Int. J. Electrochem. Sci., 12 **(2017)** 10359 – 10368, doi: 10.20964/2017.11.10

International Journal of
ELECTROCHEMICAL
SCIENCE
www.electrochemsci.org

An Electrochemical Aptasensor for Thrombin Based on a Novel Polyamidoamine Dendrimer-Streptavidin Supramolecular Architecture

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Received: 11 June 2017 / *Accepted:* 23 August 2017 / *Published:* 12 October 2017

This work reports the development of an electrochemical aptasensor for thrombin based on a polyamidoamine dendrimer-streptavidin supramolecular architecture prepared by the cyclic voltammetric electrodeposition of polyamidoamine dendrimer (PAMAM) followed by the drop coating of streptavidin on a glassy carbon electrode. Biotinylated aptamer probe was immobilised on the platform through biotin-streptavidin interaction. The aptasensor preparation was monitored with voltammetry and electrochemical impedance spectroscopy. The biosensor response to thrombin was evaluated with square wave voltammetry (in phosphate buffer solution and in ferro/ferric cyanide probe) and electrochemical impedance spectroscopy. Linear responses in the concentration range between 1 - 200 ng mL^{-1} and detection limit in the sub 0.02 ng mL^{-1} (defined as ($C_L = 3S_B/m$) were calculated. The selectivity of the aptasensor was evaluated using interfering proteins such as bovine serum albumin (BSA), lysozyme and haemoglobin. The relative response obtained shows insignificant effect from the interfering proteins with BSA, lysozyme and haemoglobin having a relative response of 9.2%, 8.7% and 7.6% respectively.

Keywords: Biosensor; thrombin; polyamidoamine dendrimer; streptavidin; supramolecular.

1. INTRODUCTION

The identification and quantification of proteins in their specified environments has always been an important and difficult task in clinical applications. Thrombin is a multifunctional serine protease that acts as the central enzyme in the coagulation cascade [1,2]. Thrombin is involved in various important physiological and pathological processes including procoagulant, anticoagulant, inflammation and cell-signalling and activation through the exertion of a host of highly regulated

action on the blood, the vessel walls and wide variety of cells [3-5]. In response to vascular injury, the blood clotting process occurs as a part of a physiological response that stops bleeding [5], by generating thrombin, that subsequently deposit fibrin, which serves to stabilise the developing clot [6]. Thus, offsetting the balance of the haemostatic system either due to the absence or abnormality of thrombin can result in a spectrum of events, such as bleeding, thrombosis and Alzheimer diseases [7-9]. Therefore, detection techniques for thrombin that are rapid, simple, sensitive, selective and cost-effective are of paramount significance for the understanding, diagnosis, treatment and prevention of many diseases.

Biorecognition molecules that are useful in the sensing and the detection of thrombin have been predominantly based on antibodies. Although antibodies are exceptionally used as recognition elements, they possess some limitations which includes production that relies on the initiation of an animal immune, sensitivity to temperature, subjection to irreversible denaturation and limited shelf life [9,10]. To overcome the limitations of antibodies as recognition elements, aptamers have been successfully adapted. Aptamers are short synthetic ribo- and deoxyribonucleic acids that can bind to almost any target and are potentially well suited for high-throughput discovery [11]. They are chemically synthesised via *in vitro* selection processes (SELEX). Aptamers have found extensive application in analytical and diagnostic applications due to numerous advantages they possess over antibodies such as ease of separation and modification, high chemical and thermal stability and resistant against denaturisation [12].

Numerous methods and strategies have been developed for aptamer based protein detections such as electrophoresis [13], affinity chromatography [14], enzyme-linked immunosorbent assay (ELISA) [15], electrochemiluminescent [16], fluorescence [17], electrochemical detection [18] and so on. Among these techniques, electrochemical detection has attracted particular interest in the development of aptasensors as it is simple, relatively low cost, highly sensitive and compatible with nanofabrication technologies [19-20]. Various materials have been used to enhance the sensitivity and selectivity of electrochemical aptasensors through the modification of the electrode. These materials include nanomaterials such as carbon nanotubes, metal nanoparticles, metal oxide nanoparticles, quantum dots, magnetic beads, polymer nanotubes or nanostructure and most recently, dendrimers [21-28]. In electrochemical aptamer based biosensor, nanomaterials form an excellent, biocompatible immobilisation interface for signal amplification.

Dendrimers are a new class of polymeric macromolecules which are highly branched and globular in shape with biocompatible properties [29]. The sizes and shapes (globular 3D) of dendrimer compare well with biomolecules such as DNA duplexes and hence their suitability for biosensor application [30]. Their self-assembly by supramolecular interactions also mimic the molecular level-organised biological structures. Polypropylenimine (PPI) and polyamidoamine (PAMAM) are major examples of dendrimers that are widely employed in biomedical research as nanocarriers for genes and drugs [31] and as platforms for biosensor development [29-35].

This work describes the preparation of an electrochemical aptamer based biosensor for the detection of thrombin. A glassy carbon electrode was modified with PAMAM followed by exploiting the supramolecular interactions between PAMAM – streptavidin and streptavidin – biotin.

2. EXPERIMENTAL

2.1. Materials and apparatus

The synthetic aptamer for thrombin with the sequence 5'-GGT TGG TGT GGT TGG-3' was obtained from Inqaba biotechnical industries (South Africa). Streptavidin, bovine serum albumin (BSA), haemoglobin bovine, lysozyme, generation 3 (G3) polyamidoamine dendrimer and other chemicals were purchased from Sigma-Aldrich (South Africa). Ultrapure water with resistivity 18.2 MΩ was used in the preparation of redox active probe and buffer solutions. Stock solutions of thrombin aptamer were prepared in Tris-EDTA buffer while the working solutions were prepared in phosphate buffer pH 7.2.

2.2. Instrumentation

Zahner Zenium electrochemical workstation running on Thales software (Zahner, Germany) was used to carry out electrochemical measurements. A 3 mL electrochemical cell with a convectional three electrode setup was used with a bare or modified GCE as working electrode, a platinum wire as counter electrode and a Ag/AgCl (3 M KCl) as reference electrode. Square wave voltammetric (SWV) measurements were performed by applying an amplitude of 20 mV and frequency of 15 Hz. Impedance (EIS) measurements were carried out at a 250 mV biased potential, a voltage amplitude of 10 mV within a frequency range from 100 KHz to 100 mHz. The Randle's equivalent circuit was used to obtain the impedance fitting parameters. High resolution scanning electron microscope (HR-SEM) was taken with ZEISS Auriga FIB-SEM (Germany) operated from 1-10 keV.

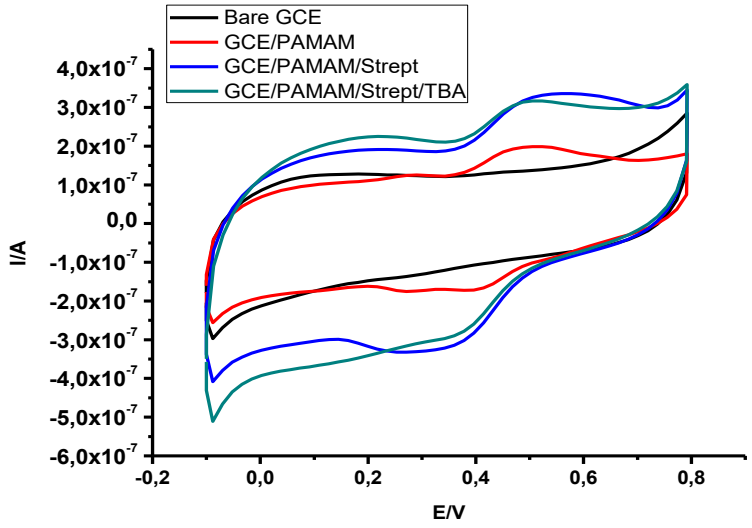
2.3. Platform preparation and biosensor development

The PAMAM modified glassy carbon electrode (GCE) was fabricated by electrodeposition from a 10 mM generation 3 (G3) PAMAM dendrimer solution by cyclic voltammetry (CV) from a potential window of -200 mV to 1200 mV at 50 mVs^{-1} scan rate for 15 scans. This was followed by rinsing gently with ultrapure water. The electrode was labelled GCE/PAMAM. The GCE/PAMAM electrode was further modified with streptavidin by drop coating with a 50 µL volume of 100 $\mu g\ mL^{-1}$ streptavidin at pH 7.5. The electrodes (at each stage of the process) was electrochemically characterised in 10 mM phosphate buffer saline (PBS) of pH 7.5 and in 5 mM (1:1) $[Fe(CN)_6^{3-/4-}]$ redox probe prepared in 10 mM PBS solution.

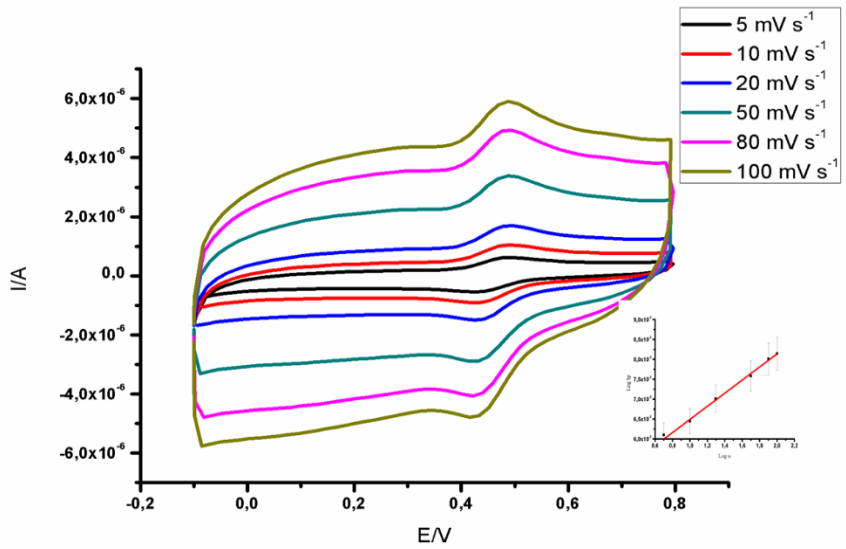
The immobilisation of thrombin aptamer on the dendrimer-streptavidin electrode (GCE/PAMAM/Strept) was performed by drop coating with 50 µL of 2 µM biotinylated thrombin aptamer for 1 h at room temperature and subsequently rinsed gently with ultra-pure water and 10 mM phosphate buffer solution of pH 7.2 respectively to remove the unbound aptamer. Thus, the biosensor (GCE/PAMAM/Strept/TBA) was fabricated and stored at 4 °C when not in use.

3. RESULTS AND DISCUSSION

3.1 Electrochemical characterisation of the platform and its morphology



a



b

Figure 1. (a) CV of the modified electrodes and the aptasensor in 10 mM PBS (b) CV of GCE/PAMAM/Strept at different scan rates in 10 mM PBS.

The modification of the glassy carbon electrode with PAMAM was through the attachment of the amine group onto the electrode surface [35-37]. The modified electrode was characterised with by cyclic voltammetry (Fig 1a) where a redox pair at formal potential of 459 mV can be observed. The presence of this peak suggests that PAMAM is electroactive. The further modification of GCE/PAMAM electrode with streptavidin via electrostatic attraction by exploiting the isoelectric point of streptavidin (Fig 1a) shows an increase in current after the immobilization. The PAMAM – streptavidin supramolecular architecture was favourable towards the flow of charge at the electrode interface after immobilization of streptavidin [38]. The log-log plot of peak current against scan rate gave a correlation coefficient $r^2 = 0.9937$ which is also characteristic of reversibility and surface bound

kinetics. Hence, the PAMAM-Strept interaction produced a reversible conjugate which is stable as highlighted from the peak potentials which were stable at different scan rates.

3.2. Electrochemical characterisation of the stepwise fabrication of the aptasensor

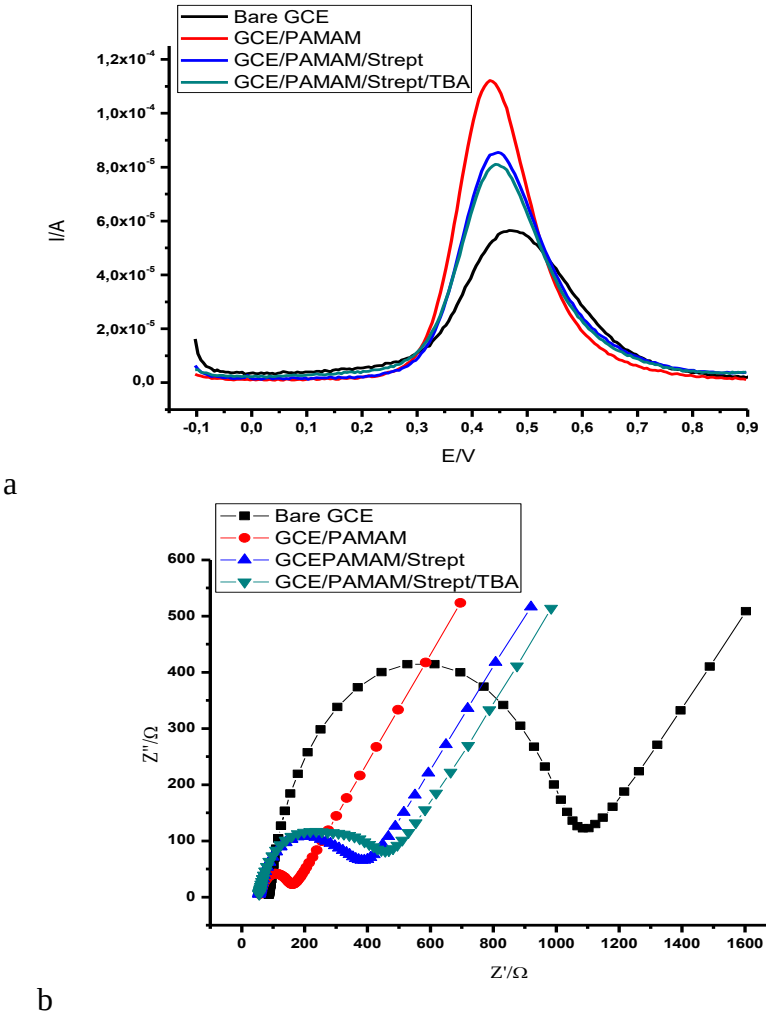


Figure 2. (a) SWV characterisation of the GCE/PAMAM/Strept/TBA modified aptasensor in 5 mM $Fe(CN)_6^{3-/4-}$ (b) Impedimetric characterisation of the GCE/PAMAM/Strept/TBA aptasensor in 5 mM $Fe(CN)_6^{3-/4-}$ redox probe

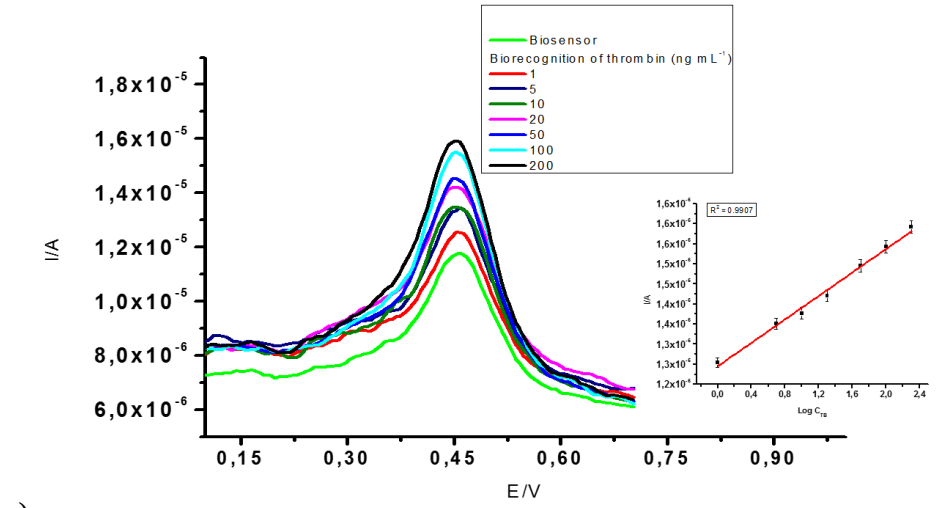
The aptasensor was prepared by immobilising the thrombin binding aptamer probe on the dendrimer streptavidin platform. SWV and impedance spectroscopy were used to monitor the electrochemical responses of the modified electrode in $Fe(CN)_6^{3-/4-}$ redox probe. After the immobilisation of the aptamer, a significant decrease in peak current of the $Fe(CN)_6^{3-4-}$ is observed in Fig 2a. This can be attributed to the electrostatic repulsion between the negatively charged deoxyribose-phosphate back bone of the aptamer and $Fe(CN)_6^{3-/4-}$ redox probe. The interfacial properties of the modified electrode were also investigated using electrochemical impedance spectroscopy. The Nyquist plot consists of a semicircle and a linear portion with the semicircle portion

representing the electron transfer resistance at higher frequencies and the linear portion corresponds to the diffusion resistance. The Nyquist plots in Fig 2b shows the modified electrode at different modification stages with the charge transfer resistant R_{ct} for the dendrimer modified electrode (168 Ω) lower than that for bare electrode (958 Ω) due to the faster electron transfer kinetics of $Fe(CN)_6^{3-/4-}$ anions on the electrode surface. After immobilisation with aptamer, the impedimetric response shows an increase in R_{ct}, which confirms the successful supramolecular self-assembly process of the aptamer on the electrode surface.

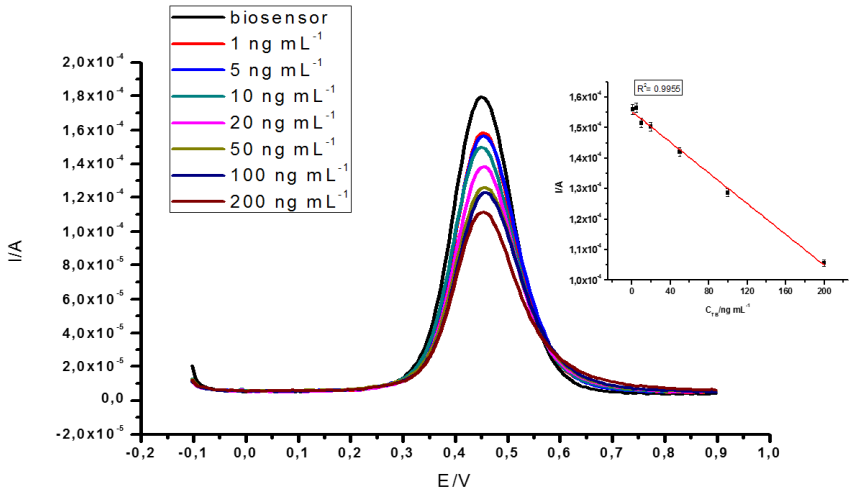
3.3. Voltammetric and Impedimetric response of the aptasensor towards different concentrations of thrombin

To assess the performance of the prepared aptasensor under optimised experimental conditions, standard samples of thrombin at different concentrations ranging from 1 ng mL^{-1} to 200 ng mL^{-1} were prepared in PBS pH 6.0 and the aptasensor was incubated for 1 hour in a series of thrombin concentrations. Square wave voltammetry was used to monitor the change in current signal in 10 mM PBS pH 7.5 and an increase in current peak with increasing thrombin concentration was observed (Fig 3a), with a dose-response curve for thrombin (inset) showing a linear response from 1 ng mL^{-1} to 200 ng mL^{-1} with a detection limit of 0.011 ng mL^{-1}. Affinity biosensors (genosensors, aptasensors, immunosensors) are usually measured with impedance spectroscopy owing to the non-electrocatalytic nature of the bio-recognition event. Voltammetric measurements are employed when the bioreceptor is enzyme tagged or modified so that an indirect measurement of the event can be taken via the enzyme-substrate reaction [39]. It is interesting to note in this report that the thrombin-aptamer binding process could be monitored directly without the use of redox tag or an enzyme tag. This phenomenon, though not fully understood, is attributed to the supramolecular alignment existing in the dendrimer/streptavidin/aptamer/thrombin architecture. This voltammetric response in PBS thus introduces a new concept to biosensor design. In $Fe(CN)_6^{3-/4-}$ redox probe (Figure 3b), a decrease in current peak signal is observed with the increase in thrombin concentration. This can be attributed to the adsorption of thrombin on the sensor surface which insulates the electrode slowing down the rate of electron transfer. More so, this demonstrates the binding capabilities of thrombin to the aptamer which blocks the electronic transmission. There is a linear relationship between the peak current and thrombin concentration within the range 1-200 ng mL^{-1} with a detection limit of 0.02 ng mL^{-1} using $C_L = 3S_B/m$ (where C_L, S_B and m are the limit of detection, standard deviation of the blank and slope of the calibration graph, respectively).

Electrochemical impedance spectroscopy was used to investigate a concentration range of the target analyte from 1 ng mL^{-1} to 200 ng mL^{-1}. The EIS response was measured after the addition of successive aliquots of different concentrations of thrombin in the presence of reversible $Fe(CN)_6^{3-/4-}$ redox probe in PBS pH 7.4.



a)



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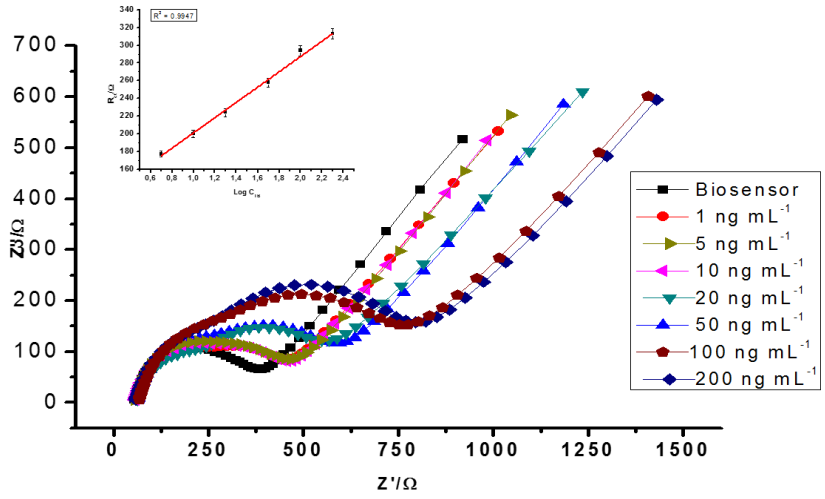

c)

Figure 3. (a) Square wave voltammetric response of the aptasensor in different concentrations of thrombin ranging from 1 ng mL^{-1} to 200 ng mL^{-1} in 10 mM PBS, (inset; Plot of I_{pa} vs log concentration of thrombin). (b) SWV of electrochemical aptasensor for thrombin detection at different concentrations ranging from 1 ng mL^{-1} to 200 ng mL^{-1} in 5 mM $Fe(CN)_6^{3-/4-}$.(inset; calibration plot of I_{pa} vs thrombin concentration). (c) Impedimetric response of the aptasensor and its target at different concentrations, (inset; Plot of R_{ct} vs log concentration of thrombin).

The different Nyquist plots obtained with an increase in thrombin concentration (Fig 3b) were modelled using the Randle's equivalent circuit. There is a linear variation of R_{ct} with thrombin concentration when the aptamer binds to thrombin molecule from 5 ng mL^{-1} to 200 ng mL^{-1} and a correlation coefficient of 0.9907. The resulting complex remained on the electrode surface hindering the transfer of electrons, thus diminishing the overall conductivity of the electrode [40].

3.4. Selectivity of the biosensor

The selectivity of the GCE/PAMAM/Strept/TBA based aptasensor was evaluated using interfering proteins - BSA, lysozyme, and haemoglobin - that also co-exist with thrombin in the blood (Fig 4). The relative response was obtained by dividing the current signals of the interfering proteins by the signal of thrombin and then multiplying by 100%. Little effect was obtained for the interfering proteins with BSA, lysozyme and haemoglobin having a relative response of 9.2%, 8.7% and 7.6% respectively. This further confirms the excellent specificity of the proposed aptasensor.

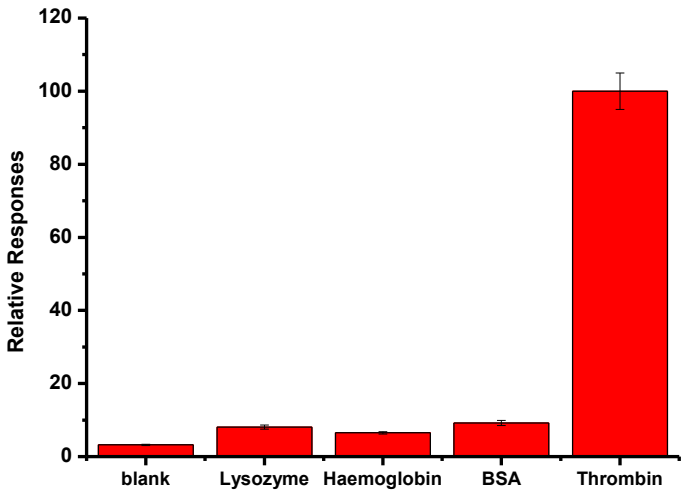

Figure 4. Specificity responses of the aptasensor towards 100 ng mL^{-1} BSA, 100 ng mL^{-1} Lysozyme, 100 ng mL^{-1} haemoglobin and 100 ng mL^{-1} thrombin

4. CONCLUSION

This work describes the design of a novel aptamer-based supramolecular biosensor for the detection and quantification of thrombin, envisaging the development of new strategies for early detection and diagnosis of cardiovascular diseases. The glassy carbon electrode was modified with PAMAM dendrimer by electrodeposition, which further lends itself to supramolecular architecture with streptavidin. The thrombin aptasensor was prepared by immobilising the thrombin binding aptamer probe on the dendrimer streptavidin platform. The performance of the aptasensor was evaluated in standard samples of thrombin at different concentrations ranging from 1 ng mL^{-1} to 200

ng mL^{-1} prepared in PBS pH 6.0. The supramolecular dendrimer-streptavidin platform allowed the biosensor performance to be evaluated in buffer solution by voltammetry. This is not a well reported approach of measurement in direct affinity sensor. The biosensor also demonstrated selectivity in the presence of other proteins.

ACKNOWLEDGMENTS
Financial supports from the following South African institutions are gratefully acknowledged: Faculty of Science, University of Johannesburg; Centre for Nanomaterials Science Research, University of Johannesburg; National Research Foundation CPRR Grant number 98887 and Free-Standing Master's Bursary.

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