2005) was adopted in the real sample analysis. The ELISA response for17β-estradiol in water sample and spiked wastewater sample, the amount of 17β-estradiol recovered and the precision and reproducibility ELISA experiments were determined. The average 17β-estradiol level determined in the samples of wastewater effluents was found to be 6.32 pg/mL (see Table 7). Similar study with aptasensor gave 7.2 pg/mL (see Table 4). It may suggest that the aptasensor is more sensitive than the ELISA method. However, the difference could be considered to be within the limit of experimental error.

Table 7. Typical recovery test results for wastewater effluent sample determined by ELISA.

17β-estradiol Concentration, pg/mL			
Spike amount	**Determined**	**Expected**	**% Recovery**
0	6.2	0	0
10	16.0	15.8	97.7
100	116.1	105.1	110.6
500	520	499	104.2

Table 8. Cross reactivity data of 17β-estradiol ELISA.

Compounds	Cross reactivity (%)
17β-Estradiol	100
Estrone	0.93
Estriol	0.17
Ethynylestradiol	0.02
β-Estradiol 17-(β -D-glucronide)	0.00
2-Methoxyestradiol	1.67

4.16.2.1 Precision and reproducibility of 17β-estradiol ELISA

Based on the intra-assay and inter-assay covariance, the precision and reproducibility evaluations for wastewater effluent samples have intra-assay values of 4.25-10% and inter-assay values of 2.77-14.2%. These values were in the same range as those of the aptasensor.

4.16.2.2 Cross reactivity of 17β-estradiol ELISA

The cross-reactivity results measured for five similar hormonal compounds listed above were in agreement with what is reported in the literature and in the kit. The trend was similar to what was observed for the aptasensor.

4.16.3 Conclusion on ELISA validation

The ELISA study gave results that are similar to those of obtained with the aptasensor system. Since immunoassay method is highly specific and sensitive, the similarity between the ELISA values and those of the aptasensor confirmed the long held understanding that aptamers are as sensitive as antibodies and perhaps may be more reliable as they are more stable under relatively harsh conditions. In previous reports the biosensor parameters have been compared with other biotechnological methods for determining 17β-Estradiol, including yeast estrogen screen (YES) (Zhang *et al.,* 2011) and estrogen receptor-mediated chemically activated luciferase gene expression (ER-CALUX) assay (Bornman *et al.,* 2007). Detailed results of this work package will be presented in the annual report. The study will be useful to Local authorities, DWAF and DEAT for developing Guidelines for ELISA methods involving anti-estrogen antibody.

5 CONCLUSIONS

A star co-polymer G2PPT-co-PEDOT has been synthesized electrochemically and this was employed as a platform for the immobilization of bio-receptor (aptamer) to develop an electrochemical detection method for the detection of 17β-estradiol an endocrine disrupting chemical. This system was able to distinguished chemical that exhibited similar structure to 17β-estradiol such as naphthalene, estrone and 17α-ethylestradiol. The obtained result confirmed that aptamer are appropriate bioreceptor for selective detection of small molecules. Sensor validation tests including cross reactivity, recovery tests, precision and reproducibility tests were performed in accordance to the USA Environmental Protection Agency's Standard Operating Procedure for the analysis of steroid hormones in aqueous samples (U.S. EPA 2005). The aptasensor exhibited high specificity for E2 in all samples studied. The ENDOTEK aptasensor analytical parameters show that it is more sensitive than the YES, ELISA, ER-CALUX, DELFIA and the solid state extraction method. The aptasensor storage stability was investigated over a period of 1 month within which only an 18% decrease in the original response was observed. The sensor is suitable for onsite application and responds much faster than the chromatographic and ELISA methods. The aptasensor is suitable for applications in water containing high E2 concentration as well as those that have very low. The nanosensors analytical parameters DLR and DL of 0.05-307 pg/mL and 0.043 pg/mL makes the sensor suitable to be deployed for testing the proposed quality criteria of 0.4 pg/mL E2 in Swiss and EU wastewater effluents (Robert *et al.* 2011). Not only is the sensor adequate determining the levels of E2 reported for various water systems in South Africa, it is also suitable for the determination of E2 levels in food and serum. The ENDOTEK sensor will also be suitable for measuring deviation from acceptable daily intake (ADI) of E2 through all sources. The ADI limit for E2 is 0.05 μg/kg bw as determined by the Food and Agriculture Organisation of the United Nations (FAO/WHO 1999). For someone weighing 100 kg, the intake limit will be 0.5 pg/mL which is within the analytical detection range of the aptasensor.

Estrogen receptor α-recombinant protein (ER-α) and 76-mer biotinylated aptamer biosensor systems are characterized by fast response time, high sensitivity and their use require minimal sample pre-treatment procedures and certainly no derivatisation steps as have been shown to be the case with chromatographic methods. The aptasensor's analytical parameters such as DLR and DL of 0.05-307 pg/mL and 0.043 pg/mL were validated with chromatographic studies. As observed previously in this report it is possible to remove 99.9% of the total E2 originally present in an influent during water treatment, depending of course on the treatment procedure in place. A variety and variation of concentrations of estrogens exist in different water samples which further justifies the case for the development of biosensors for real time determination of levels of estrogens in samples.

6 RECOMMENDATIONS

The ENDOTEK project demonstrates the suitability of aptamers as the biological sensing element or bioprobe in sensor development. The superiority of DNA aptamer over enzymes and antibodies lie in the fact that DNA aptamer are inherently more environmentally stable than enzymes and antibodies. It will therefore be possible to produce biosensor systems that have longer shelf life with DNA aptamers. In its present form, the project and its findings represent a proof of concept. Further work is required in development of sensor devices that will integrate the mechanics, electronics and electrochemical microfluidics reactor cell with the electrochemical signal transduction microchip. Also further work is required to develop new aptameric biosensor for other e-EDCs including 17alpha-ethynylestradiol, estriol and estrone, as well as combinatorial aptamer biosensor that will be used for the determination of the total e-EDC content of a water sample. Considering that only 78% of 17alpha-ethynylestradiol (EE), the major component of birth control pill, is removed by water treatment plants, it is very urgent to develop aptasensor devices for monitoring EE level in water for domestic usage. Another important research to undertake is the development of electrochemical ELISA library for the major estrogen. Commercial ELISA systems are mainly based on UV-Vis measurements.

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APPENDIX

Appendix I: Original project title

Ultra-sensitive electrochemical nanobiosensor array devices for real-time determination of estrogenic endocrine disruptors in municipal wastewater (ENDOTEK)

Appendix II: List of Publications and other Technology-Transfer Actions

(Publications listed here on amperometric sensors for endocrine disruptors, aptasensor for cyanotoxins and dendritic polymer sensor systems, represent the applications of various technologies developed in the ENDOTEK project.)

1. Rasaq A. Olowu, Omotayo Arotiba, Stephen N. Mailu, Tesfaye T. Waryo, Priscilla Baker, Emmanuel Iwuoha. Electrochemical aptasensor for endocrine disrupting 17β-estradiol based on a poly(3,4-ethylenedioxylthiopene)-gold nanocomposite platform. *Sensors* 10 (2010) 9872-9890.

2. Rasaq A. Olowu, Avril Williams, Peter M. Ndangili, Rachel F. Ngece, Stephen N. Mailu, Peter Baker, Emmanuel Iwuoha. Impedimetry and microscopy of electrosynthetic poly(propylene thiophenoimine)-co-poly(3,4 ethylene dioxythiophene) dendritic star copolymer. *International Journal of Electrochemical Science* 6 (2011) 1855-1870.

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12. Emmanuel I. Iwuoha, Rasaq Olowu, Priscilla Baker, Tesfaye Waryo, Abd Baleg, Nazeem Jahed. Smart nano-dimensional dendritic aptasensor for real-time determination of estrogenic 17β-estradiol, *The International Conference and Exhibition on Biosensors & Bioelectronics (Biosensors & Bioelectronics-2012)***,** *(Analyze Novel Approaches of Biosensors & Bioelectronics);* hosted by the OMICS Group, was held on May 14-16, 2012 in Embassy Suites Las Vegas, USA.

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Appendix III: Conference presentations

1. Rasaq Olowu (South Africa), Pricilia Baker, Emmanuel Iwuoha. Electrochemical multichannel transduction of poly(propylenethiopenoimine)-co-poly(3,4-ethylenedioxythiopene) aptameric nanobiosensor for estrogeneous endocrine disruptors. *The 62nd Annual Meeting of the International Society of Electrochemistry on ''Electrochemical Frontiers in Global Environment and Energy*", 11-16 September, 2011, Niigata, Japan.

2. Ezo Nxusani, 3-Mercaptopropionic acid capped Ga_2Se_3 nanocrystal-CYP3A4 biosensor for the determination of 17-alpha-ethinyl estradiol in water. *2nd International Symposium on Electrochemistry*, 19-20 July 2012, University of Western Cape.

3. Rasaq A. Olowu. Poly(propylenethiophenoimine)-co-poly(3,4ethylenedioxythiopene) dendritic star copolymer aptameric nanobiosensor for estrogeneous 17β-estradiol. *2nd International Symposium on Electrochemistry*. 19-20 July 2012, University of Western Cape.

4. Peter Munyao Ndangili, Jijana N. Abongile, Priscilla G.L. Baker, Emmanuel I. Iwuoha, Stephen N. Mailu, Fanelwa R. Ngece, Rasaq A. Olowu, Tesfaye T. Waryo, Avril Williams. Gallium-induced electronic properties of surface capped chalcogenic (selenide) quantum dots electrochemical genosensors for 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS). *10th Spring Meeting of the International Society of Electrochemistry*, 15 to 18 April, 2012, Perth, Australia.

5. Emmanuel Iwuoha, Priscilla Baker, Abd Almonam Baleg, Nazeem Jahed, Abongile Jijana, Peter Ndangili, Ezo Nxuzani, Rasaq Olowu, Tesfaye Waryo. Smart nano-dimensional dendritic aptasensor and quantum dots-linked estrogen receptor alpha-recombinant protein biosensor for real-time determination of estrogenic 17β-estradiol. *13th Topical Meeting of the International Society of Electrochemistry Advances in Electrochemical Materials Science and Manufacturing*. 7-10 April, 2013, Pretoria, South Africa.

6. Abongile Nwabisa Jijana, Priscilla Baker, Emmanuel Iwuoha, Tesfaye Waryo, Spectroelectrochemically active carboxylic acid capped semiconducting nano-matrices of metal selenides integrated ER-α, receptor-sensor for estrogens. *13th Topical Meeting of the International Society of Electrochemistry Advances in Electrochemical Materials Science and Manufacturing.* 7-10 April, 2013, Pretoria, South Africa.

7. Peter Munyao Ndangili, Jijana N. Abongile, Priscilla G.L. Baker, Emmanuel I. Iwuoha, Stephen N. Mailu, Fanelwa R. Ngece, Rasaq A. Olowu, Tesfaye T. Waryo, Avril Williams. Gallium-induced electronic properties of surface capped chalcogenic (selenide) quantum dots electrochemical genosensors for 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS). *13th Topical Meeting of the International Society of Electrochemistry Advances in Electrochemical Materials Science and Manufacturing.* 7-10 April, 2013, Pretoria, South Africa.

8. Emmanuel Iwuoha, Ezo Nxusani, Tesfaye Waryo, Priscilla Baker. Estrogen Receptor α-Recombinant protein-sensitised quantum dots biosensors for estrogenic endocrine disrupting 17β-Estradiol. *Symposium of the Analytical Division of Sociedade Portuguesa de Quimica*, 14-15 April 2014, (*SPQ Analítica 2014*) Coimbra, Portugal.

9. Emmanuel Iwuoha. Engineering smart polymeric and biocompatible quantum dots platforms for nanobiosensor systems. *The 5th Conference of the Southern and Eastern African Network of Analytical Chemists,* 9-13 June 2014, Mombasa, Kenya.

Appendix IV: Capacity building

Students Training from the ENDOTEK Project

Student	Degree	Project information	Degree status
Ms Abongile Jijana Student No.; 2505749 South African (Female)	MSc	Novel tin selenide quantum dots biosensors for water estrogens.	Graduated March 2011
Ms Nolubabalo Matinise Student No.: 2440924) South African (Female)	MSc	Synthesis, characterisation and sensor application of PGM oxide-polymer composites.	Graduated March 2011
Ms Virginia B. Matyholo Student No.: 2324617 South African (Female)	MSc	Enzyme-based biosensors for the determination of 17-β-estradiol and other estrogens.	Completed June 2011
Mr Milua Masikini Student No.; 2914873	MSc	Synthesis of metal oxide nanomaterials in presence and absence of beta-cyclodextrin and characterization for development of electrochemical sensors for phenolic endocrine disruptor compounds.	Graduated September 2011
Ms Ezo Nxusani Student No.: 2505749 South African (Female)	MSc	3-mercaptopropionic acid-capped gallium selenide QDs-CYP3A4 biosensor for environmental 17α-ethinylestradiol.	Graduated March 2012
Ms Sinazo Qakala Student No.: 2346099 South African (Female)	MSc	Development of rapid test systems of enzyme and aptamer biosensors for water estrogens.	Graduated March 2014
Dr Rasaq A. Olowu Student No.: 2919248 Nigerian (Male)	PhD	Multichannel robotic Au|G2PPT-co-PEDOT|76-mer-ssDNA-Aptamer biosensor for 17β-estradiol in treated wastewater samples.	Graduated March 2012
Dr Peter M. Ndangili Student No.: 2773828 Kenyan (Male)	PhD	Electrochemical/optical modulation of selenide and telluride ternary alloy quantum dots nanobiosensors for 17β-estradiol.	Graduated September 2012
Dr Mawethu Bilibana Student No.: 2324593 South African (Male)	PhD	Application of aptasensor technology in the determination of cyanotoxins in water (Aptatoxisens technology).	Graduated September 2014
Ms Abongile Jijana Student No.: 2505749 South African (Female)	PhD	A 17β-estradiol biosensor constructed with tin selenide quantum dots incorporating estrogen receptor α-recombinant protein (Receptorsens).	Completed PhD in December 2014