



Electrically active magnetic nanoparticles as novel concentrator and electrochemical redox transducer in *Bacillus anthracis* DNA detection

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ABSTRACT

Magnetic polymer nanostructures are a new class of multifunctional nanomaterials that are recently being explored in biosensor devices. In this paper, for the first time we report the novel application of electrically active magnetic (EAM) nanoparticles as concentrator of DNA targets as well as electrochemical transducers for detection of the *Bacillus anthracis* protective antigen A (*pag A*) gene. The EAM nanoparticles are synthesized by chemical polymerization and have dimensions of 80–100 nm. The biosensor detection encompasses two sets of DNA probes that are specific to the target gene: the detector probe labeled with the EAM nanoparticles and the biotinylated capture probe. The DNA targets are double hybridized to the detector and the capture probes and concentrated from nonspecific DNA fragments by applying a magnetic field. Subsequently, the DNA sandwiched targets (EAM-detector probe–DNA target–capture probe–biotin) are captured on streptavidin modified screen printed carbon electrodes through the biotinylated capture probes. Detection is achieved electrochemically by measuring the oxidation–reduction signal of the EAM nanoparticles. Preliminary results indicate that the biosensor is able to detect the redox signal of the EAM nanoparticles at DNA concentrations as low as 0.01 ng/ μ l.

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1. Introduction

Bacillus anthracis is the etiological agent of anthrax, a zoonotic disease, which may be developed by all mammals, particularly humans. Anthrax is initiated by the entry of the spores into the host through skin lesions, insect bites, ingestions of contaminated meat or inhalation of airborne spores. The inhalational form of the disease is the most dangerous with mortality rates approaching 100% (CDC, 2001). The use of anthrax as biological weapons in the United States in 2001 has elucidated that such infectious pathogen can have considerable socio-economic impacts.

Nanomaterial based biosensors are evolving as promising alternatives for rapid, inexpensive, sensitive and accurate identification of infectious pathogens such as *B. anthracis*. Recent advances in nanotechnology have stemmed the creation of diverse nanostructures, e.g. nanowires, nanotubes, nanoparticles, nanospheres, nanorods, and nanopores. Due to their extreme small sizes, these nanostructures exhibit unique properties, such as increased surface area, mechanical strength, chemical reactivity, electrical conductivity, magnetism and optical characteristics. These characteristics have offered enormous prospects in designing novel methods of sig-

nal transduction in biosensor devices. Current literature shows numerous applications of different nanostructures in biosensor devices for the detection of pathogenic microorganisms that are of importance to food and environmental safety, biosecurity, and medical diagnostics. Some examples of applications include silicon nanowire arrays for detection of influenza A virus (Patolsky et al., 2004), conducting polymer nanowires for *Bacillus cereus* detection (Pal et al., 2007), single walled carbon nanotube based field effect transistors for *Escherichia coli* detection (So et al., 2008), gold nanoparticles in biobarcode based detection of *Salmonella Enteritidis* (Zhang et al., 2009) and magnetic nanoparticles for detection of Human Papillomavirus (HPV) DNA sequences (Wang and Li, 2008).

Magnetic nanoparticles are characterized by their ability to aggregate and resuspend in response to variations in external magnetic field. This unique property has allowed their exploitation in separation and pre-concentration processes, and in biosensors. Magnetic separation techniques are widely used in bioengineering and biomedical applications such as magnetic resonance imaging, gene delivery, drug delivery, diagnostics, immunoassays and biosensors (Lee et al., 2006; Ludwig et al., 2006; Xu and Sun, 2007). In biosensor detection, magnetic pre-concentration can be advantageous in the elimination of nonspecific adsorption of interfering biomolecules and in reducing analysis time and stress-induced damages to biomolecules.

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Magnetic polymer nanostructures, where magnetic nanoparticles are embedded in polymer matrices, have attracted recent interests due to their novel magnetic, electrical and optical properties along with their mechanical strength, dielectric tunability and non-corrosiveness (Lee et al., 2006; Poddar et al., 2004). In such multifunctional nanostructures, the conductive polymer, polyaniline, has been widely investigated (Dallas et al., 2006; Li et al., 2007) for their robust nature, controllable electrical and chemical properties, and simple chemical and electrochemical synthetic procedures (Stejskal and Gilbert, 2002).

The magnetic and electrical properties of electrically active magnetic (EAM) nanoparticles synthesized from aniline monomer and iron-oxide nanoparticles have been exploited in magnetic concentration and biosensing of bacterial cells from complex matrices in previous publications of the authors (Pal et al., 2008; Pal and Alocilja, 2009). This paper describes the novel application of EAM nanoparticles as a pre-concentrator and an electrochemical transducer in a DNA based biosensing mechanism. Experimental results are presented for structural and electrochemical characterization of the EAM nanoparticles and electrochemical detection of dually hybridized *B. anthracis* *pag A* gene fragments on EAM nanoparticles. The sensitivity of the biosensor has also been included in this paper. Till date, literature shows no report of such multifunctional nanoparticles in the dual function of a magnetic concentrator and an electrochemical transducer in DNA detection.

2. Experimental

2.1. Chemicals and reagents

Aniline, iron (III) oxide nanopowder, sodium chloride (NaCl), sodium phosphate (monobasic and dibasic), sodium acetate, phosphate buffered saline tablets (0.01 M), formamide, ethylene diamine tetra acetic acid (EDTA), isopropanol, ethanol, sodium dodecyl sulfate (SDS), proteinase K, hydrochloric acid (HCl), Trizma base, ethidium bromide, gel loading solution and streptavidin from *Streptomyces avidinii* were purchased from Sigma Aldrich (St. Louis, MO). AccuPrime™ Taq DNA Polymerase system, Ultrapure™ DNase RNase free water and Ultrapure™ agarose were purchased from Invitrogen Corporation (Carlsbad, CA). EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) was obtained from Pierce (Rockford, IL).

2.2. Selection of *B. anthracis* DNA primers and probes

The forward and reverse primers for PCR, and the capture and detector probes for biosensor detection were designed and selected from the protective antigen (*pag A*) gene of *B. anthracis* (Song et al., 2005). The specificity of the primer and the probe sequences were analyzed using the Basic Local Alignment Search Tool (BLAST). The sequence information of the *B. anthracis* primer pairs and the probes are given below:

Forward primer: 5'-AAAATGGAAGAGTGAGGGTG-3', 20 bases, positions 3284–3303.

Reverse primer: 5'-CCGCCTTCTACCAGATTTA-3', 20 bases, positions 3383–3402.

Detector probe: 5'-GGAAGAGTGAGGGTGGATACAGGCTCGA-ACTGGAGTGAAGTGTACCGCA-3', 50 bases, positions 3289–3338.

Capture probe: 5'-GGAAAAGATTAAATCTGGTAGAAAGCGCG-3', 30 bases, positions 3373–3402.

The detector probe was phosphorylated at the 5' end and the capture probe was biotinylated at the 3' end. All oligonucleotide

sequences were purchased from Integrated DNA Technologies, Inc. (Coralville, IA).

2.3. *B. anthracis* culturing, DNA isolation and PCR

B. anthracis Sterne strain obtained from the Michigan Department of Community Health (Lansing, MI) was used in this experimental study. The bacterial culture was grown in trypticase soy broth at 37 °C for 24 h and the cells were enumerated by microbial plating in trypticase soy agar (II) plates containing 5% sheep blood (BD Biosciences, MD). Genomic DNA was isolated from 1 ml of a 24 h enrichment culture of *B. anthracis* following a modified protocol for mammalian DNA extraction (Laird et al., 1991). PCR amplification of the isolated target DNA was performed using a DNA thermocycler (Eppendorf, Westbury, NY). The 50 µl PCR mixture consisted of template DNA, forward and reverse primers, PCR buffer [1×], 2'-deoxynucleoside 5'-triphosphate (dNTP) mix [0.2 mM], magnesium chloride (MgCl₂) [1.5 mM], and 1 µl of AccuPrime™ Taq DNA Polymerase. The PCR amplification was run under the following conditions: 95 °C for 2 min; 35 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s; and 72 °C for 5 min. The amplified product was purified using a PCR purification kit (QIAGEN, Valencia, CA) and characterized by gel electrophoresis in ethidium bromide-stained 2.5% agarose gels (EmbiTec, CA). The concentration of the purified PCR DNA was finally determined using a spectrophotometer (NanoDrop™ 1000, ThermoScientific, Wilmington, DE) and was diluted as needed.

2.4. EAM nanoparticle synthesis and characterization

The EAM nanoparticles (NPs) were synthesized by a chemical polymerization process using gamma iron (III) oxide (γ-Fe₂O₃) nanoparticles as a template and coated with the electrically active polyaniline (Sharma et al., 2005). The magnetic and electrical properties of the NPs were confirmed from Superconducting Quantum Interference Device (SQUID) and Four Point Probe measurements. The structural morphology and size distribution of the NPs were studied using a 200 kV Field Emission Transmission Electron Microscope (JEOL 2200). The presence of the polymer was confirmed by studying the absorbance spectra of the EAMs using a UV–vis–NIR scanning spectrophotometer (UV-3101PC, Shimadzu, Kyoto, Japan). One milliliter of the EAM NP suspension in water was transferred into a quartz cuvette (10 mm path length) and the absorbance was measured by scanning the sample for a wavelength range of 300–1000 nm using a step size of 1 nm.

2.5. DNA probe labeling with EAM nanoparticles

The EAM NPs were labeled with the phosphorylated detector DNA probes (Ph-PRO) using phosphoramidate linkage between the amine groups of the polymer and the phosphate group of the oligonucleotides (Zhu et al., 2006). The NPs were dispersed in 10 mM sodium acetate buffer (pH 5.2) by sonication for 10 min and mixed with the Ph-PRO probes [22.5 µM] and 0.1 M 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC). The mixture was incubated with shaking in a rotational hybridization oven at room temperature for 4 h (Amerex Instruments Inc., CA). The Ph-PRO DNA probe labeled EAM NPs (PRO-EAMs) were then separated by a magnetic separator and washed repeatedly with acetate buffer and DNase RNase free water to remove the unbound probes.

The conjugation efficiency of the DNA probes with the NPs was determined using 6-carboxy fluorescein (6-FAM™) labeled Ph-PRO probes (at 3' end). Attachment of Ph-PRO detector probes to the EAM NPs was confirmed by fluorescence measurements in a Microplate Fluorometer Reader (Victor³, Perkin Elmer, MA). Fluor-

rescence of the pure 6-FAMTM labeled Ph-PRO probe solution and the supernatant solution containing the unreacted probes after magnetic separation of the PRO-EAMs was observed by excitation at 495 nm and detection of emission at 520 nm. The NP concentrations were varied at 0.1, 1, 10 and 20 mg/ml while keeping the Ph-PRO probe concentration fixed. The fluorescence measurements were also confirmed by spectrophotometric UV–vis absorbance measurements at 260 nm.

2.6. Dual hybridization and concentration of target DNA

The PCR amplified DNA targets were hybridized dually with the detector probe labeled EAM NPs (PRO-EAMs) and the biotinylated capture probes (PRO-Bio) (Song et al., 2006). Before hybridization, the purified PCR product was denatured by heating at 95° for 10 min in the thermocycler and cooling in icebath for 5 min, followed by a second cycle of heating at 95° for 5 min and cooling for 5 min. One hundred microliters of the denatured PCR product were diluted in 35 μ l of hybridization buffer composed of 2 M NaCl and 0.2 M phosphate buffer (pH 7.4) and added to appropriate concentrations of the PRO-EAMs and the PRO-Bio probes; the mixtures were hybridized at 45°C for 1 h in a rotational hybridization oven. The resulting EAM–target–biotin DNA hybrids were washed twice with tris ethylene diamine tetraacetic acid (TE) buffer (1 $\times$, pH 7.4) for 1 min by magnetic separation of the EAM NPs to remove the unbound target DNA. This was followed by washing the EAM NPs with 20% formamide in 0.01 M phosphate buffered saline (PBS) at 50°C for 1 min by magnetic separation to remove the nonspecifically bound DNA targets. Finally, the concentrated EAM–target–biotin DNA hybrids were resuspended in 40 μ l of DNase RNase free water.

2.7. Sensor design and surface modification

A screen printed three-electrode sensor (Gwent Group, UK) was used in the electrochemical detection of DNA targets. The sensor chip had an overall dimension of 22 mm $\times$ 12 mm with a circular working electrode of 4 mm diameter and a partially circular (270°) common reference and counter electrode of 1.5 mm in width (Fig. 1(a)). Screen printed carbon acted as working electrode and screen printed silver/silver chloride (Ag/AgCl) acted as the common reference and counter electrode. The working electrode surface was modified with streptavidin for target binding. Forty microliters of streptavidin solution (1 mg/ml) prepared in 0.1 M phosphate buffer (pH 7.0) were added onto the working electrode surface and incubated for 2 h at room temperature. The electrodes were then dried at ambient temperature for an additional 30 min and were ready for use in biosensor experiments. Approximately, the working electrode surface was coated with 0.8 μ g/mm² of streptavidin.

2.8. Electrochemical detection of EAM nanoparticles and EAM–target–biotin DNA hybrids

Electrochemical detection and characterization of straight EAM NPs were performed on the bare working electrode surface using cyclic voltammetry (CV) in a Potentiostat/Galvanostat (Princeton Applied Research, OakRidge, TN) with two vertex potentials in the ramp mode. One hundred microliters of the NP solution (concentration: 100 μ g/ml) in 0.1 M HCl were applied to the electrode surface and allowed to equilibrate for 5 min. The EAM solution was scanned at a scanning potential between –0.4 and +1.0 V and at scan rates ranging from 20 to 200 mV/s.

The EAM–target–biotin DNA hybrids obtained following dual hybridization were detected on streptavidin modified working electrode surface using CV. The concentrated DNA hybrids were allowed to react with the avidin modified electrode surface for

15 min at room temperature and rinsed with DNase RNase free water three times to remove any unbound EAM NPs from the surface and dried at ambient temperature for 15 min. One hundred microliters of 0.1 M HCl solution were added to the electrode surface and allowed to equilibrate for 5 min. The CV scans were performed using the same scanning potential window as that of the bare EAMs at a scan rate of 20 mV/s.

2.9. Biosensor sensitivity analysis

For sensitivity analysis, the purified PCR products were serially diluted to concentrations ranging from 10^{–1} to 10^{–5} ng/ μ l using DNase RNase free water. Each PCR dilution was then dually hybridized with the PRO-EAM and the PRO-Bio probes and the DNA hybrids were electrochemically detected on the streptavidin modified screen printed electrode surface. A blank control consisting of straight EAM NPs (concentration: 1 mg/ml) suspended in DNase RNase free water was tested for comparison. Base line curves for the bare working electrode and the streptavidin modified electrode were also obtained for each experiment.

The CV data obtained as a current vs. potential (*I* vs. *E*) curve was used to determine the oxidation (anodic) and reduction (cathodic) peak potentials. The mean and standard deviation of the anodic peak current signal for all target concentrations were estimated on the basis of data from three replicates and the differences between the means were statistically analyzed using single factor analysis of variance (ANOVA).

3. Results and discussion

3.1. Biosensor detection principle

The principle of detection of the EAM based electrochemical DNA biosensor is illustrated in the schematic in Fig. 1(b). The detection involves an electrochemical sandwich assay engaging a detector DNA probe and a capture DNA probe. The detector probe is labeled with EAM NPs (PRO-EAM) and the capture probe is labeled with biotin (PRO-Bio). The DNA targets are dually hybridized with the PRO-EAM and the PRO-Bio probes resulting in EAM–target–biotin DNA hybrids. The DNA hybrids are concentrated and separated from other noncomplementary sequences and unreacted DNA using a magnetic separation stand. The concentrated DNA target hybrids are then added directly to the surface of streptavidin modified screen printed electrodes for anchoring the hybrids on the electrode surface using streptavidin–biotin interactions. After a brief incubation period, the electrode surface is washed to remove the excess EAM NPs and the unbound DNA hybrids. The target DNA is finally detected electrochemically on the electrode surface exploiting the redox properties of the EAM NPs.

3.2. Characterization of EAM nanoparticles

The TEM image of the EAM NPs in Fig. 2(a) shows that the NPs have an average size of about 80–100 nm. Although some inhomogeneity exists in the NP shape, it is evident from the TEM image that electrically active polyaniline coats the surface of the iron-oxide cores which is essential for the biosensor design as the detection principle is based on the electrochemical properties of the polymer. The presence of the polymer as well as the magnetic core was determined by four point probe and DC SQUID measurements which showed the NPs to have a saturation magnetization value of 44.1 emu/g and an electrical conductivity of 3.3 S/cm (Pal and Alocilja, 2009). The high electrical conductivity observed in the NPs is imparted by the conducting polymer, polyaniline, in the EAM. The presence of polyaniline in EAM NPs was further confirmed

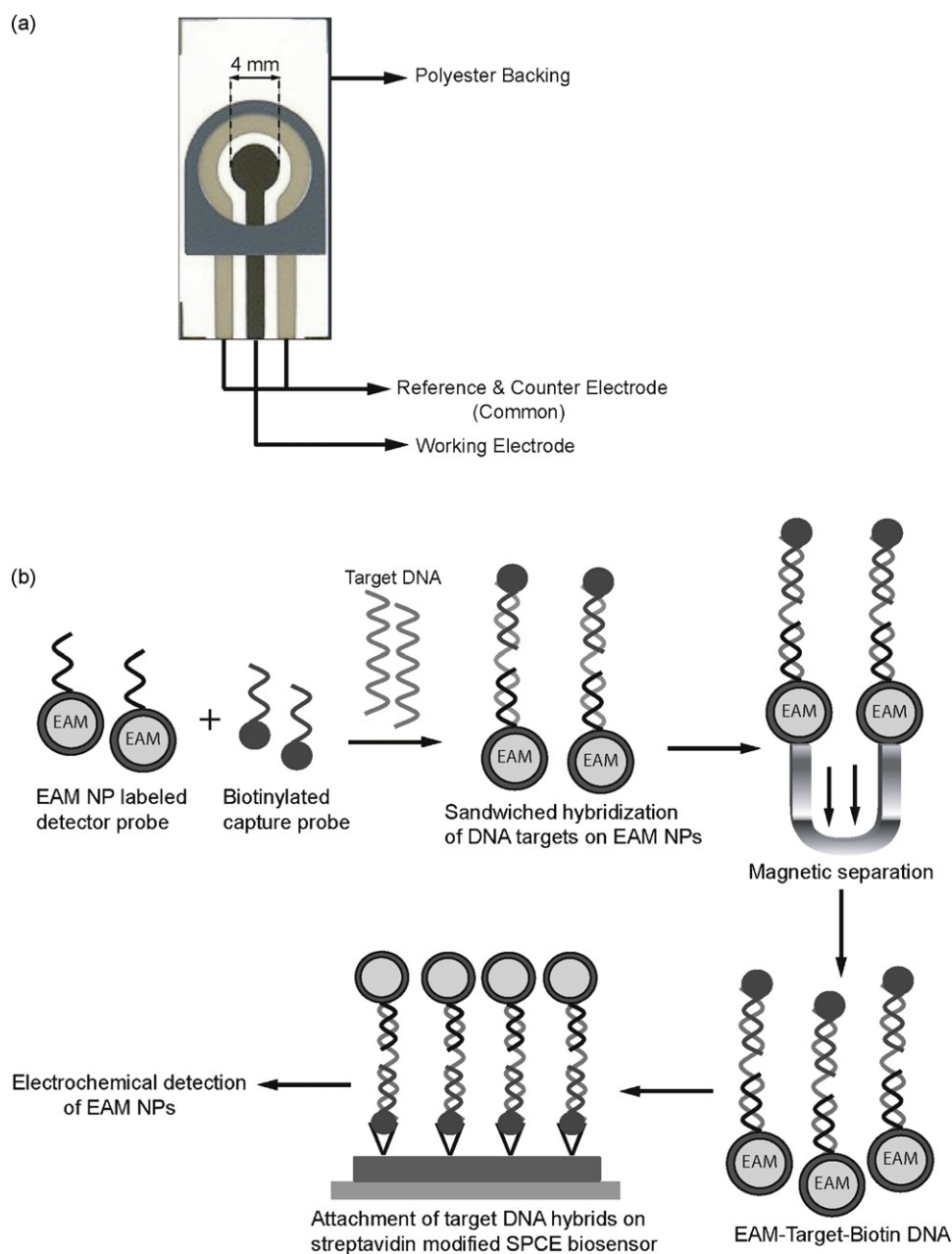


Fig. 1. Schematic illustration of (a) screen printed three-electrode sensor and (b) EAM based electrochemical DNA biosensor detection principle.

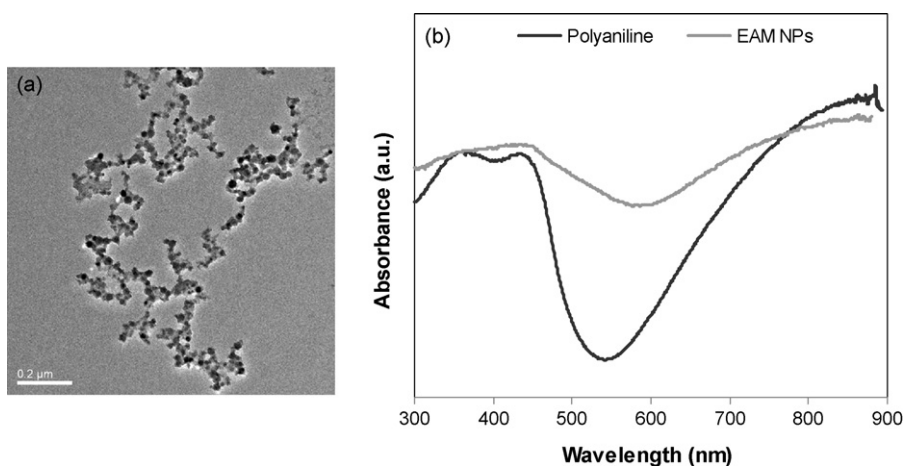


Fig. 2. EAM characterization: (a) TEM image of the EAM NPs and (b) UV-vis absorbance spectra of pure polyaniline and EAM NPs.

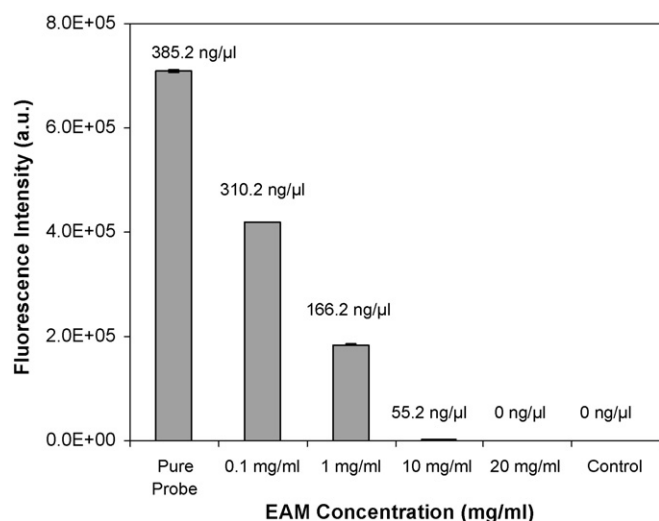


Fig. 3. Fluorescence signal of pure Ph-PRO probes and unreacted Ph-PRO probes after magnetic separation from EAM NPs (mean fluorescence intensity $\pm$ SD, $n = 3$) (SD = standard deviation, n = no. of replicates).

by the UV–vis spectral analysis results in Fig. 2(b). As observed in the figure, the characteristic peaks of pure polyaniline appear at 356, 433 and 862 nm. The peak at 356 nm can be attributed to π – π^* transition of the benzenoid ring, while the peaks at 433 and 862 nm are associated with polaron– π^* and π –polaron band transitions of polyaniline, respectively (Dallas et al., 2006; Lv et al., 2005; Stafstrom et al., 1987). For the EAM NPs, characteristic absorption peaks are observed at 441 and 864 nm that can be related to the polaron– π^* and π –polaron band transitions of polyaniline. For the EAM NPs, the polaron– π^* peak shows an 8 nm shift and the π –polaron peak shows a 2 nm shift from that of pure polyaniline. The red shifts observed in the spectrum can be explained by interactions between the Fe_2O_3 nanoparticles and the polymer backbone. However, the π – π^* transition peak of the polymer is not properly distinguished in the absorbance spectrum of the EAM NPs. The absorbance data also confirms that the polymer is present in the doped (conductive) state in the EAM NPs (Ohira et al., 1987).

3.3. Confirming EAM–DNA probe labeling

The biomodification of the EAM NPs with DNA probes (Ph-PRO) was confirmed by fluorescence and spectrophotometric studies. Fig. 3 shows the fluorescence intensity measurements of the pure 6-FAM labeled Ph-PRO probe solution [22.5 μM] and that of the unreacted probes in the supernatant after magnetic separation of the PRO-EAMs (probe labeled EAMs) at different concentrations of the NPs. As evident in the figure, the 6-FAM labeled Ph-PRO probes (pure probe) show the highest fluorescence signal due to the absence of any EAM NPs in the solution. The supernatants from the labeling process after the magnetic separation of the PRO-EAMs show significantly lower fluorescence signal than that of the pure probe at different EAM concentrations thus indicating the attachment of the probes to the NPs. A linear decrease in the fluorescence signal is also observed as the NP concentration increases from 0.1 to 20 mg/ml which is expected since an increased EAM NP concentration would result in a greater number of attachment sites (terminal amine groups of polyaniline) for the phosphorylated probes. The spectrophotometric measurements of single-stranded DNA (ss-DNA) concentration for the pure probes and the supernatants further confirm the attachment of the probes to the EAM NPs. The ss-DNA concentration for the pure probe is 385.2 ng/ μl , whereas for the supernatants from PRO-EAMs, the ss-DNA concentration decreases and is in the range of 310.2–0 ng/ μl . A final EAM

concentration of 1 mg/ml is chosen for the hybridization of DNA targets since the EAM NPs appear to be saturated with the DNA probes at this concentration.

3.4. Electrochemical characterization of the EAM nanoparticles

Electrochemical characterization of the synthesized EAM NPs was performed on the working electrodes using CV before proceeding to the detection of EAM–target–biotin DNA hybrids. The electrochemical property of the conducting polymer (polyaniline) in the EAM NPs is strongly dependent on doping the polymer with the ions present in the electrolyte and therefore its redox-activity is prevalent in the protonated (doped) state that occurs at around $\text{pH} < 4$ (Abdullin et al., 2007; Mazeikiene et al., 2003). For this reason, 0.1 M HCl was chosen as the electrolyte in all CV scans of the EAM NPs to ensure the electrically active state of the polymer in the NPs. Additionally, the potential window (–0.4 to +1.0 V) chosen for the CV experiments was favorable for the redox process of the polymer in the NPs over that of the γ - Fe_2O_3 core which is electrochemically reduced at potentials lower than –0.4 V (Dubois and Chevalet, 2003). Fig. 4(a) shows the cyclic voltammograms of the EAM NPs in 0.1 M HCl scanned from –0.4 to +1.0 V at scan rates ranging from 20 to 200 mV/s. As evident in the figure, two well-defined redox peaks (the anodic and cathodic peaks are indicated as E_{pa} and E_{pc} in the figure) of the EAM NPs are visible at the different scan rates. The two redox peaks obtained can be attributed to the characteristic electrochemical behavior of polyaniline (Ambrosi et al., 2008; Zhang et al., 2007; Gospodinova et al., 1996). The broad anodic peak at +0.118 V (at 20 mV/s scan rate) corresponds to the switching of the leucoemeraldine base form to the emeraldine salt form of polyaniline in the EAM NPs. The sharper anodic peak at +0.600 V (at 20 mV/s scan rate) is conventionally attributed to the oxidation of the emeraldine form to the pernigraniline salt form of the polymer (Zhang et al., 2007). The corresponding cathodic peaks for the reduction process are observed at +0.530 and –0.070 V, respectively. The effect of scan rate on the rate of electron transfer to the electrode surface by the EAM NPs is also evident in Fig. 4(a). With an increase in the scan rate, the anodic peak potential of the EAM NPs is shifted to a more positive potential, the peak current is increased, and the cathodic peak potential is shifted to a negative potential. Fig. 4 (b) shows a plot of the dependence of the anodic peak current and the cathodic peak current vs. the scan rate. As observed in the figure, the peak current exhibits a linear relationship with increasing scan rate thus suggesting surface controlled behavior and diffusion controlled system (Bard and Faulkner, 2001). However, the ratio of the peak currents at different scan rates $I_{\text{pa}}/I_{\text{pc}} \neq 1$ which indicates a quasi-reversible chemistry of the EAM NPs and the electrode process (Bard and Faulkner, 2001; Ram et al., 1999).

3.5. Detection of *B. anthracis* DNA

The DNA hybridization conditions were determined from fluorescence assays and the data is presented in the supplemental information (Figs. S1 and S2). The optimal hybridization time and temperature for dual hybridization of the DNA targets with the capture and detector probes as determined from supernatant fluorescence measurement was 30 min at 45 °C. Fig. 5 shows the electrochemical response of the concentrated EAM–target–biotin DNA hybrids from undiluted PCR products (7.3 ng/ μl) captured on streptavidin modified working electrode following dual hybridization. The electrochemical responses of the bare electrode and streptavidin modified electrode were compared as control. As evident, the CV of the EAM captured targets shows the two characteristic redox peaks of the EAM NPs that are absent in the control. The anodic peak potentials are located at +0.110 and +0.597 V,

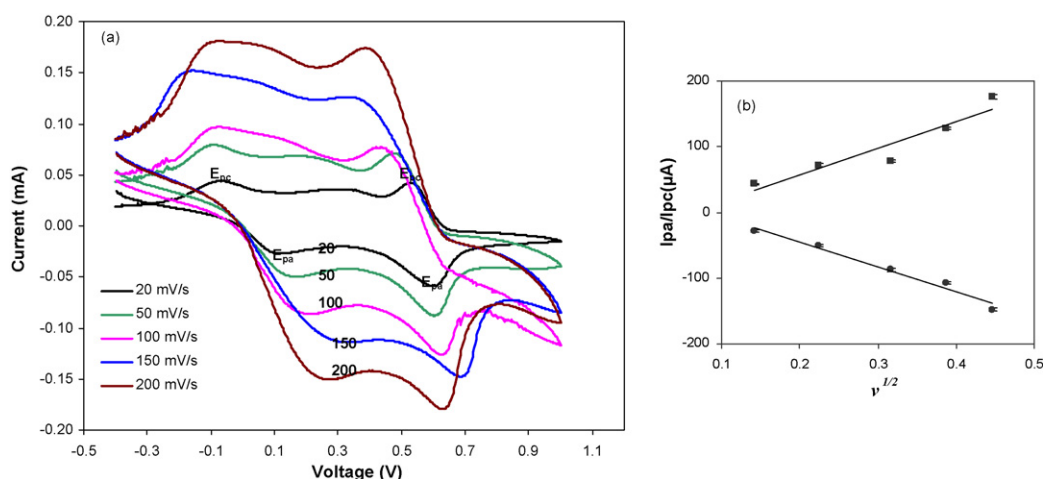


Fig. 4. (a) Cyclic voltammograms of EAM NPs in 0.1 M HCl at scan rates of 20, 50, 100, 150 and 200 mV/s. and (b) plots of anodic and cathodic peak current vs. scan rate (mean current $\pm$ SD, $n = 3$).

whereas the cathodic peak potentials are located at +0.530 and -0.070 V, similar to observations with bare EAM NPs in 0.1 M HCl. The presence of the redox peaks in the CV response demonstrates that the EAM NPs are electrochemically active after the probe labeling and sandwiched hybridization processes and also confirms the dual function of the EAM NPs, i.e. successful magnetic capture of target DNA and electrochemical detection of the concentrated EAM–target–biotin DNA hybrids.

3.6. Sensitivity analysis of *B. anthracis* DNA targets

Fig. 6(a) shows the electrochemical response of the biosensor in PCR target concentrations ranging from 0.001 to 10 ng/ μ l and the control. The control solution consisted of probe labeled EAM NPs hybridized with 0 ng/ μ l of the PCR target solution. As observed in the CV response, the two characteristic redox peaks of the EAM NPs are present at different concentrations of the PCR target. A gradual decrease in the intensity of the redox peaks is noted with a decrease in the target concentration. The anodic peak current at +0.59 V is maximum (17 μ A) for the highest PCR target concentration (10 ng/ μ l) and decreases to 1.95 μ A at a target concentration of 0.01 ng/ μ l which is expected as a low target concentration would

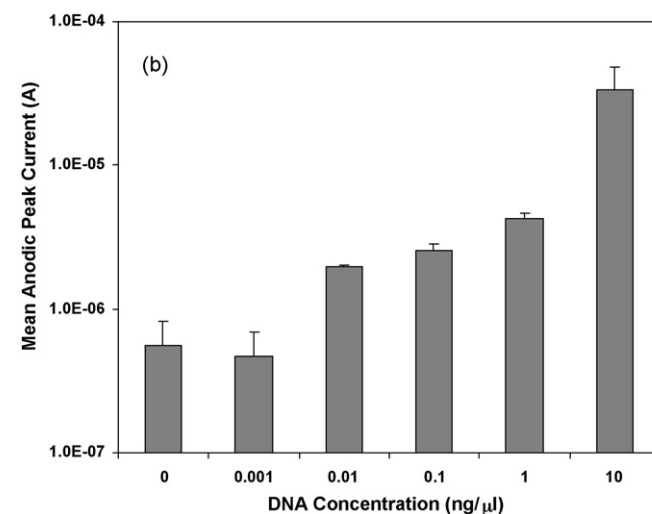
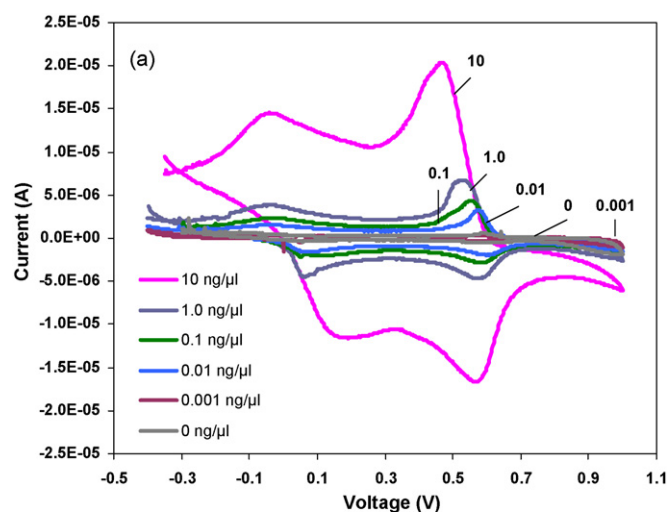


Fig. 6. (a) Electrochemical response of the biosensor in PCR target concentrations ranging from 0 to 10 ng/ μ l in 0.1 M HCl at 20 mV/s and (b) CV mediated anodic peak current at different target DNA concentrations (mean current $\pm$ SD, $n = 3$).

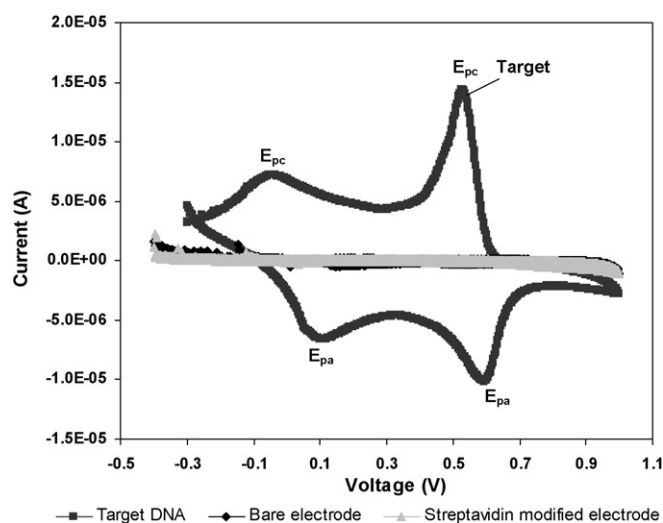


Fig. 5. Cyclic voltammograms of the bare screen printed electrode, the streptavidin modified electrode and EAM captured target DNA hybrids on the electrode in 0.1 M HCl at 20 mV/s.

imply fewer EAM–target DNA–biotin hybrids bound to the electrode surface. The anodic peak currents observed for the control and DNA concentration of 0.001 ng/μl are 0.84 and 0.29 μA, respectively. The presence of EAM redox peaks in the CV scans also confirms the ability of the EAM NPs to capture and detect DNA targets from low DNA concentrations.

Preliminary sensitivity analysis of the detected *B. anthracis* DNA targets was performed from the CV results. Although both redox peaks of EAM NPs were evident at lower target concentrations in Fig. 6(a), the anodic peak current (oxidation peak) at the peak potential of +0.59 V was chosen for the sensitivity analysis due to minimal shifts observed in the peak current values in repeated observations. Fig. 6(b) shows the mean anodic peak current values observed for the different target concentrations from three experimental replicates. A hundredfold increase in the anodic current signal is observed as the target DNA concentration increases from 0 to 10 ng/μl. At a potential of 0.59 V, the mean anodic peak current value from three experimental trials is $33.6 \times 10^{-6} (\pm 14.07 \times 10^{-6})$ A for the highest PCR target concentration (10 ng/μl) and decreases to $1.99 \times 10^{-6} (\pm 0.03 \times 10^{-6})$ A at the target concentration of 0.01 ng/μl. The mean anodic peak currents for the control and the lowest tested concentration of 0.001 ng/μl are $0.55 \times 10^{-6} (\pm 0.27 \times 10^{-6})$ A and $0.47 \times 10^{-6} (\pm 0.21 \times 10^{-6})$ A, respectively. The slightly higher current values observed for the control as compared to 0.001 ng/μl of target implies no detection at this concentration and can be attributed to signal from non-specifically adsorbed EAM NPs on the electrode surface in both cases.

The sensitivity result obtained from the CV response was statistically analyzed by one-way single factor analysis of variance (ANOVA) of the anodic peak current signal for the different DNA concentrations at a significance of 95% ($P < 0.05$) using the SAS software. The lowest DNA concentration that showed an anodic peak current signal significantly different ($P < 0.05$) from the control was considered as the detection limit of the sensor. The statistical results indicated that the different target DNA concentrations had a significant effect ($P < 0.0001$) on the anodic peak current signal. The anodic peak current for DNA concentrations ranging from 10 to 0.01 ng/μl were significantly different from control with P -value < 0.0001 , whereas for DNA concentration of 0.001 ng/μl the P -value was 0.6943. Therefore, the lower limit of detection of the EAM based DNA sensor was determined to be 0.01 ng/μl of DNA from the ANOVA results. Furthermore, the signal-to-noise ratio (S/N) of the mean anodic peak current at 0.01 ng/μl DNA concentration and the control is greater than 3 ($S/N = 1.99 \times 10^{-6} / 0.55 \times 10^{-6}$). Additionally, the ANOVA of the current signal between different DNA target concentrations (10–0.01 ng/μl) was significantly different ($P \leq 0.05$) from each other indicating the feasibility of quantitative estimation of target DNA using the EAM based biosensor.

4. Conclusion

This study explores the potential of EAM nanoparticles as a magnetic concentrator of DNA targets and a novel nanostructured transducer in the electrochemical detection of DNA. The EAM based DNA biosensor is fabricated and implemented in the detection of *B. anthracis* pag A gene. The sensitivity of the biosensor as determined from cyclic voltammetry measurements is 0.01 ng/μl of DNA in a total detection time of 60 min. These preliminary results indicate that the EAM nanoparticles hold promise in electrochemical biosensing applications. Future research work would involve evaluating the specificity of the biosensor and advancing the detection process through different electrochemical techniques. Furthermore, the potential of the biosensor as a field based device for diagnostic and biosecurity applications will also be explored.

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Appendix A. Supplementary data

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