

Protein A immunosensor for the detection of immunoglobulin G by impedance spectroscopy

Burak Derkus · Kaan Cebesoy Emregul ·
Hidayet Mazi · Emel Emregul · Tugrul Yumak ·
Ali Sinag

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Abstract A novel highly sensitive electrochemical impedimetric Protein A immunosensor for the determination of immunoglobulin G (IgG) was developed by immobilization of Protein A within a newly synthesized, and characterized polymer, *poly(maleicanhydride-alt-decene-1)*. TiO₂ nanoparticles (10–30 nm) were synthesized, characterized with X-ray diffraction, transmission electron microscopy and Brunauer–Emmett–Teller surface analysis. The electron transfer between IgG and the *poly(maleicanhydride-alt-decene-1)*-TiO₂-Protein A is quasireversible with a formal potential of 225 mV vs Ag|AgCl. The response of the *poly(maleicanhydride-alt-decene-1)*-TiO₂-Protein A immunosensor was proportional to IgG concentration with a correlation coefficient of 0.9963. The detection limit and linear range was 0.57 ng mL⁻¹ and 0.0062–500 µg mL⁻¹, respectively. Impedance measurements showed that synthesized TiO₂ nanoparticles have better conducting properties compared with commercial degussa P25 TiO₂ nanoparticles. The nonspecific binding of anti-MBP was 10 %. The label-free impedimetric immunosensor provided a simple and sensitive detection method for the specific determination of IgG in human serum.

Keywords Poly(maleicanhydride-alt-decene-1) · TiO₂ nanoparticle · Immunoglobulin G · Impedance spectroscopy · Immunosensor

Introduction

Immunoglobulin G (IgG) which plays a major role in host defense against extracellular infections is primarily involved in immune memory. IgG comprises approximately 70–80 % of the total serum immunoglobulin content due to its high production rate and long half-life relative to the other immunoglobulin species [1]. Immunoglobulin therapy is the basis for treatment in the majority of primary immune deficiencies. Intravenous immunoglobulin is administered for various indications and generally considered a safe therapy. But the adverse effects including anaphylactoid reactions, renal, pulmonary, dermatological adverse effects, hyperviscosity, aseptic meningitis, arthritis, cerebral infarction, haemolysis and leucopenia and transmission of infectious agents can alternately be observed [2, 3]. To prevent the adverse effect, the serum IgG level must be monitored for clinical efficacy.

Immunosensors are based on antibody-antigen interactions and many studies have been reported for various applications in food industry, environmental monitoring, biotechnology, pharmaceutical chemistry and especially clinical diagnostics in previous years [4–7]. Modification agent plays an important role in the development of sensors. It acts both as an immunosensing interface and electrochemical surface, where a specific immunoreaction occurs. An effective immunosensing interface should be able to attach proteins without blocking their biological functions. Numerous strategies have been developed to

B. Derkus · K. Cebesoy Emregul · E. Emregul (✉) ·
T. Yumak · A. Sinag
Department of Chemistry, Science Faculty, Ankara University,
Tandoğan, Ankara 06100, Turkey
e-mail: eemregul@yahoo.com

H. Mazi
Department of Chemistry, Science Faculty,
Gaziantep University, Gaziantep, Turkey

immobilize antibodies for immunosensor design, such as direct physisorption [8], covalent binding [9, 10] and affinity binding via the biotin/avidin interaction [11].

S. aureus Protein A (PrA) binds specifically to the Fc region of immunoglobulin molecules, especially IgG [12]. Several examples of immunosensing devices based on the immobilization of PrA have been reported. The benefits are a proper orientation of the bound antibody on surfaces [13] and easy immunosensor regeneration [14]. Rönmark et. al. [15] describe the use of the affibody technology platform for the identification of PrA-derived variants showing IgA- rather than IgG-binding, thus expanding the available repertoire of tools for detection and recovery of native or recombinant IgA from different sources. Zengin and Caykara [16] were fabricated a multilayer system consisting of 3-glycidoxypropyldimethylmethoxysilane (GPDS), poly(dimethylsiloxane) bis 3-aminopropyl terminated (PDMS) and PrA on a silicon wafer surface for oriented immobilization of IgG. It was shown that the existence of PrA in the multilayer system had a strong influence on the binding properties of IgG not only in the efficiency of binding, but also in its specificity. Cao et.al [17] immobilized Staphylococcal PrA on magnetic cellulose microspheres for immunological capture of IgG and prepared affinity media for the purification of IgGs. They purified rabbit IgG from serum with an overall recovery of 73.18 % and purity of 90.27 %. Feng et.al. [18] presented a method for oriented antibody immobilization based on the formation of an orderly, organized aggregation of IgG and staphylococcal PrA. Following the chelation of His-tag with Ni^{2+} , antibodies were immobilized on a solid surface in a three-dimensional (3D) manner and exposed the analyte receptor sites well, which resulted in an enhancement of the signal. Protein A was used as a binding material to immobilize antibodies onto gold-derivatized transducers for the construction of a well-defined antibody surface [19]. They tried to indirectly bind PrA onto sensors through hyperbranched polymer. The fabricated quartz crystal microbalance immunosensor was applied to detect hepatitis B surface antigen in solutions that ranged from 0.71 to 300 $\mu\text{g mL}^{-1}$.

Electrochemical impedance spectroscopy (EIS) is a powerful, nondestructive and informative technique, which can be used to study the electrical properties of almost any sensing device interface [20]. In recent years, the potential application of this technique to the immunosensors for a broad set of applications has been examined [21–24]. EIS is capable of investigating electrode processes and determining diffusion kinetics as well as mass-transport parameters [25]. The electrochemical complex impedance (Z) can be represented as a sum of the real (Z_{real}) and imaginary (Z_{imag}) components that originate generally from the resistance and capacitance of an electrolytic cell,

respectively. Bode plot and Nyquist plot are the commonly used two types of impedance graphs. While semi-circle part of the nyquist plot gives information about capacitance and resistance, linear part shows diffusive effects.

Copolymers which have newly functional groups, can be used in any further incorporation of drugs or other bioactive agents [26]. Covalent linkages between polymer chains can be established by the reaction of functional groups with complementary reactivity, such as an amine, carboxylic acid or an isocyanate OH/NH_2 reaction [27]. The copolymers of maleic acid and its derivatives have a wide range of applications in various fields, including biomaterials and drug delivery systems [28]. Maleic anhydride (MA) copolymers are known for their biomedical applications as drugs and as drug or enzyme carriers [29]. The alternating copolymers of MA can be regarded as pre-activated polymers due to the presence of anhydride moieties susceptible to the reaction with a primary amine of a biomolecule [30]. The balance between repulsion and hydrophobic interactions is sensitive to environmental pH and temperature, and therefore variation of these parameters produce controllable conformational changes [31].

In recent years, nanomaterials have attracted extensive interest for their unique properties in various fields (such as catalytic, electronic, and magnetic properties) in comparison with their bulk counterparts. Oxide nanoparticles are often used to immobilize biomolecules due to their biocompatibility, while semiconductor nanoparticles are often used as labels or tracers for electrochemical analysis [32]. TiO_2 nanoparticles, the best known semiconductor with its 3.2 eV band gap energy, were used for several electrochemical applications [33–35]. Nanostructured TiO_2 is an ideal sensing material as it is usually easy to fabricate, has a large specific surface area, good biocompatibility, good chemical and photochemical stabilities and negligible protein denaturation [36].

IgG which is present in all body fluids, is the most abundant type of antibody. Based on the level of IgG present in the body, physicians are able to draw conclusions about the functioning of the immune system and related infections or autoimmune diseases. Administration of intravenous immunoglobulin has various indications and generally considered as a safe therapy. To prevent the adverse effect of excess IgG, the serum IgG level must be monitored for clinical efficacy. The ultimate objective of this study is to design an immunosensor which is capable of satisfying the desired properties. For this purpose, a new alternating copolymer of MA with 1-decene was synthesized and characterized with Fourier Transform Infrared Spectroscopy (FT-IR) for the development of an immunosensor. Protein A was immobilized onto poly(maleicanhydride-alt-decene-1), *poly(MA-alt-D-1)*, using N-hydroxysuccinimide (NHS) crosslinker. For a detailed

characterization *poly(MA-alt-D-I)*, crosslinker, PrA and TiO_2 concentrations were optimized using EIS and cyclic voltammetry (CV). Nanoparticles were used for the immunosensor development to benefit from high surface area and enhance the electron transfer rate. To this end, TiO_2 nanoparticles were synthesized and characterized with TEM, XRD, and BET and used for the development of *poly(MA-alt-D-I)*- TiO_2 -PrA immunosensor. The surface morphologies of the *poly(MA-alt-D-I)*, *poly(MA-alt-D-I)*-PrA, *poly(MA-alt-D-I)*- TiO_2 , *poly(MA-alt-D-I)*- TiO_2 -PrA films were investigated with SEM.

Experimental

Materials

Staphylococcal Protein A, human IgG from serum, human IgG ELISA kit, bovine serum albumin, NHS, potassium ferrocyanide, and potassium ferricyanide were purchased from Sigma (St Louis, MO, USA). Sodium dihydrogen orthophosphate, disodium hydrogen orthophosphate, sodium chloride were obtained from Merck. De-ionized water was purified using a MilliPore Simplicity unit to a resistivity $\geq 18.2 \text{ M}\Omega\text{cm}$. Electrochemical measurements were carried out with Gamry Instrument using Framework Version 5.50 software.

Titanium isopropoxide (TTIP), isopropyl alcohol (IPA), and Evonik Degussa 25-P commercial TiO_2 were obtained from Sigma–Aldrich and used without further purification.

MA monomer (Aldrich, Germany) was purified before recrystallization and sublimation under vacuum. Decene-1 (Aldrich, Germany) comonomer was distilled under moderate vacuum before use. Benzoyl peroxide (BPO; Merck) was recrystallized twice in chloroform solution. The solvents (1,4-dioxane, methanol, chloroform, and diethyl ether) were all analytical grade (Sigma–Aldrich).

Electrolysis cell and electrodes

A three-electrode electrochemical cell comprised of a working, counter, and reference electrode was used for all experiments. The working and counter electrodes which have 1 cm^2 surface area were platinum and the reference electrode Ag/AgCl was introduced into the cell via Luggin capillary. The surface of the working electrode was polished using $0.05 \mu\text{m}$ alumina slurry prior to each experiment.

Synthesis of copolymer

Alternating copolymerization of MA with 1-decene was carried out in 1,4-dioxane in nitrogen atmosphere with BPO

as a radical initiator at 70°C to conversions of 10–12 %. The copolymers produced were precipitated by diethyl ether and purified twice by reprecipitation from the 1,4-dioxane solution into diethyl ether and by washing with n-hexane. Purified copolymers were then dried in vacuum at 40°C to a constant weight. Copolymers were synthesized in the given reaction conditions: $[\text{M}]_{\text{total}} = 3.0 \text{ mol/L}$, monomer molar ratio 50:50, $[\text{BPO}] = 6.7 \times 10^{-3} \text{ mol/L}$, 70°C .

Synthesis of TiO_2 nanoparticles

The hydrothermal synthesis of TiO_2 nanoparticles was performed in an autoclave. The autoclave has an internal volume of 75 mL and the temperature can be controlled by an external apparatus. The reactor was loaded with appropriate amount of TTIP and IPA, and the autoclave was put on a heater and temperature kept constantly at 150°C for 6 h. Then, the autoclave was cooled to room temperature. The product was washed with distilled water and ethanol and centrifuged. The obtained product was dried in an oven at 70°C for 4 h. The particles were characterized by XRD, TEM and BET.

The XRD analysis of TiO_2 nanoparticles were carried out on a Rigaku D/Max–2200 ULTIMAN X-ray diffractometer using $\text{Cu K}\alpha$ radiation $\lambda = 1.5418$. TEM micrograph belongs to TiO_2 nanoparticles were taken by an FEI Tecnai G2 (at 200 kV) transmission electron microscope. The BET analyses of the samples were taken by Quantachrome Corporation, Autosorb-6.

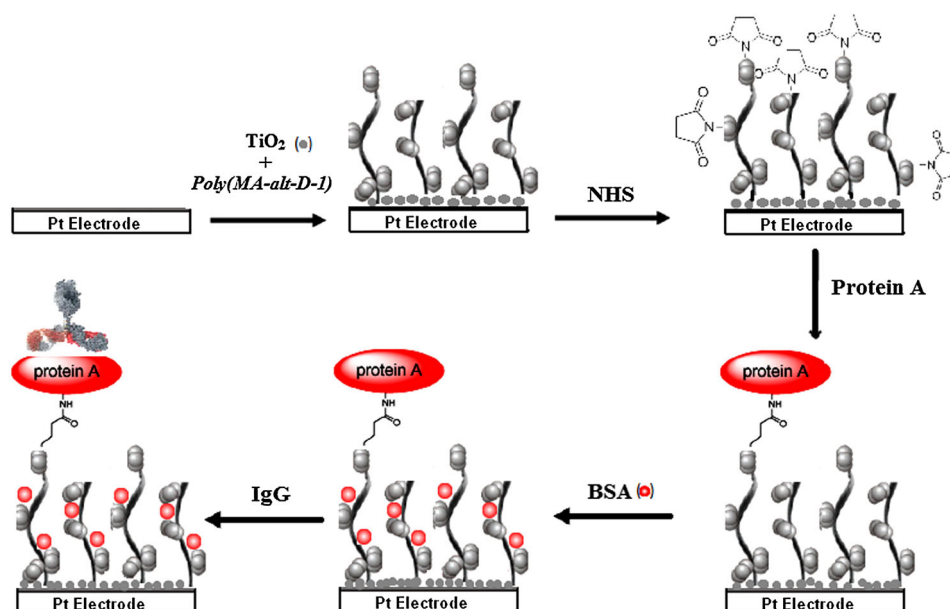
Preparation of Protein A immunosensor

Stock solution of *poly(MA-alt-D-I)* was prepared by dissolving polymer (0.002 g) in 1 mL 1,4-dioxane followed by addition of TiO_2 (0.0002 g) to the polymer solution. Each surface of the electrode was covered with polymer- TiO_2 solution (75 μL) and let to dry for 30 min. The polymer coated electrode was washed with PB (0.05 M, pH 7.4), then 25 μL of PB (0.05 M, pH 7.4) containing PrA (0.1 mM), and NHS (3.5 mM) was added to each electrode surface. The PrA electrode was left at room temperature for 2 h. After treated with 1 % BSA solution to prevent non specific binding, the electrode was washed with PB (0.05 M, pH 7.4). The stepwise fabrication process of the immunosensor is schematically illustrated in Scheme 1.

Measurements

FT-IR measurements of MA, 1-decene, and *poly(MA-alt-D-I)* and FT-IR, XRD, TEM, and BET analyses of TiO_2 were performed for characterization. The surface morphologies of the *poly(MA-alt-D-I)*, *poly(MA-alt-D-I)*- TiO_2 , *poly(MA-alt-D-I)*- TiO_2 -PrA, and *poly(MA-alt-D-I)*- TiO_2 -PrA

Scheme 1 Schematic illustration of the immobilization of PrA on *poly(MA-alt-D-I)*-TiO₂ modified Pt electrode using NHS as coupling agents



films were investigated with SEM. Different concentration of IgG was dropped on *poly(MA-alt-D-I)*-TiO₂-PrA electrode surface after washing the immunosensor carefully with 0.05 M pH 7.4 PBS and incubated at 37 °C for 30 min. Following a rinse with PBS (0.05 M, pH 7.4), the electrochemical measurements were carried out in PBS (0.05 M, pH 7.4) containing 0.1 M KCl, 5 mM K₃[Fe(CN)₆]/K₄[Fe(CN)₆], pH 7.4.

The principle of detection for *poly(MA-alt-D-I)*-TiO₂-PrA electrode lies in the changes in impedance spectra of the redox probe after the attachment of IgG to the immobilized PrA. The EIS measurements were recorded within the frequency range of 0.01 Hz to 100 kHz at 220 mV open circuit potential (Eoc). All the experiments for the development of immunosensor were performed three times using EIS and CV.

Determination of nonspecific binding

The nonspecific binding was also studied due to its importance for the application in immunosensors. For this purpose, anti-myelin basic protein (anti-MBP) was tested. Different concentrations of anti-MBP were used to determine the nonspecific binding and show the specificity of the developed immunosensor.

Application of the immunosensor in real samples

The *poly(MA-alt-D-I)*-TiO₂-Protein A immunosensors were applied to CSF and serum to evaluate the appropriateness of immunosensors in real samples. Serum samples were centrifuged for 5 min with 3,000 rpm prior to use. The utilization of the immunosensors for the analysis of real

samples was evaluated by analysis of IgG in the blank (artificial CSF), control samples (artificial CSF containing IgG and artificial serum containing IgG), CSF and serum of relapsing–remitting MS patients. Measurements were carried out following the same procedure as in Sect. 2.7. The determination of IgG in CSF and serum was also performed with ELISA.

Results and discussion

Characterization of *poly(MA-alt-D-I)*

FT-IR analysis

The MA, 1-decene and, *poly(MA-alt-D-I)* were characterized by FT-IR (Fig. 1). The absorption bands of MA are shown in Fig. 1a. The absorption bands at 3,122, 3,058, 1,774, 1,631 and 1,056 cm⁻¹ are assigned to asymmetrical C–H stretching vibration (CH₂ = CH), symmetrical C–H stretching vibration (CH₂ = CH), C = O stretching band, C = C stretching band and, C–O–C a symmetrical stretching band, respectively. The absorption bands of 1-Decene are shown in Fig. 1b. The absorption bands at 3,078, 2,957, 2,923, 2,854, 1,641, 1,465 and 1,378 cm⁻¹ are assigned to an asymmetrical C–H band (CH₂ = CH), a C–H symmetrical band, a symmetrical C–H stretching band, a C = C stretching band, an asymmetrical C–H bending band and, a symmetrical C–H bending band, respectively. The absorption bands of *poly(MA-alt-D-I)* are illustrated in Fig. 1c. The absorption bands between 2,855 and 2,925 cm⁻¹ are assigned to symmetrical and asymmetrical C–H stretching vibration of CH (MA unit), CH,

CH₂ and CH₃ (D-1 unit); absorption bands at 1,776 and 1,731 cm⁻¹ are assigned to symmetrical and asymmetrical C = O stretching vibration of MA unit and band at 1,706 cm⁻¹ assigned to C = O stretching vibration of hydrolyzed MA unit, respectively. The absorption bands at 1,081 and 1,440 cm⁻¹ are assigned to C–O–C stretching vibration of MA and scissor vibrations of CH₂ groups of

D-1 units, respectively [37–39]. The observed other bands are 1,216 cm⁻¹ (CH₃ bending of D-1) and 923 cm⁻¹ (CH₃ rocking in D-1). The morphology of the polymer layers was not studied in detail but the obtained values for the constant of complex formation (K_c)(0,15) and the monomer reactivity ratios (r_1 and r_2) (0.047 ± 0.05) indicate a donor–acceptor type of alternating copolymerization for the complexed MA-H-1 pair.

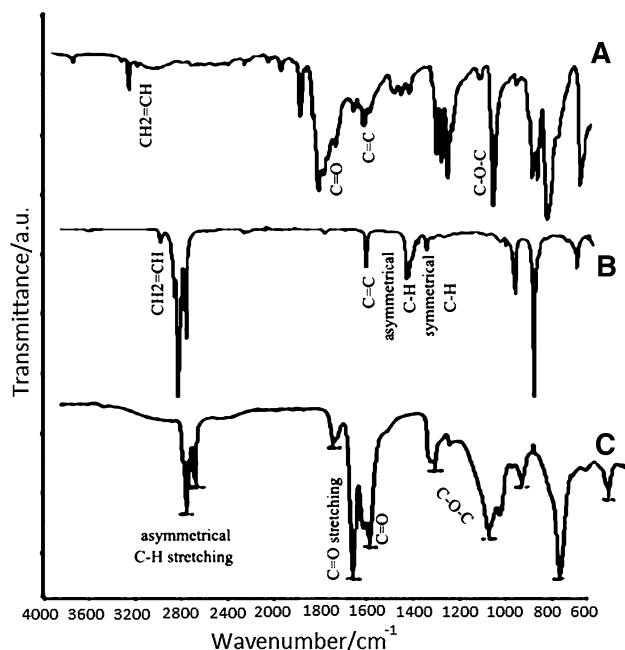
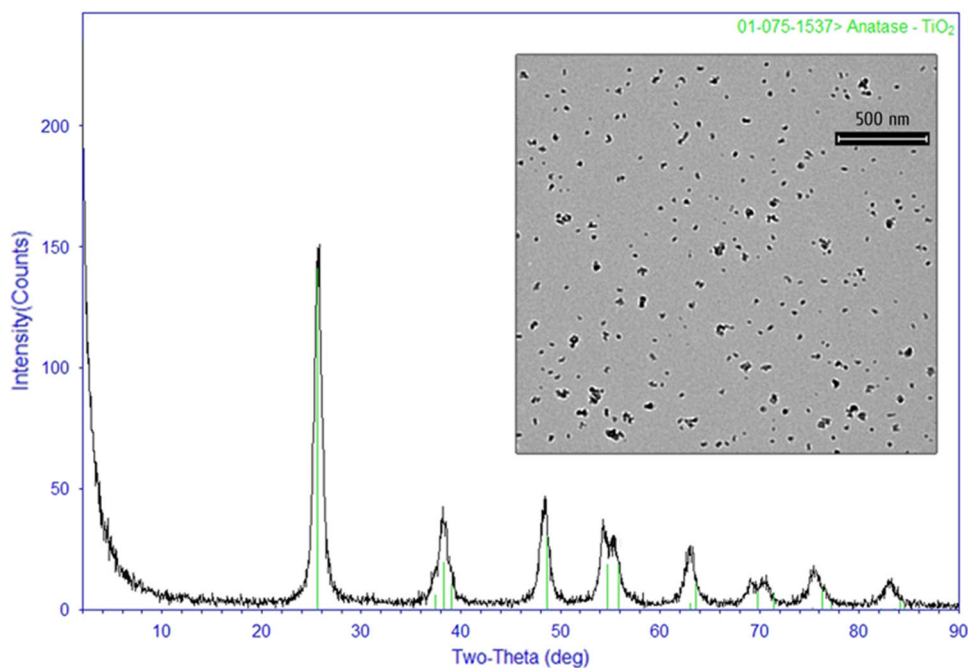


Fig. 1 FT-IR spectrums of: Maleic Anhydride (a), 1-Decene (b), poly(MA-alt-D-1) (c)

Fig. 2 XRD pattern of synthesized nanoparticles. Inset shows TEM image of TiO₂ nanoparticle



Characterization of TiO₂ nanoparticles

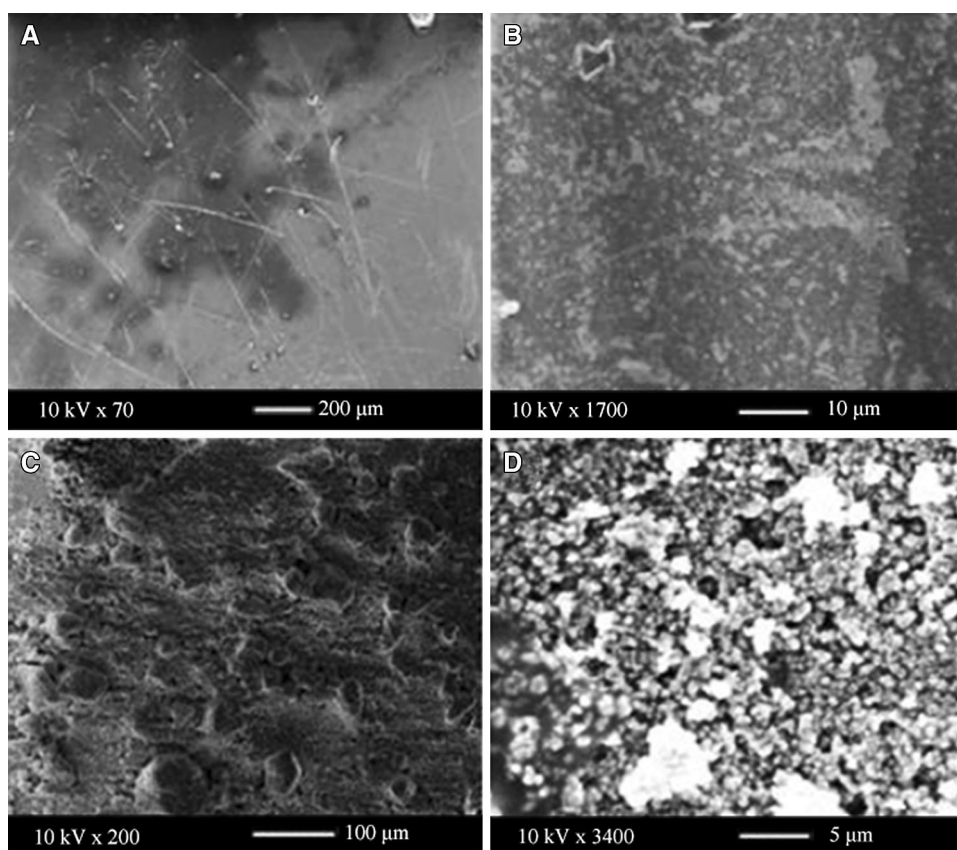
XRD measurements

Figure 2 shows the XRD pattern of synthesized TiO₂ nanoparticles. Phases were identified using the library - Joint Committee on Powder Diffraction Standards (JPCDS) database for anatase (JPCDS No. 71-1167) and rutile (JPCDS No. 76-1941). Compared to Degussa P25, which contains anatase and rutile phases, the XRD patterns of the synthesized TiO₂ samples have revealed only anatase phase. The crystalline size of the TiO₂ samples can be calculated from the width of the XRD peak using the Scherrer equation.

$$D_{101} = \frac{0.89\lambda}{\beta \cos \theta} \quad (1)$$

The strongest peak at $2\theta = 25.3^\circ$ (corresponding to the {101} plane) was used to calculate the average crystallite size of the samples [40, 41]. The average crystallite size of P25 is given as ~ 25 nm by Degussa, and the average crystallite size of hydrothermally synthesized TiO₂ nanoparticles is 10 nm.

Fig. 3 SEM images of immunosensor: *poly(MA-alt-D-I)* (a), *poly(MA-alt-D-I)*-PrA (b), *poly(MA-alt-D-I)*-TiO₂ (c), *poly(MA-alt-D-I)*-TiO₂-PrA (d) at 10 kV



TEM analysis

TEM image of synthesized TiO₂ nanoparticles is shown in inset of Fig. 2. As seen in the image, the TiO₂ nanoparticles are of spherical shape between 10 and 30 nm with a mean value at 15 nm and softly agglomerated by van der Waals forces [42].

BET analysis

BET surface areas of the synthesized TiO₂ nanoparticles and Evonik Degussa P-25 were investigated. BET surface areas of synthesized TiO₂ nanoparticles and Evonik Degussa P-25 were 244, 50 m²g⁻¹, respectively. The synthesized TiO₂ nanoparticles have larger surface area compare to Evonik Degussa P-25.

Optimization and characterization of Protein A immunosensor

Surface characterization with SEM

The scanning electron microscope (SEM) images of *poly(MA-alt-D-I)*, *poly(MA-alt-D-I)*-PrA, *poly(MA-alt-D-I)*-TiO₂, *poly(MA-alt-D-I)*-TiO₂-PrA were characterized by SEM studies (Fig. 3). The image of the *poly(MA-alt-D-I)*

shows that the *poly(MA-alt-D-I)* shows a straight surface morphology (Fig. 3a). When PrA were assembled onto the films of *poly(MA-alt-D-I)* polymer (Fig. 3b), the straight structure of the *poly(MA-alt-D-I)* became blurry, indicating that PrA biomolecules were immobilized successfully on the surface of electrodes. Fig. 3c displays the immobilization of TiO₂. The enhancement of surface area after the seeding of TiO₂ nanoparticles to the polymer, increased the PrA immobilization as seen in Fig. 3d.

Optimization of immunosensor

Different parameters affecting the immunosensor performance namely *poly(MA-alt-D-I)* matrix (0.05–0.5 mg), NHS crosslinker concentration (0.875–20 mM), TiO₂ nanoparticle (0.01–0.05 mg) and PrA concentration (0.01–0.20 mM) during the fabrication process of immunosensor were studied. The obtained optimum values for *poly(MA-alt-D-I)* matrix, NHS concentration, TiO₂ nanoparticle, PrA concentration were 0.30 mg (Fig. 4a), 3.5 mM (Fig. 4b), 0.02 mg (Fig. 4c), and 0.1 mM (Fig. 4d), respectively. The incubation time (1–60 min) was also studied. The optimum incubation time was found as 30 min. The optimization experiments for each parameter were performed using both EIS and CV.

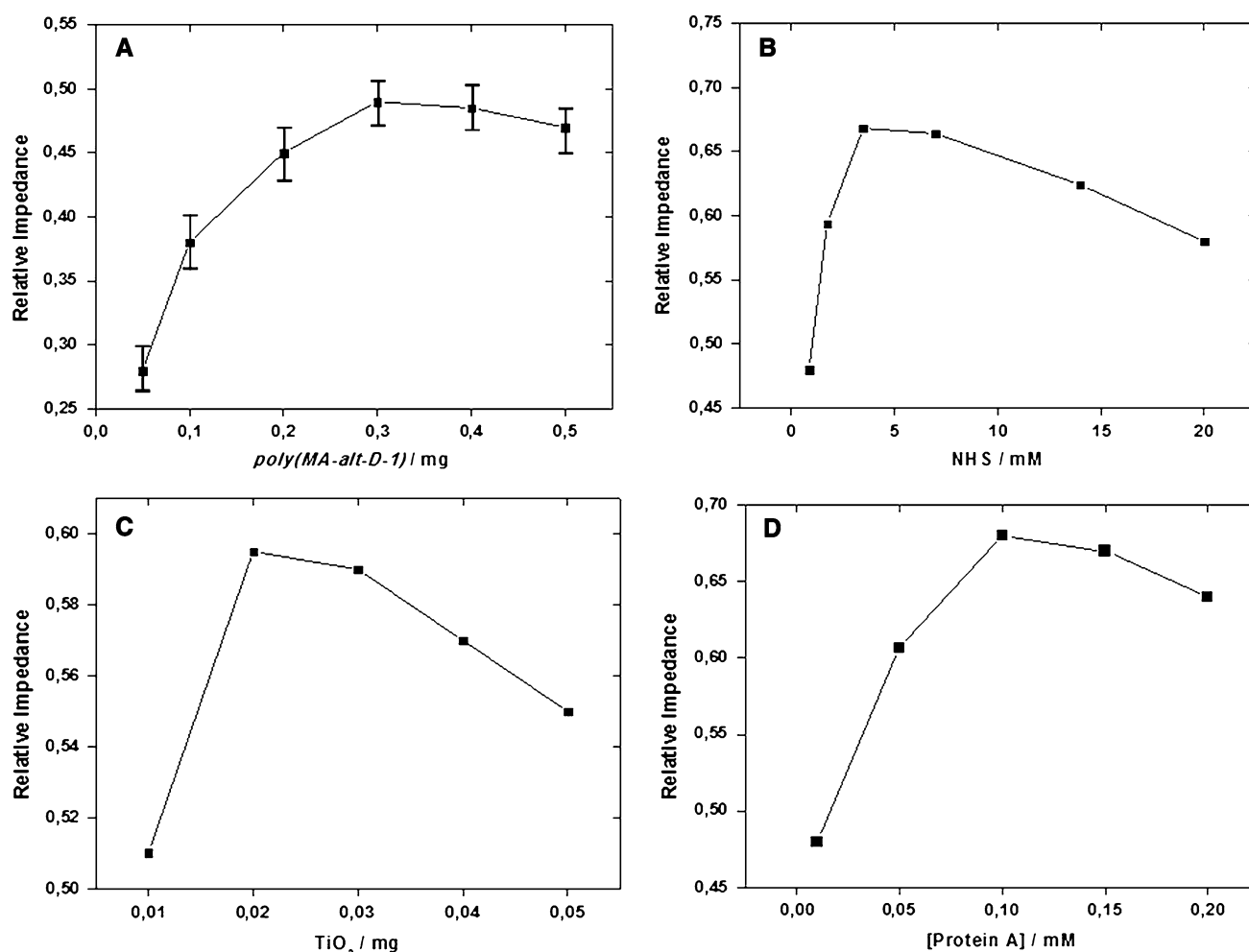


Fig. 4 Effect of **a** Poly(MA-alt-D-I) concentration, **b** NHS concentration, **c** TiO_2 concentration, and **d** PrA concentration on poly(MA-alt-D-I) - TiO_2 -PrA immunosensor

Cyclic voltammetry

Cyclic voltammetry of electroactive species, $\text{Fe(CN)}_6^{4-/3-}$, is an available tool for evaluating the kinetic barrier of the interface [43, 44]. Fig. 5a shows the CV graphs of $\text{Fe(CN)}_6^{4-/3-}$ at bare Pt electrode, poly(MA-alt-D-I) modified electrode, poly(MA-alt-D-I) - TiO_2 modified electrode, poly(MA-alt-D-I) - TiO_2 -PrA electrode, and IgG incubated poly(MA-alt-D-I) - TiO_2 -PrA electrode. The formal potential ($E^0 = (E_{p,a} + E_{p,c})/2$) of PrA where $E_{p,a}$ and $E_{p,c}$ are the anodic and cathodic peak potentials, respectively, confined on poly(MA-alt-D-I) - TiO_2 -PrA surface is estimated to be 225 mV vs Ag/AgCl. The peak separation (ΔE_p , defined as $\Delta E_p = E_{p,a} - E_{p,c}$) of PrA (149 mV at 100 mV s^{-1}) and the asymmetric anodic and cathodic peak currents indicate that the electron transfer between IgG and the poly(MA-alt-D-I) - TiO_2 -PrA is quasireversible [45–47]. After modifying the electrode with poly(MA-alt-D-I) , the peak belonging to $\text{Fe(CN)}_6^{4-/3-}$ redox probe disappears.

Poly(MA-alt-D-I) film layer acts as the inert electron transfer blocking layer, and thus prevents the diffusion of $\text{Fe(CN)}_6^{4-/3-}$ to the electrode surface. On the contrary, when TiO_2 nanoparticles were seeded to the electrode surface, the voltammetric peak of $\text{Fe(CN)}_6^{4-/3-}$ was increased. This is originated from high surface area of nanoparticles which increases and promotes the electron transfer rate [48]. The nanoparticles tend to act like nanoscale electrodes thus promoting the electron transfer between the analyte and the electrode surface. Immobilization of PrA decreased the current response. The immobilization of PrA on the electrode surface creates extra resistance and thus decrease electron transfer of $\text{Fe(CN)}_6^{4-/3-}$.

Electrochemical impedance spectroscopy

EIS is an effective technique for probing the features of surface-modified electrodes. The impedance curve include a semicircle part and a linear part. The semicircle diameter

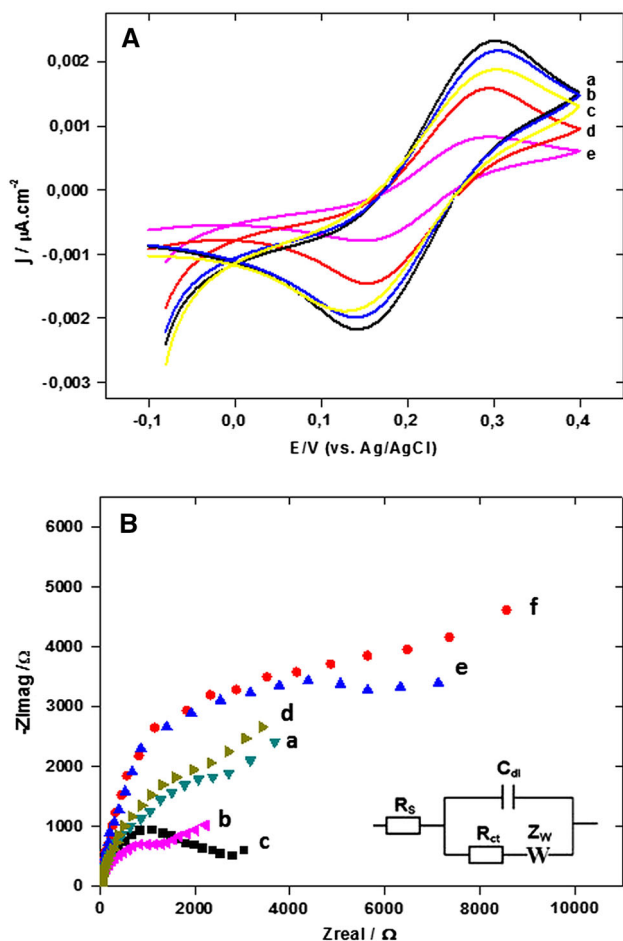


Fig. 5 **A** Cyclic voltammograms of: *poly(MA-alt-D-I)*-TiO₂ electrode (**a**), Bare Pt electrode (**b**), *Poly(MA-alt-D-I)*-TiO₂-PrA electrode (**c**), IgG incubated (150 μg mL⁻¹) *poly(MA-alt-D-I)*-TiO₂-PrA electrode (**d**), *poly(MA-alt-D-I)* modified electrode (**e**). **B** Nyquist diagrams of: bare Pt electrode (**a**), *poly(MA-alt-D-I)* modified electrode (**d**), *poly(MA-alt-D-I)*-TiO₂ electrode (**b**), *poly(MA-alt-D-I)*-Dequssa electrode (**c**), *poly(MA-alt-D-I)*-TiO₂-PrA electrode (**e**), IgG incubated *Poly(MA-alt-D-I)*-TiO₂-PrA electrode (**f**) Solution composition: 0.1 M KCl, 5 mM K₃[Fe(CN)₆]/K₄[Fe(CN)₆], pH 7.4. The frequency range 0.01 Hz–100 kHz at 220 mV open circuit potential (*E*_{oc}), Scan rates 100 mVs⁻¹

at higher frequencies corresponds to the charge transfer resistance (*R*_{ct}), and the linear part at lower frequencies corresponds to the diffusion process.

Nyquist plot was obtained and used to determined relative change in surface-charge resistance. Interfacial *R*_{CT} in Nyquist plot of impedance was obtained from real (*Z'*) and imaginary (*-Z''*) impedance at different frequencies using the following equations [Bard and Faulkner (2000)] for a parallel RC circuit.

$$Z(\omega) = Z' + jZ'' = R_s + \frac{R_p}{(1 + j\omega C_d)} \quad (2)$$

$$Z' = R_s + \frac{R_p}{(1 + \omega^2 R_p^2 C_d^2)} \quad (3)$$

$$-Z'' = \frac{\omega R_p^2 C_d}{(1 + \omega^2 R_p^2 C_d^2)} \quad (4)$$

where, *R*_s was the electrolyte solution resistance and *R*_p was polarization resistance. *R*_p obtained at zero potential was described as surface-charge resistance (*R*_{CT}). The frequency associated with maximum *Z''* and *R*_{CT} were used to calculate *C*_d using the following equation.

$$R_{cd} C_d = \frac{1}{2\pi f_{\max}} \quad (5)$$

Warburg resistance (*Z*_w) was also obtained in Nyquist plot and an equivalent circuit to describe the electrical response at electrode labeled in Fig. 4b. Warburg impedance was expressed by an intercept of straight line having a slope of unity and can be derived from the following equation.

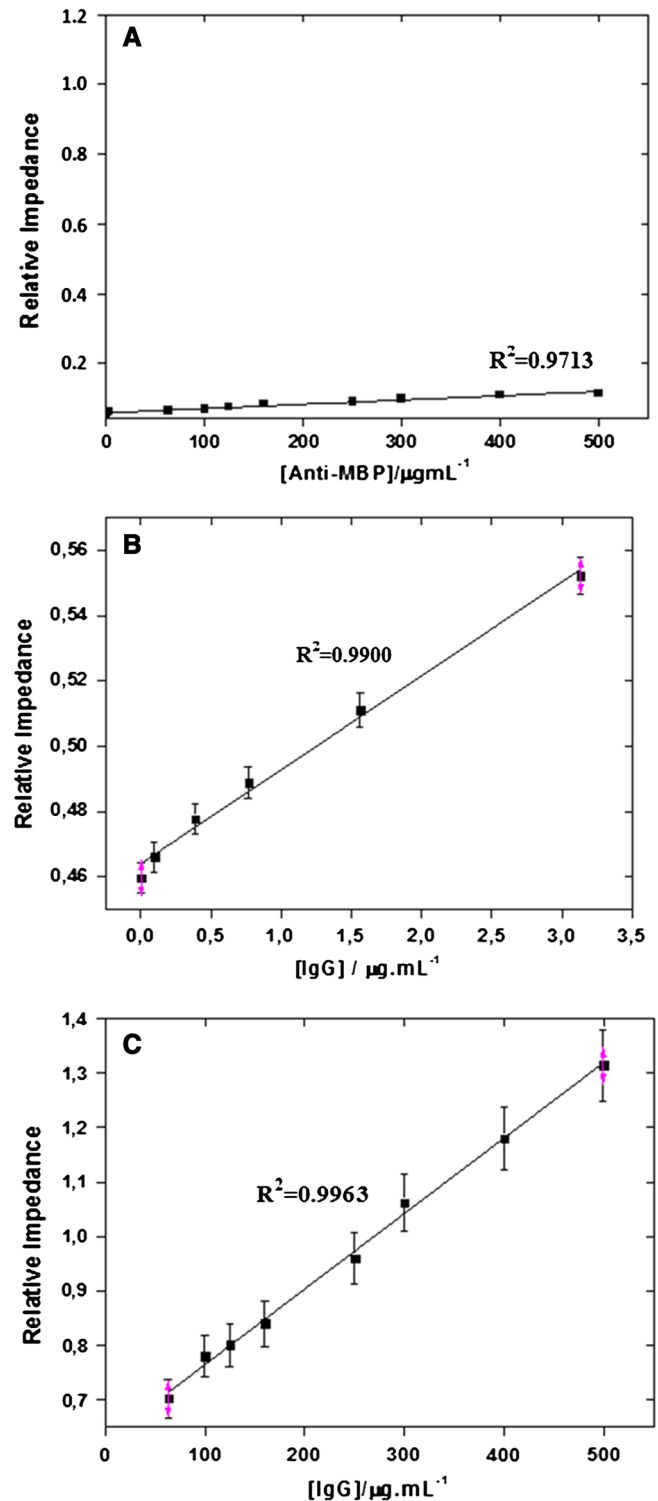
$$W_{\text{int}} = R_s + R_p - R_p^2 \omega^2 C_d \quad (6)$$

$$Z_w(\omega) = W_{\text{int}} + \frac{R_p \lambda}{\sqrt{2\omega}} (1 - j) \quad (7)$$

where $\lambda = (k_f/\sqrt{D_0} + k_b/\sqrt{D_R})$, *k*_f and *k*_b were forward and backward electron-transfer rate constants, *D*₀ and *D*_R were the diffusion coefficient of oxidant and reductant.

Figure 5b shows the AC impedance spectra of Fe(CN)₆^{4-/3-} at bare Pt electrode, *poly(MA-alt-D-I)* modified electrode *poly(MA-alt-D-I)*-TiO₂ electrode, *poly(MA-alt-D-I)*-Dequssa electrode, *poly(MA-alt-D-I)*-TiO₂-PrA electrode and, IgG incubated electrode obtained in 0.05 M pH 7.4 PB. When the electrode is covered with *poly(MA-alt-D-I)*, *R*_{ct} of *poly(MA-alt-D-I)* electrode shows a slight increase compared to the bare Pt electrode. This implies that *poly(MA-alt-D-I)* are successfully assembled on the Pt surface and has some conducting properties. With the incorporation of nanoparticles onto the *Poly(MA-alt-D-I)* Pt surface, *R*_{ct} decreases dramatically due to the large surface area and superior magnetic properties of TiO₂, accelerating the electron transfer rate [49, 50]. The *R*_{ct} for *Poly(MA-alt-D-I)*-TiO₂ electrode is lower than *Poly(MA-alt-D-I)*-Dequssa electrode due to the large surface area of TiO₂ compared to Dequssa. Subsequently, when the PrA was immobilized on the surface of *Poly(MA-alt-D-I)*-TiO₂ electrode, the EIS showed a large increase in circle diameter, suggesting that the PrA formed an external barrier, preventing any surface reaction. Therefore, from changes of *R*_{ct}, immobilization of PrA on the electrode surface was confirmed. Further increase in *R*_{ct} after IgG incubation indicates the formation of an immunocomplex layer [45].

Fig. 6 **a** Nonspecific binding of anti-MBP (0.0062–500 $\mu\text{g mL}^{-1}$ concentration range) to *poly(MA-alt-D-I)*-TiO₂-PrA immunosensor, **b** Calibration graph of the *poly(MA-alt-D-I)*-TiO₂-PrA immunosensor for 0.0062–3.125 $\mu\text{g mL}^{-1}$ concentration range, and **c** Calibration graph of the *poly(MA-alt-D-I)*-TiO₂-PrA immunosensor for 62.5–500 $\mu\text{g mL}^{-1}$ concentration range



IgG determination

The interaction of immobilized PrA with antibody was evaluated using EIS in 5 mM $[\text{Fe}(\text{CN})_6]^{4-/3-}$ solution containing 0.1 M KCl (pH 7.4). The variation of the relative impedance (RI), $R_{\text{ct}}(i) - R_{\text{ct}}(0)/R_{\text{ct}}(0)$, was used as

response. $R_{\text{ct}}(0)$ is the charge transfer resistance when PrA is immobilized on the electrode surface, while $R_{\text{ct}}(i)$ is the value of the charge transfer resistance after binding of antibody to PrA.

The nonspecific binding was tested using anti-MBP to obtain corrected calibration curves. For this purpose,

different concentrations of anti-MBP ($0.0062\text{--}500\text{ }\mu\text{g mL}^{-1}$) were used to determine the nonspecific binding and show the specificity of the developed immunosensor (Fig. 6a). The nonspecific binding of anti-MBP was 10 %. PrA binds specifically to the Fc region of immunoglobulin molecules, especially IgG. Linear regression equations were expressed for *poly(MA-alt-D-I)*-TiO₂-Protein A immunosensor as $\text{RI} = (0.059 \pm 0.012) + (0.00012 \pm 5.88 \times 10^{-5}) \text{ Anti-MBP}/(\mu\text{g mL}^{-1})^{-1}$ for anti-MBP with a correlation coefficient of 0.9713. Corrected calibration curves were obtained using relative impedance changes for $0.0062\text{--}3.125\text{ }\mu\text{g mL}^{-1}$ (Fig. 6b) and $62.5\text{--}500\text{ }\mu\text{g mL}^{-1}$ concentration ranges (Fig. 6c). Linear regression equations were expressed for *poly(MA-alt-D-I)*-TiO₂-Protein A immunosensor as $\text{RI} = (0.625 \pm 0.0089) + (0.0014 \pm 3.2 \times 10^{-5}) \text{ IgG}/(\mu\text{g mL}^{-1})^{-1}$ for high IgG concentrations and $\text{RI} = (0.46 \pm 0.0018) + (0.028 \pm 0.0012) \text{ IgG}/(\mu\text{g mL}^{-1})^{-1}$ for low IgG concentrations. The response of the *poly(MA-alt-D-I)*-TiO₂-Protein A immunosensor was proportional to IgG concentration with a correlation coefficient of 0.9900 for low and 0.9963 for high concentrations. The detection limit was found as 0.57 ng mL^{-1} using IUPAC formula. The developed immunosensor exhibits high analytical performance with a wide linear range ($0.0062\text{--}500.0\text{ }\mu\text{g mL}^{-1}$).

The analytical performance of the proposed immunosensor has been compared with those of other IgG immunosensors reported (Table 1). The comparative data suggested superiority of the present sensor over some earlier reported methods, especially the detection limit. The *poly(MA-alt-D-I)*-TiO₂-Protein A immunosensor also has a wide linear range compared to the other developed immunosensors [52–57]. The label-free impedimetric immunosensors provide a simple and sensitive detection method for the specific determination of IgG.

Table 1 Comparison of different immunosensors for the determination of IgG

Detection methods	Detection limit (ng mL ⁻¹)	Linear range (μg mL ⁻¹)	Reference
Amperometric	30	0.002–2.5	[51]
Amperometric	180	0.51–30.17	[52]
Amperometric	25	0.03–1	[53]
Surface plazmon resonance	150	0.15–40.00	[54]
Fluorescence	10	0.01–5	[55]
Differential pulse voltammetry	0.009	$10^{-5}\text{--}10^{-2}$	[56]
Impedance	300	0.3–7	[50]
Impedance	6.2		This work

Table 2 Comparison of human serum and CSF IgG levels determined using two methods

Samples	Serum	CSF
Immunosensor (mg mL ⁻¹) ^a	0.265	0.181
ELISA (mg mL ⁻¹) ^a	0.274	0.198
Relative deviation (%)	−3.328	−6.070

^a The average value of three successive determinations

Immunosensing of IgG in human serum and cerebrospinal fluid (CSF)

To investigate the reliability of the designed immunosensor for clinical analysis, human serum and CSF specimens were examined utilizing the proposed immunosensor and the results were compared with those obtained by the standart sandwich ELISA method (Table 2). The relative deviations of proposed immunosensor and ELISA were suggested that there was no significant difference between the results given by two methods. Therefore, the proposed immunosensor could be reasonably applied in the clinical determination of IgG in biological samples.

Conclusion

A sensitive label-free and rapid electrochemical PrA immunosensor for detection of IgG has been developed. For this purpose, a novel copolymer of MA was synthesized, purified, characterized and used for PrA immunosensor. To increase immunosensor sensitivity, TiO₂ nanoparticles were synthesized in autoclave and characterized using TEM, XRD and BET. EIS measurements showed that synthesized TiO₂ nanoparticles have better conducting properties compared with degussa P25. Addition of TiO₂ in *poly(MA-alt-D-I)* enhanced the surface morphology. R_{ct} of *poly(MA-alt-D-I)*–TiO₂ film was reduced by one order relative to only *poly(MA-alt-D-I)*. The immunosensor was fabricated by immobilization of PrA with NHS crosslinker on *poly(MA-alt-D-I)*-TiO₂ matrix. Successive immobilization of PrA on *poly(MA-alt-D-I)*-TiO₂ electrodes had increased R_{ct} values. The designed *Poly(MA-alt-D-I)*-TiO₂-PrA impedimetric immunosensor has capability to determine IgG with a wide linear range ($0.0062\text{--}500\text{ }\mu\text{g mL}^{-1}$), and low detection limit (0.57 ng mL^{-1}). The formal potential of Protein A immunosensor is estimated to be 225 mV vs Ag/AgCl. The electron transfer between IgG and Protein A immunosensor is quasi-reversible due to the peak separation of Protein A (149 mV at 100 mV s^{-1}) and the asymmetric anodic and cathodic peak currents. The specificity of the developed immunosensor was tested using anti-MBP ($0.0062\text{--}500\text{ }\mu\text{g mL}^{-1}$). The nonspecific binding of anti-MBP was 10 %. The developed

immunosensor exhibited high analytical performance with a wide linear range ($0.0062\text{--}500\text{ }\mu\text{g mL}^{-1}$), and high sensitivity. The application of the designed immunosensor for clinical analysis, human serum and CSF specimens were investigated using the developed immunosensor. IgG in human serum and CSF was also determined with ELISA. The results showed that the immunosensor can be used for the clinical analysis of IgG in real samples. The label-free impedimetric immunosensor provided a simple and sensitive detection method for the specific determination of IgG. The developed immunosensor is less time consuming than the ELISA due to the immunoreaction without the need of sample treatment steps. Therefore, *Poly(MA-alt-D-I)-TiO₂-PrA* impedimetric immunosensor will become a powerful tool for the monitoring of IgG in many autoimmune disease. Thus, the present sensing methodology represents a very attractive alternative to the existing methods due to simplicity, high sensitivity and inexpensive instrumentation.

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