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Conducting polyamic acid membranes for sensing and site-directed immobilization of proteins

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ABSTRACT

A biosensor platform based on polyamic acid (PAA) is reported for oriented immobilization of biomolecules. PAA, a functionalized conducting polymer substrate that provides electrochemical detection and control of biospecific binding, was used to covalently attach biomolecules, resulting in a significant improvement in the detection sensitivity. The biosensor sensing elements comprise a layer of PAA antibody (or antigen) composite self-assembled onto gold (Au) electrode via *N*-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) linking. The modified PAA was characterized by Fourier transform infrared (FTIR), ^{1}H nuclear magnetic resonance (NMR), and electrochemical techniques. Cyclic voltammetry and impedance spectroscopy experiments conducted on electrodeposited PAA on Au electrode using ferricyanide produced a measurable decrease in the diffusion coefficient compared with the bare electrode, indicating some retardation of electron transfer within the bulk material of the PAA. Thereafter, the modified PAA surface was used to immobilize antibodies and then to detect inducible nitric oxide synthase and mouse immunoglobulin G (IgG) using enzyme-linked immunosorbent assay (ELISA), surface plasmon resonance (SPR), and amperometric techniques. ELISA results indicated a significant amplified signal by the modified PAA, whereas the SPR and amperometric biosensors produced significant responses as the concentration of the antigen was increased. Detection limits of 3.1×10^{-3} ng/ml and 2.7×10^{-1} ng/ml were obtained for SPR and amperometric biosensors, respectively.

An emerging area of research is the implementation of new materials for the development of a novel class of biosensors, biochips, nanosensors, and other transducers [1–3]. Novel materials are needed to improve the mechanical and biochemical stability of the sensor while improving the immobilization scheme to achieve biostability and spatial control of biomolecules. An ideal electrode material for the construction of biosensors must possess the following characteristics [2,3]: (i) biocompatibility with the biological element, (ii) absence of diffusion barriers, (iii) stability with changes in temperature, pH, ionic strength, and/or macroenvironment, (iv) sufficient sensitivity and selectivity for the analyte of interest, and (v) ease of mass production. Moreover, the ideal material either should possess the necessary functional groups needed for the attachment of biomolecules or can be easily functionalized. In addition, an ideal electrode material must be characterized by good conductivity to ensure rapid electron transfer.

Creating an oriented layer of protein molecules at such a surface requires suitable attachment chemistry with control over the conformation and spacing of the molecules to give a packing density that allows the analyte to access the binding sites. To achieve this, we developed a biosynthetic hybrid material by cross-linking polyamic acid (PAA)[1] chain with goat anti-mouse using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS). The techniques described here, therefore, are driven by the purpose of producing a versatile platform that can be used for different bimolecular immobilization. This approach

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[1] *Abbreviations used:* PAA, polyamic acid; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; NHS, *N*-hydroxysuccinimide; Au, gold; $K_3Fe(CN)_6$, potassium ferricyanide; BSA, bovine serum albumin; NaCl, sodium chloride; H_2SO_4, sulfuric acid; NaOH, sodium hydroxide; NaH_2PO_4, sodium dihydrogen phosphate; Na_2HPO_4, disodium hydrogen phosphate; H_2O_2, hydrogen peroxide; KCl, potassium chloride; Ag/AgCl, silver chloride; DMSO, dimethyl sulfoxide; ACN, acetonitrile; AP, alkaline phosphatase; iNOS, inducible nitric oxide synthase; PNPP solution, *p*-nitrophenyl phosphate disodium; CV, cyclic voltammetry; ELISA, enzyme-linked immunosorbent assay; EIS, electrochemical impedance spectroscopy; NMR, nuclear magnetic resonance; SPR, surface plasmon resonance; ODA, 4,4-oxydianiline; PMDA, pyromellic dianhydride (1,2,4,5-benzenetetracarboxylic anhydride); DMF, *N,N*-dimethylformamide; FTIR, Fourier transform infrared; PBST, phosphate-buffered saline with Tween 20; IgG, immunoglobulin G; DEA, diethanolamine; HSA, human serum albumin; Ab, antibody.

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establishes a versatile material for direct selective covalent attachment of antibodies to a modified gold (Au) electrode surface, facilitating an immunoassay platform construction for electrochemical signal assessment strategies in the study of antibody–antigen recognition events.

PAA, a precursor of polyimide, possesses these desirable characteristics. PAA is a conducting polymer that has amide and carboxylic groups in its polymer backbone [4]. PAA can be dissolved in both aqueous and organic solvents and can be easily processed [5]. Due to its solubility in dipolar aprotic solvents and the functional groups on its polymer backbone, PAA has many potential interesting material applications [4]. We have used the unique reactivity of PAA as a novel electrode material by preventing the cyclization of the reactive soluble intermediate into polyimides at low temperature [5]. The ability to prevent the cyclization process has enabled the design of a new class of electrode materials [6]. PAA is a precursor of polyimides with cation complexing properties [7–10]. An average functionality (the average number of functional groups per monomer molecule) in PAA can vary from 160 to 600 depending on its molecular weight [7,9].

We have recently shown that these functional groups act as molecular anchors in the formation of a series of novel, self-standing, flexible, phase-inverted PAA membranes [11–13]. These have similar morphologies as the typical phase-inverted membranes with a thin, dense top layer supported by a porous sublayer. The surface pore size of these membranes can be controlled by the synthesis conditions, method of desolvation, and concentrations of polymer composition. The phase-inverted PAA derivatives were found to have a number of unusual but practically favorable properties. The resulting polymers were found to be thermally and chemically (except strong bases) stable; they exhibit flexibility, high tensile strength, and electrooptical properties. Unlike thermally cured derivatives of these films, which were transparent with the highest absorption band from 280 to 350 nm, the phase-inverted membranes were opaque. So, their fluorescence and vibrational properties were studied. Results showed that the emission intensities increased with increasing excitation wavelengths until they reached their maximum with an excitation wavelength at 460 nm and then decreased at longer excitation wavelengths. The phase-inverted PAA membranes were also found to be Raman active [11].

Explanations for these properties have been hypothesized in terms of favorable electronic properties associated with aromatic moieties, the presence of reactive functional groups, and the ability of the PAA functional units to self-assemble into reversible polymeric networks.

Here, we demonstrate that the carboxylic and amide groups available in PAA can be used for appropriate covalent attachment of biomolecules, making them potential candidates for biosensor applications [5,14]. The development of probes for the detection of biomolecules using conducting polymers is of considerable interest [15–17]. Polypyrrole and other conducting polymers have been investigated extensively regarding their applications in chemical sensors and biosensors [15,17,18]. Although carboxylic acids can be introduced into conducting polymers such as PANI by simple reflux methods or anchored into poly-SNS conducting polymer and then used for immobilization of biomolecules [17], there are significant limitations. First, the deposition of biologically active material onto these surfaces is achieved almost exclusively through *physical adsorption* or entrapment via the weak bonds such van der Waals forces, ionic binding, and hydrophobic interaction. Second, this mode of immobilization produces biosensors that are characterized by poor analytical performance, especially with regard to operational and storage stability. Third, the immobilized biomaterials are subject to variation when exposed to changes in pH, temperature, and ionic strength. Finally, the extreme hydrophobicity and insolubility of the polymeric matrix triggers protein adsorption and makes it less than ideal material for practical sensing applications [19].

In contrast, PAA is soluble in both organic and aqueous solvents and binds specifically to biomolecules through its pendant carboxyl groups that are in close proximity to the transducer surface. The carboxylic acid groups in the PAA structure are part of a rigid section of the polymer with a mild degree of rotation about the ether linkages that allow for locked-in orientation during immobilization. These locked-in functional groups in the side chains facilitate in situ surface reactions (e.g., water-soluble EDC and NHS linkage) for optimization of sensor properties. These characteristics allow the design of electron transfer pathways between the immobilized biomolecule and the electrode surface.

Materials and methods

Materials

Potassium ferricyanide [$K_3Fe(CN)_6$] and bovine serum albumin (BSA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sodium chloride (NaCl), sulfuric acid (H_2SO4), sodium hydroxide (NaOH), sodium dihydrogen phosphate (NaH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), hydrogen peroxide (H_2O_2), and potassium chloride (KCl) were purchased from J.T. Baker Chemicals (Philipsburg, NJ, USA). Silver chloride (Ag/AgCl) electrode, Au electrodes, and platinum wire as auxiliary electrode were purchased from BASi Analytical Instruments (West Lafayette, IN, USA). Dimethyl sulfoxide (DMSO), acetonitrile (ACN), and alkaline phosphatase (AP) were purchased from Sigma–Aldrich (Milwaukee, WI, USA). Mouse anti-iNOS (inducible nitric oxide synthase) and iNOS enzyme were purchased from Cayman Chemicals (Ann Arbor, MI, USA). NHS, EDC, and *p*-nitrophenyl phosphate disodium (PNPP solution) were obtained from Pierce (Rockford, IL, USA).

Instrumentation

Cyclic voltammetry (CV) experiments were carried using a BAS 50W electrochemical workstation (Bioanalytical Systems, USA) having a three-electrode electrochemical cell and a gold electrode as the working electrode. All enzyme-linked immunosorbent assay (ELISA) experiments were carried out on 96-well microtiter plates. The microplate reader (KC4) was obtained from Bio-Tek Instruments (Winooski, VT, USA). Amperometric measurements were carried out using a BAS 100B electrochemical analyzer (Bioanalytical Systems). Electrochemical impedance spectroscopy (EIS) measurement and characterization of the modified/unmodified PAA were carried out using a PGZ402 VoltaLab Analyzer (Radiometer Analytical, France). Nuclear magnetic resonance (NMR) analysis was carried out on a Bruker AM 360 spectrometer operated by Tecmag NTNMR software. Surface plasmon resonance (SPR) measurements were carried out on a double-channel cuvette-based SPR system (Autolab Espirit, Eco Chemie, Netherlands) equipped with software (ESPIRIT Kinetic Evaluation, version 4.1.0) for data analysis.

Methods

Synthesis of PAA

The PAA was synthesized in organic medium using 4,4-oxydianiline (ODA) and 1,2,4,5-benzenetetracarboxylic anhydride (pyromellic dianhydride, PMDA) precursors as reported previously [5]. Briefly, to prepare the PAA, 2.0024 g (0.01 mol) of ODA and 157 ml of ACN were stirred until solvation. Then, 50 ml of ACN containing 2.1812 g of PMDA (0.01 mol) was added drop by drop for

more than 1 h, and the solution was stirred for more than 24 h. The resulting precipitates were filtered through a membrane under suction and finally dried at room temperature. The amount of PAA obtained was 3.21 g, producing 85% yield. This homogeneous powder was found to be soluble in *N,N*-dimethylformamide (DMF) and DMSO. Scheme 1 shows the structure of the PAA that was synthesized.

Modification and characterization of PAA

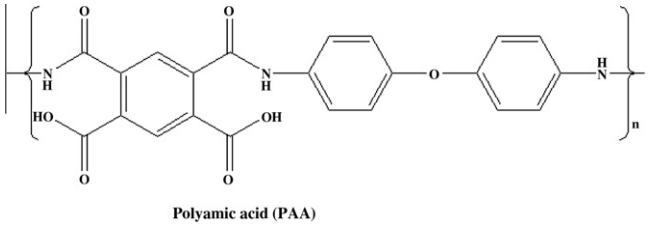
The synthesized PAA was modified using EDC and NHS protocols. Briefly, 5 mg/ml PAA was dissolved in DMSO. Then, 1 ml each of 8 mM NHS and 5 mM EDC was added and left to react for 1 h at room temperature. Scheme 2 shows the reaction. The sample was then subjected to NMR, Fourier transform infrared (FTIR), and elecrochemical characterization.

NMR characterization. We carried out ^{1}H NMR characterization to confirm the modification of the PAA by the EDC/NHS chemistry as follows. The PAA synthesized above was dissolved in DMSO, and EDC/NHS was added and left to react for 1 h. The product was then subjected to ^{1}H NMR characterization. For comparison, PAA, EDC, and NHS—all dissolved in DMSO—were also characterized using ^{1}H NMR. According to ChemDraw predictions, the presence of the characteristic peaks at around 2.64 to 2.75 ppm, which were attributed to the attached NHS, indicated that the PAA modification was successful because those peaks were not present in the unmodified PAA.

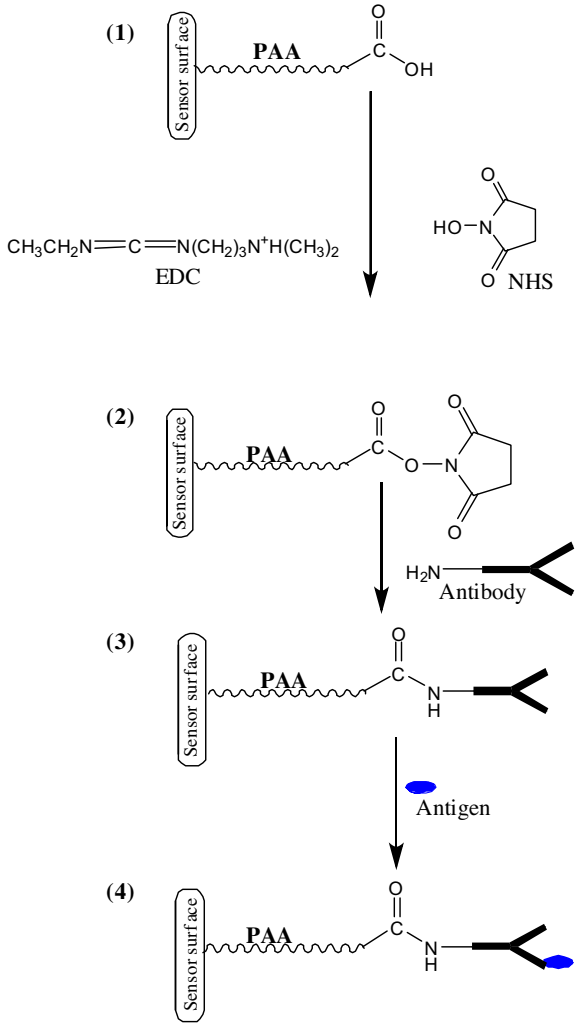
FTIR characterization. The FTIR spectrum of the modified and unmodified PAA was recorded over the range of 4000 to 450 cm^{-1}. The unmodified PAA was recorded as powder, whereas the modified PAA was dissolved in DMSO. FTIR of EDC and NHS was also performed to compare with the modified PAA.

Electrochemical characterization

CV characterization. CV was used to characterize the electrochemistry of the synthesized PAA. The experiments were carried using BAS 50W electrochemical workstation (Bioanalytical Systems). Gold electrode was used as the working electrode, and it was first cleaned using alumina slurries of 0.1, 0.3, and 0.05 μm to provide a shiny finish. The electrode was then sonicated with piranha solution (sulfuric acid/hydrogen peroxide, 3:1, v/v) and finally rinsed with distilled water to remove any impurities from the surface. The reference electrode used was Ag/AgCl, and the counter electrode was a platinum wire (Alfa Aesar). The as-synthesized PAA was dissolved in DMSO at 5 mg/ml and electrochemically deposited onto the Au electrode using 50 cycles between −500 and 1000 mV at 50 mV/s. The PAA-modified Au electrode was further characterized using CV at different scan rates in 2 mM K_3FeCN_6 with 0.1 M KCl as the supporting electrolyte. This was carried out to investigate the effect of the PAA on the redox activity of K_3FeCN_6. Prior to the electrodeposition of PAA, the CV of the bare electrode at different scan rates (0.01–0.1 V/s) was performed. The PAA-modified Au electrode was further modified using EDC/NHS for 1 h, after which the CV at different scan rates was carried out. The diffusion coefficients were calculated at this stage and compared for all of the electrodes.



Scheme 1. Structure of PAA.



Scheme 2. Schematic of biorecognition strategy employed for site-directed immobilization of proteins using PAA. Step 1: Covalent attachment to activated surface groups. Step 2: Surface linkage and activation using EDC/NHS chemistry. Step 3: Attachment of antibody (Ag). Step 4: Antigen recognition.

Impedance characterization. EIS of the modified and unmodified PAA was carried out using a PGZ402 VoltaLab Analyzer (Radiometer Analytical) together with a similar three-electrode cell arrangement and electrolyte combination as CV. An AC amplitude of 10 mV and a frequency of 1 kHz to 50 mHz with 50 steps per frequency decade were applied, and the potential was stepped between −50 and 100 mV at 50-mV intervals. The impedance data were modeled as equivalent electrical circuits using ZView software.

ELISA

The experimental setup was as outlined in Scheme 2. Briefly, the ELISA used polystyrene with 96 wells that were modified with the PAA. The total liquid volume in each well was 100 μl. Each well was coated with a layer of the PAA by incubating the plate with 100 μl of 5 mg/ml PAA dissolved in DMSO overnight. PAA-covered wells were washed three times by flooding the wells with PBST buffer (phosphate-buffered saline with Tween 20) and tap-dried. The PAA-coated wells were ready for use in a protein immobiliza-

tion platform. Selected wells were cross-linked with water-soluble carbodiimide in PBS buffer (pH 7.2). The carboxylic acid groups of the PAA were activated using 5.0 mM EDC in the presence of 8.0 mM NHS. The reaction was maintained for 2 h at room temperature. After the removal of the cross-linking solution, the wells were washed three times with PBST and tap-dried. Mouse immunoglobulin G (IgG) was used as the model analyte to investigate the feasibility of PAA as a template for biomolecule immobilization. After the carboxylic ends of the PAA were activated using the EDC/NHS, the capture antibody (goat anti-mouse antibody) was incubated overnight in the wells. This was followed by washing the plates with the PBST and incubating the wells with BSA to block the free non-antigenic sites of the antibody.

A similar washing procedure was followed with 100 μl of different concentrations of the antigen (mouse IgG) in PBS–BSA buffer applied to the wells and incubated overnight at 4 °C. Three wells, which were treated similarly as the sample wells but contained no antigen, were added as control. The plates were subsequently washed, and the 100 μl of 1 μg/ml secondary antibody (rabbit anti-mouse antibody) was applied except for the blank. Following the 2-h incubation at ambient temperatures, another round of washing was carried out before adding 100 μl of goat anti-rabbit IgG–AP in Tris buffer (pH 8.0) excluding the blanks. This step was followed by additional incubation for 1 h at room temperature. After the final rinsing with Tris buffer (pH 8.0), 100 μl of 1 mg/ml PNPP solution prepared in 10% diethanolamine (DEA) buffer was added except for the blank and the plates were incubated for 30 min at room temperature. The blank wells were filled with 100 μl of DEA buffer before measuring the optical densities of the solutions at 405 nm using a Synergy HTRDR multidetection microplate reader.

SPR-based sensor using the modified PAA

The feasibility of using the PAA for biomolecule immobilization was further tested using the SPR. The procedure used is described in Ref. [19]. Briefly, a self-assembled layer of PAA was affixed to the SPR Au disk surface via adsorption by overnight incubation in PAA/DMSO solution, followed by thorough rinsing with triply distilled water. The PAA-covered sensor disk was then either used immediately or stored at 4 °C for later use. Antibody was immobilized on the sensor surface by using the carboxylic groups of the self-assembled monolayer of the PAA, which was activated using 8 mM EDC and 5 mM NHS. The model analyte was iNOS; thus, 1 μg/ml of the capture antibody (mouse anti-iNOS) that was prepared in acetate buffer (coupling buffer) that replaced the NHS ester was formed following activation of the carboxylic groups to form an amide bond, thereby covalently linking the antibody to the sensor surface (Scheme 2). The unreacted NHS ester and any free non-antigenic sites of the antibody were blocked by injecting 1 mg/ml BSA to the surface. HCl (0.1 M) was used as the regeneration solution as optimized in the literature [19]. Different concentrations of the antigen (iNOS enzyme, 3.35×10^1–1.08×10^3 ng/ml) prepared in PBS–BSA buffer (pH 7.6) were used as the analyte for the interaction with the immobilized capture antibody. The results were compared with those using mercaptoundecanoic acid, a common reagent used in SPR experiments to aid in the immobilization of the capture antibody.

Amperometric detection using the modified PAA

The antibody-modified PAA electrode was used for antibody immobilization (Scheme 1) and as biosensor for iNOS. This was first modified using EDC/NHS for 1 h. The antibody to iNOS was deposited on the surface of the modified electrode and left to immobilize for 1 h at room temperature. Using the same three-electrode cell arrangement, the electrode was rinsed with PBS buffer and then used to detect iNOS using the amperometric technique at a fixed potential of 300 mV. To assess the biosensor response to different concentrations of the antigen, 10 μl of 2.7×10^4 ng/ml iNOS enzyme was injected into the cell and the resulting current reduction was measured. PBS buffer (5 ml) was

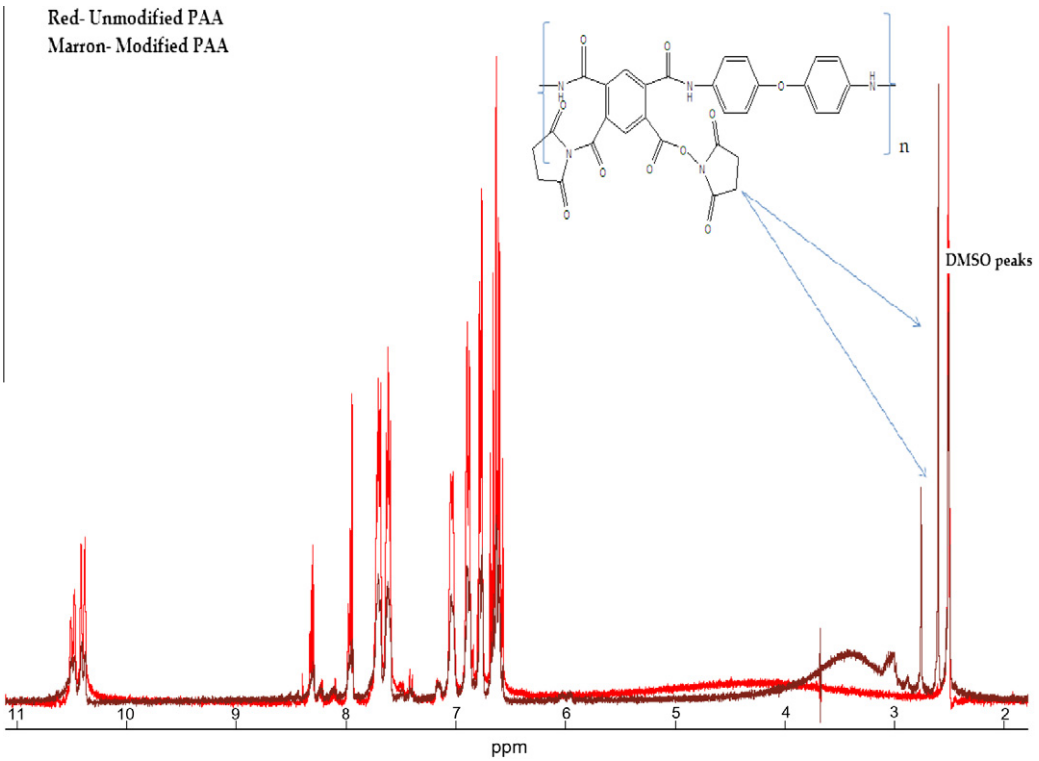


Fig.1. ^{1}H NMR spectra of the modified (maroon) and unmodified (red) PAA. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Summary of the chemical shifts of modified and unmodified PAA.

Chemical shift (ppm)	Functional group
2.64–2.75	NHS protons
6.5–8.5	Aromatic protons
10.42	-NH-

used as the supporting electrolyte. The PAA-modified electrode was also tested using mouse IgG. The sensor design for coating goat anti-mouse antibody onto the PAA electrode is shown in Scheme 2. The selectivity of this system was studied using human serum albumin (HSA) and BSA.

Results and discussion

The objective of this work was to investigate the feasibility of modifying the carboxylic groups available in PAA for site-directed immobilization of biomolecules and as template for electrochemical sensors. The carboxylic groups of the PAA were modified using NHS and EDC covalent chemistry to allow further biomolecular linking and signal amplification. Solution of PAA was first synthesized from the polycondensation of ODA and PMDA [5,20]. The resulting powdered polymer was subsequently modified to create the site-directed immobilization of the antibody (Scheme 2). This sensor was tested for the detection of iNOS enzyme using amperometric and SPR techniques.

Uniqueness of PAA

Polyamic acids refer to a class of polymeric compounds with amide, carbonyl, and carboxylic acid functional groups (Scheme 1) that can easily bond to other groups [4,5]. With an average functionality varying between 160 and 600 depending on its molecular weight, several derivatives of PAA could be prepared by tailoring a random sequence to the polymer chain using either a diamine or dianhydride with other additives such as metal nanoparticles and polysiloxanes. PAA films are usually developed by thermal or sputtered metal vapor deposition, chemical vapor deposition, electrochemical procedure, and the Langmuir–Blodgett technique. The thickness of the resulting layer can be controlled by varying the solvent composition, pH, and concentration of the monomer. The type of solvent used to dissolve nonaqueous PAA mixture

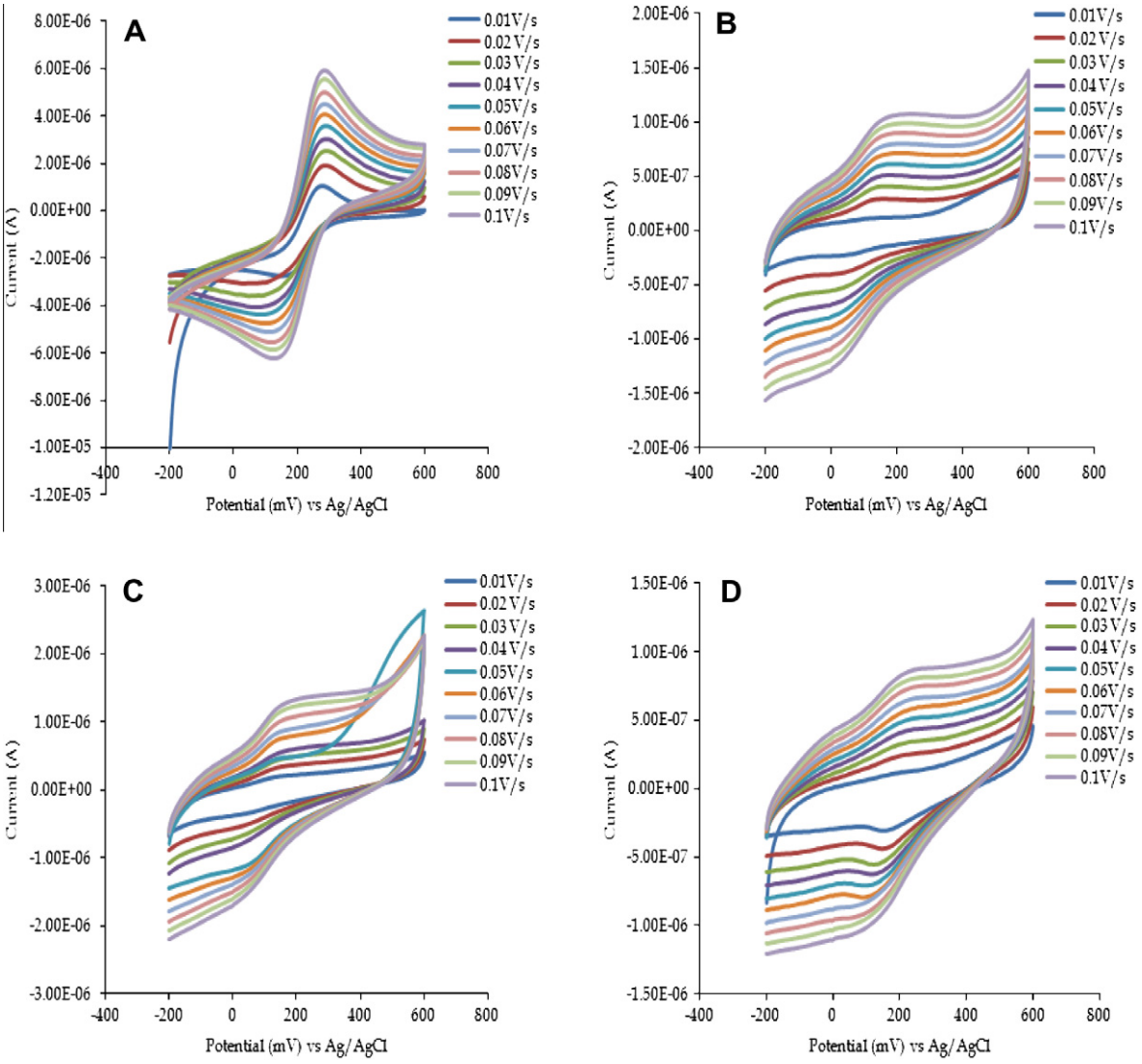


Fig.2. Cyclic voltammograms of PAA at Au electrode in K_3FeCN_6 at different scan rates: (A) bare electrode; (B) Au electrode–PAA; (C) Au electrode–PAA–EDC/NHS; (D) Au electrode–PAA–EDC/NHS–Ab. Scan rates between 0.01 and 0.1 V; potential range between −200 and 600 mV (vs. Ag/AgCl).

could influence the nature of the functional groups retained in the resulting polymer. For example, when the emulsions are dissolved in protonated solvents such as *N,N′*-dimethylacetamide or DMF, the carboxyl groups are retained [4,5]. However, when deprotonated solvents such as ethanol and acetone are employed, imidization occurs to produce polyimides. We have shown that the imidization of PAA to polyimide can be avoided to retain the carboxyl groups and have further derivatized these groups as a template for nanoparticles or as reducing agents for the reduction of chromium(VI) to chromium(III) [11–13]. In this work, we demonstrate that PAA can provide a selective surface for the immobilization of proteins, and we compare the results with those obtained using polystyrene surfaces in ELISA and SPR sensor chips.

Characterization of the modified PAA

NMR characterization

^{1}H NMR analysis of the modified PAA was undertaken to confirm whether the PAA has been successfully modified. According to ChemDraw predictions, the modified PAA should have characteristic peaks at around 2.64 to 2.75 ppm that are attributed to the protons from the attached NHS. These peaks are absent in the unmodified PAA. The NMR shown in Fig. 1 indicates a comparison of ^{1}H NMR spectra of the unmodified and modified PAAs. The spectrum shows the presence of amine protons at around 10.42 ppm and aromatic protons between 6.5 and 8.5 ppm for both modified and unmodified PAAs (Table 1). As predicted, the results show two peaks at around 2.6 and 2.75 ppm. These peaks were absent in the unmodified PAA, suggesting that the NHS had been attached. Ideally, the protons in the NHS are at the same chemical environment and should appear as one peak. If the carboxylic groups of the PAA are modified, the attached NHS should shift to a different chemical environment. As predicted, Fig. 1 shows the presence of two peaks for the protons, which could indicate that both carboxylic groups in the PAA were modified. This could lead to an added advantage in that more antibodies will be immobilized to increase the antigen binding sites. These results indicate that the modification of the carboxylic groups of the PAA using EDC/NHS chemistry was successful.

FTIR characterization

The FTIR spectra of the modified and unmodified PAAs were recorded over the range of 4000 to 450 cm^{-1}. The absorption bands that occur at around 3274 cm^{-1} (broad) and 1642, 1602, and 1377 cm^{-1} indicate the presence of the amide group, whereas the bands occurring at around 2610 cm^{-1} (broad) and 1716 cm^{-1} can be assigned to the vibrational modes of carboxylic acid. A spike appearing at 3044 shows the existence of NH. The strong peak at around 1100 cm^{-1} is associated with a stretching vibration of the ether group. The spectrum of the unmodified PAA was very much in agreement with published results [5], with peaks observed at 1659, 1549, and 1407 cm^{-1} due to ν(CO-NH) stretching band and peaks observed at 1714 cm^{-1} and 1218 cm^{-1} due to due to ν(COOH) and ν(C–O–C) stretching bands. The peak for ν(N–H) was observed at 2955 cm^{-1}.

Electrochemical characterization

CV characterization

Relative to an unmodified Au electrode, the effect of PAA and subsequent modification with EDC/NHS on the surface of the electrode was studied using the redox activity of K_3FeCN_6 in 0.1 M KCl. PAA was electrodeposited onto Au solid electrode by using 50 cycles from −500 to 1000 mV at a 50-mV/s scan rate. The resulting PAA film on the surface of the electrode was then characterized in 2 mM K_3FeCN_6 in 0.1 M KCl at different scan rates (0.01–0.1 V/s). The oxidation and reduction peaks increased with the scan rates, indicating a diffusion-controlled reaction [21,22].

Fig. 2A shows the cyclic voltammograms of bare Au electrode in 2 mM $K_3Fe(CN)_6$. The PAA-coated Au electrode is shown in Fig. 2B, whereas the PAA–EDC/NHS-coated electrode is shown in Fig. 2C and finally the PAA–EDC/NHS–Ab (antibody)-coated electrode is shown in Fig. 2D. The cyclic voltammograms from the bare electrode show anodic and cathodic peaks of a near reversible redox

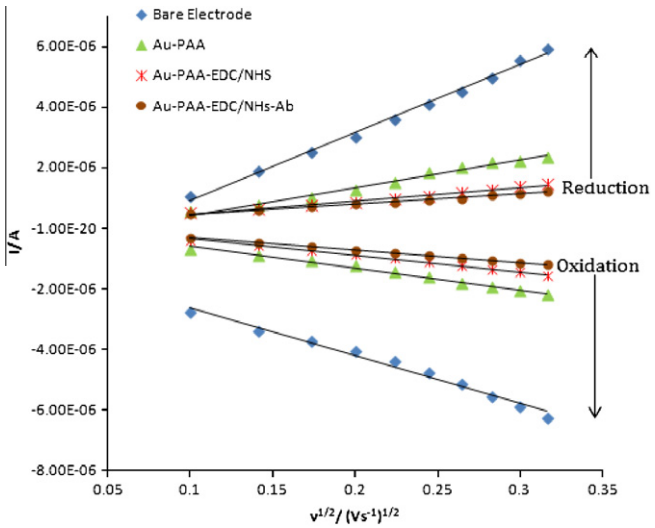


Fig.3. Plot of the I_p against the square root of the scan rates used to obtain diffusion coefficients from the slope for both anodic and cathodic processes. Scan rates between 0.01 and 0.1 V; area of Au electrode: 0.0201 cm^2; number of electrons: 1; concentration of the redox species: 2×10^{-6} mol/cm^3.

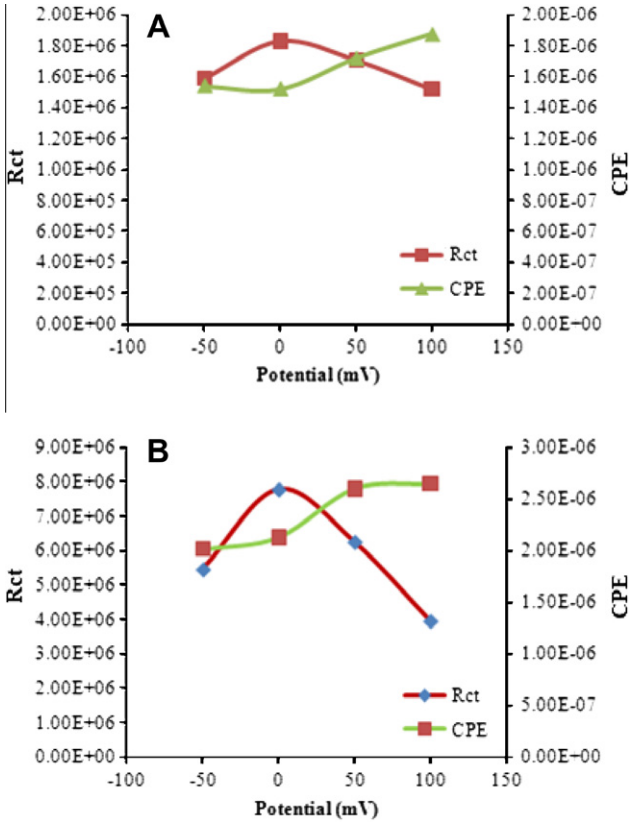


Fig.4. Plot of the interfacial capacitance (CPE) and charge transfer resistance (Rct) against potential: (A) unmodified PAA; (B) modified PAA.

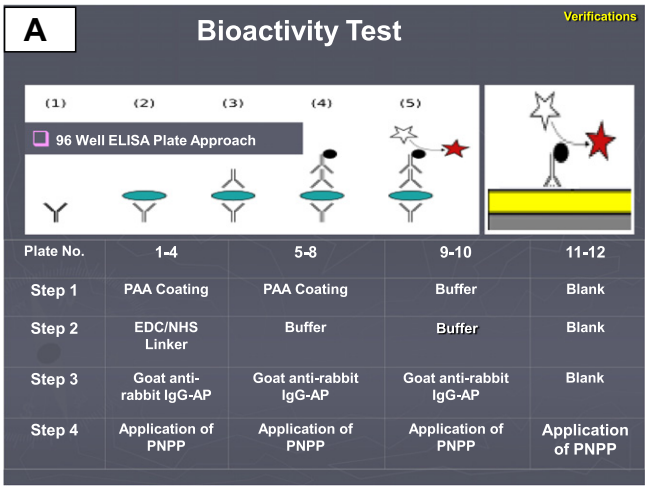


Plate No.	1-4	5-8	9-10	11-12
Step 1	PAA Coating	PAA Coating	Buffer	Blank
Step 2	EDC/NHS Linker	Buffer	Buffer	Blank
Step 3	Goat anti-rabbit IgG-AP	Goat anti-rabbit IgG-AP	Goat anti-rabbit IgG-AP	Blank
Step 4	Application of PNPP	Application of PNPP	Application of PNPP	Application of PNPP

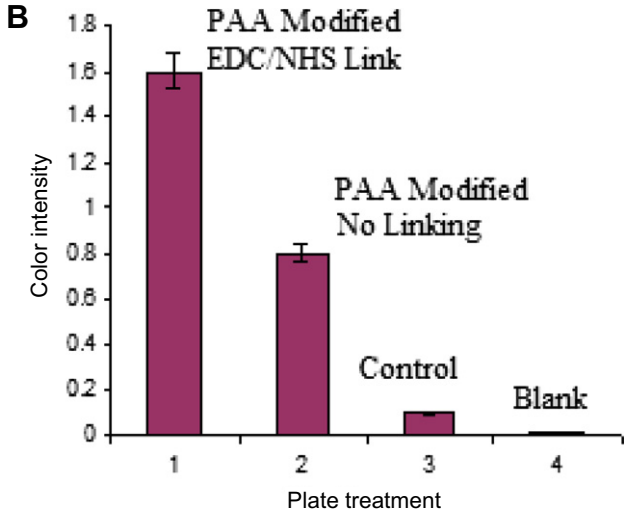


Fig.5. (A) Schematic showing the ELISA steps and how the experiment was carried out. (B) Results for the sandwich ELISA showing the bioactivity data for modified and unmodified PAA surfaces.

electrode reaction due to the redox activity of ferricyanide to ferrocyanide and vice versa. However, when the PAA (Fig. 2B) was electrodeposited, the peak currents were found to have decreased, presumably because the low conductivity of the polymer increased the resistance of the electrode by limiting the electron transfer and, hence, the current was decreased [23]. Additional current decrease was recorded on the introduction of the EDC/NHS and the antibody, presumably due to the increased resistance by the EDC/NHS and the antibody. Electron neutrality within the electroactive conducting polymer film [24] was facilitated by the movement of ions between the polymer and the electrolyte solution, and the overall process was oxidation–reduction. Overall, the process includes electron transfer, electron and counter ion transport in the conducting polymer film, and diffusion redox–ion transfer (generally linear diffusion) at the film–electrolyte interface [25].

To further understand the kinetics of the electron transfer process, the effects of varying the scan rate (v) on the peak currents and peak potentials of all the electrodes were investigated. The diffusion coefficient of each electrode was calculated using the Randles–Sevcik equation:

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

where I_p is the peak current, A is the geometric surface area (0.0201 cm^2 for Au electrode), n is the number of electrons transferred during the redox reaction (1), C is the concentration of the redox species (2×10^{-6} mol/cm^3), D is the diffusion coefficient, and v is the scan rate.

A plot of the I_p against the square root of the scan rates (Fig. 3) was used to obtain diffusion coefficients from the slope for both anodic and cathodic processes. The diffusion coefficient for the bare electrode was calculated to be 3.96×10^{-6} cm^2/s, which was close to the reported value (7.26×10^{-6} cm^2/s) in the literature [26]. The diffusion coefficients were 6.35×10^{-7} cm^2/s for the PAA-coated electrode, 1.56×10^{-7} cm^2/s for the PAA–EDC/NHS-coated electrode, and 9.55×10^{-8} cm^2/s for PAA–EDC/NHS–Ab-coated electrode. As seen in the diffusion coefficient values, the decrease from the bare electrode to the antibody-coated electrode indicated some retardation of electron transfer within the bulk material of the PAA film.

Impedance characterization

Compared with other techniques, EIS is a widely used versatile technique for characterization of organic–inorganic coatings and self-assembled monolayers adsorbed on the surface of an electrode [27]. Electron neutrality within the electroactive conducting polymer film [24] is facilitated by the movement of ions between the polymer and the electrolyte solution and the overall process of oxidation–reduction. Overall, the process includes electron transfer at the electrode–film interface, electron and counterion transport in the conducting polymer film, and diffusion redox–ion transfer (generally linear diffusion) at the film–electrolyte interface [25]. The EIS results show that the interfacial capacitance increased from 1.72 to 2.6 μ F at 50 mV (Fig. 4). They also indicate that the overall impedance of the modified film was higher. There was a lowering of charge transfer that can be associated with the modified film in the oxidized state and can be attributed to the increased interfacial distance that the electron needs to traverse as

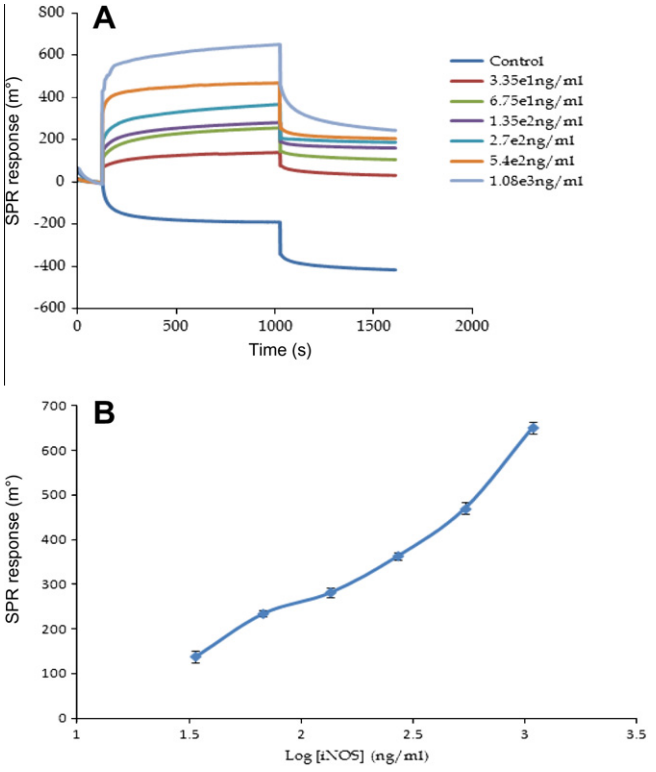


Fig.6. (A) Typical time profile of the SPR signal response for different concentrations of iNOS enzyme. (B) Standard calibration curve for the SPR immunosensor. A detection limit of 3.10×10^{-3} ng/ml was recorded based on 3 times the standard deviation of blank.

a result of the deposition of the PAA film on the surface of the electrode.

ELISA results

ELISA experiments were conducted to confirm that the immobilized antibodies retained bioactivity. ELISA is the commonly used method for examining the interaction between antibodies and the corresponding antigen. In this case, it should also provide evidence regarding the viability of the PAA functional groups to attach antibody on the surface of a well plate. To test any effect on the signal, an experiment was carried out without the modified PAA. Fig. 5 shows the results obtained and show an increase in the absorbance with increasing concentrations of the analyte for the modified and unmodified surfaces [28], indicating a biomolecular interaction of the enzymes with their respective antibodies. It was also noted that there was enhanced signal for the modified surface compared with the unmodified surface due to efficient orientation of the antibody; hence, this increased binding sites for the antigen. As predicted, these results indicate that the PAA-modified surface provides directed antibody immobilization as well as enhanced sensitivity.

SPR-based immunosensor for the detection of iNOS using the modified PAA

SPR is a surface-sensitive analytical technique based on the ability to detect small changes in the refractive index induced by molecular adsorption at a noble metal film [29,30]. This technique involves the excitation of surface plasmons using a light source at planar surfaces. It has been used extensively to characterize binding reactions without labeling requirements [31]. The apparent refractive index is determined by the mass and dielectric properties of the substances present on the sensor surface [32].

Scheme 2 shows the immobilization of the capture antibody (mouse anti-iNOS) on the SPR sensor based on the covalent attachment using the EDC/NHS chemistry. The control channel was subtracted from the signal of the sample channel. The sensor was exposed to different concentrations of iNOS enzyme as the detection antigen. Fig. 6A shows a typical time profile of the SPR signal for various iNOS enzyme concentrations after subtracting the signal from the control channel. Any possible nonspecific binding was eliminated by subtracting the signal from the control channel, thereby leaving only the specific binding of the analyte of interest. Based on the examined iNOS concentration range (3.375×101–1.08×103 ng/ml), there was an increase in the SPR signal with iNOS concentration. This increase is associated with the mouse anti-iNOS and the iNOS enzyme binding interaction on the sensor surface, as indicated by ELISAs. After the binding of the iNOS, the signal decreased due to the dissociation of the enzyme from the surface by the HCl regeneration solution. A control experiment where only buffer was used did not show any increase in the SPR signal [19] and served as a blank signal.

A standard calibration curve constructed from the SPR response signals was linear between 3.35×101 and 1.08×103 ng/ml

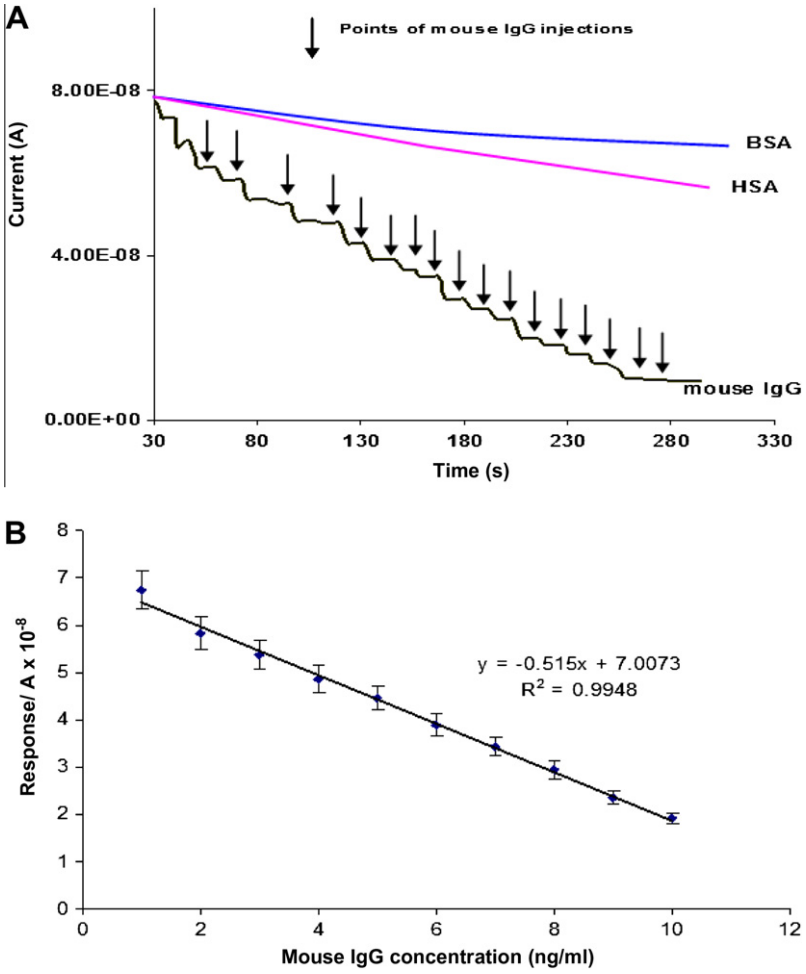


Fig.7. (A) Amperometric response of the biosensor to successive injection of 10 μl of mouse IgG at PAA-modified Au electrode surface as captured by goat anti-mouse IgG (10 mg/ml). Cross-reactivity tests using HSA and BSA were studied under similar experimental conditions. (B) Standard calibration curve of the biosensor with a detection limit of 10 ng/ml was recorded based on 3 times the standard deviation of blank.

(Fig. 6B), with a detection limit of $3.10 \times 10{-}3$ ng/ml calculated as 3 times the standard deviation of blank. These results indicate that the modified PAA can be used as a template for the immobilization of biomolecules; however, it was not found to be superior to the mercaptoundecanoic acid that is normally used in SPR experiments and was used in our previous experiments reported in literature [28]. This could be due to mercaptoundecanoic acid having thiol groups that allow direct attachment onto a gold surface, whereas the alkyl structure, once activated, facilitates attachment of the antibody while elevating it above the surface to enable better analyte access [33–35].

Amperometric-based sensor using modified PAA

The modified PAA electrode was tested using mouse IgG. A goat anti-mouse antibody immobilized on the modified PAA electrode was used to detect different concentrations of mouse IgG. Fig. 7A shows a well-defined anperometric response obtained after successful additions of 10 μg/ml mouse IgG to a continuously stirred phosphate buffer solution in a 25-ml electrochemical cell. The observed stepwise current decreases as the antigen concentration is increased, confirming that the antibody–antigen binding is specific. Selectivity using HSA and BSA shows no significant current decrease as a further confirmation that the current decrease observed with the mouse IgG is due to the binding to its antibody. Fig. 7B shows the calibration plot for the mouse IgG, with a detection limit of 10 ng/ml calculated based on 3 times the standard deviation of blank (signal before addition of the antigen). Thus, these results confirmed that the carboxylic ends of the PAA were successfully modified and subsequently used to immobilize biomolecules on a sensor surface.

Conclusion

In this work, we have demonstrated that the carboxylic end of the PAA can be modified using EDC/NHS chemistry and subsequently used to immobilize biomolecules on a sensor surface. The ^{1}H NMR results indicate that the modification of the PAA using EDC/NHS chemistry was successful. The effect of the PAA film and subsequent addition of the EDC/NHS and the antibody on the redox activity of ferricyanide solution was studied using CV and impedance spectroscopy. The results indicate that even though the PAA is conductive, it does limit the electron transfer on the surface of the electrode, as shown by the diffusion coefficients calculated using the Randles–Sevcik equation. The application of the site-directed immobilized antibodies on PAA template was tested using ELISA, SPR, and amperometric assays, and the recorded results were consistent with literature findings, thereby confirming that PAA could be modified and used as a template for biomolecule immobilization in biosensor development.

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