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Synthesis and characterization of water-insoluble statistical copolymer and its application in the development of electrochemical DNA sensor

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ABSTRACT

Water-insoluble statistical copolymer was synthesized by copolymerization of methyl methacrylate (MMA) with 2-(dimethylamino)ethyl methacrylate (DMA) via group transfer polymerization (GTP). The DMA residues of the precursor P(MMA-*co*-DMA) statistical copolymer were then quaternized by reacting with methyl iodide under mild conditions to get well-defined P(MMA-*co*-QDMA) cationic copolymer. Then, P(MMA-*co*-QDMA) copolymer was successfully used for surface modification of pencil graphite electrode (PGE) to develop a disposable DNA sensor. This P(MMA-*co*-QDMA) copolymer modified electrode (q-PGE) was examined for electrochemical monitoring of DNA by using differential pulse voltammetry (DPV) in contrast to unmodified one. The effect of both DNA concentration and sonication time was also examined based on the response of q-PGE. The detection limit was calculated to be 8.06 μg/mL at q-PGE. Electrochemical impedance spectroscopy (EIS) was used for the characterization of the surface modification of q-PGE and consequently, the results were found to be in good agreement with the voltammetric measurements.

1. Introduction

There is a growing interest in the use of polymers for the design of electrochemical biosensors and their applications for DNA monitoring. DNA can be easily and strongly immobilized onto the polymer based surfaces via interaction between DNA and functional groups of polymer [1–8]. The modification of sensor surface with different polymers can yield a signal enhancement at sensor response with another advantage of easy preparation of sensor design. Booth et al. developed an impedimetric polypyrrole-based DNA sensor by using 3-pyrrolylacrylic acid (PAA) as a monomer with polypyrrole (PPy) for development of a P(Py-*co*-PAA) copolymer [9]. Carbon nanotube/polypyrrole/antibodies polymer films were synthesized on microelectrodes by Tam and Hieu used electropolymerization process for the design of immunosensor in order to determine anti-goat IgGs [10]. Recently, Diaz-Serrano et al. investigated a polymer-based electrochemical DNA biosensor for *Salmonella* detection by using bifunctional polymeric films of polystyrene prepared via free radical polymerization [11]. Liu et al. reported polythionine/gold nanoparticles/multi-wall carbon nanotubes modified electrodes for simultaneous determination of adenine and guanine in DNA [12]. Another nucleic acid sensor was developed by modified multilayers of ssDNA, cytochrome c, L-cysteine, gold nanoparticles and chitosan [13]. An electrochemical method for the detection of DNA–PNA hybridization using ferrocene-containing cationic polythiophene on nanogold modified electrode was performed by Fang et al. [14].

Cationic copolymers adsorb very well onto both silica and mica surfaces due to electrostatic interaction [15–19]. Such polymers were also suggested as gene transfer agents due to their good complexation with DNA [19,20]. Rungsardthong et al. investigated the influence of the degree of ionization of poly [2-(dimethylamino)ethyl methacrylate] homopolymer (PDMA) weak cationic polyelectrolyte on its ability to form complexes with DNA [20]. They concluded that the increased degree of ionization for the DMA homopolymer at lower pH resulted in higher binding affinity, smaller and more compact complexes, and more efficient condensation. Herein we report successful synthesis of a water-insoluble statistical cationic copolymer P(MMA-*co*-QDMA) having hydrophobic MMA residues and hydrophilic/cationic QDMA residues and its usage in surface modification of the disposable graphite electrode (PGE) for development of electrochemical DNA sensor platforms.

As far as we know, for the application of electrochemical DNA monitoring, there is no report in the literature about the surface modification of PGE with a water-insoluble cationic copolymer. Herein, P(MMA-*co*-DMA) copolymer was first synthesized by copolymerization of methyl methacrylate (MMA) and

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2-(dimethylamino)ethyl methacrylate (DMA) via group transfer polymerization (GTP). Then, the DMA residues of the precursor P(MMA-*co*-DMA) copolymer were quaternized by reacting with methyl iodide under mild conditions to get well-defined P(MMA-*co*-QDMA) cationic copolymers. Finally, P(MMA-*co*-QDMA) copolymer was successfully used for surface modification of PGE to develop a disposable DNA sensor. For the investigation of electrochemical behavior of P(MMA-*co*-QDMA) modified PGE compared to unmodified PGE, experiments were performed by using differential pulse voltammetry (DPV) and electrochemical impedance spectroscopy (EIS) techniques in the absence/presence of DNA in optimum experimental conditions.

2. Experimental

2.1. Apparatus

All experimental measurements were carried by using an AUTOLAB—PGSTAT 302 electrochemical analysis system supplied with an FRA 2.0 module for impedance measurements and GPES 4.9 software package (Eco Chemie, The Netherlands). Both DPV and EIS were used for electrochemical measurements. The three electrode system consisted of the PGE, an Ag/AgCl/KCl reference electrode (BAS, Model RE-5B, W. Lafayette, USA) and a platinum wire as the auxiliary electrode. The EIS measurements were performed in the Faraday cage (Eco Chemie, The Netherlands).

2.2. Chemicals

The fish sperm DNA (dsDNA) was obtained from Serva. The stock solutions of dsDNA were prepared at 1 mg/mL concentration with Tris–EDTA buffer solution (10 mM Tris–HCl, 1 mM EDTA, TE, pH 8.00) and kept frozen (1000 mg L^{-1}). More dilute solutions of dsDNA were prepared with 0.50 M acetate buffer solution containing 20 mM NaCl (ABS, pH 4.80).

Other chemicals were of analytical reagent grade and they were supplied from Sigma and Merck. All stock solutions were prepared using deionized and autoclaved water.

2.3. Procedure

All measurements, including the modification of P(MMA-*co*-DMA)/nucleic acid and electrochemical detection cycle, were carried out at room temperature.

2.3.1. Synthesis of P(MMA-co-QDMA) statistical copolymer

The statistical copolymer precursor was first synthesized from methyl methacrylate and 2-(dimethylamino)ethyl methacrylate monomers, P(MMA-*co*-DMA), via group transfer polymerization (GTP) chemistry using 1-methoxy-2-methyl-1-(trimethylsiloxy)-propene as an initiator and tetrabutylammonium bibenzoate as a catalyst as reported elsewhere [21,22]. Before polymerization, both monomers were mixed in a schlenk under dry nitrogen and then added into reaction media. Gel permeation chromatography [GPC: tetrahydrofuran eluent; refractive index detector; poly(methyl methacrylate) standards] showed that the statistical copolymer had narrow unimodal distributions with M_n of 23,200, and M_w/M_n of 1.05 (see Fig. 1).

Fig. 2 shows the ^{1}H NMR spectra of the P(MMA-*co*-DMA) copolymer before quaternization (in $CDCl_3$) and after quaternization of DMA residues (in d_6-DMSO) with all the relevant signals assigned. Peak d at δ 2.1 ppm in Fig. 2a represents the six dimethylamino protons of DMA residues. After quaternization of this tertiary amine residues with MeI (see Fig. 2b), nine quaternary ammonium protons appear at δ 2.2 ppm (peak d, in d_6-DMSO).

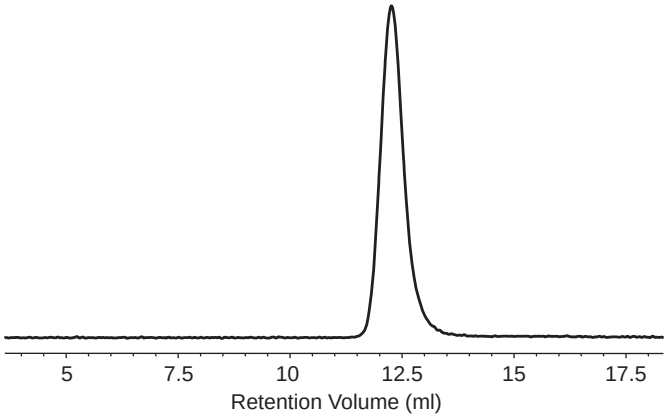


Fig. 1. GPC chromatogram of precursor P(MMA-*co*-DMA) copolymer (M_n: 23,200; M_w/M_n: 1.05).

Comparison of the peak integral of this signal with that of the oxymethylene proton signal at δ 4.1 ppm indicates 100% quaternization. The absence of any unquaternized dimethylamino protons at δ 2.1 ppm and azomethylene protons at δ 2.4 ppm in Fig. 2b confirms this calculation. DMA content was determined to be 15 mol% in the precursor P(MMA-*co*-DMA) copolymer by comparing the integral of peak "a" at δ 3.9 ppm with the integral of peak "d" at δ 2.1 ppm (see Fig. 2a). The mean degree of polymerization for each comonomer in P(MMA-*co*-DMA) statistical copolymer was determined from both GPC data and ^{1}H NMR spectra and calculated to be 181 and 32, respectively. The subscript in P(MMA_{181}-*co*-DMA_{32}) describes the calculated mean degree of polymerization for each comonomer. It is worth mentioning that if QDMA content of the P(MMA-co-QDMA) copolymer is 25 mol%, it becomes soluble in water which limits its usage in aqueous media as a surface modifier agent. Thus, to keep P(MMA-co-QDMA) copolymer insoluble in aqueous phase, the QDMA content of the copolymer was chosen to be 15 mol%.

The tertiary amine residues of the DMA units of the P(MMA-*co*-DMA) statistical copolymer (5 g) were selectively quaternized by reacting with a 3-fold excess of methyl iodide at room temperature in THF for 4 h [21,22]. The mean degree of quaternization of DMA residues was 100 mol% as determined from ^{1}H NMR spectroscopy (see Fig. 2b). The M_n of the resulting cationic copolymer P(MMA-*co*-QDMA) was calculated to be 27,750 g mol^{-1}. The resulting quaternized P(MMA-*co*-QDMA) statistical copolymer was purified by soxhlet extraction with methanol (to remove excess methyl iodide and iodine). The quaternized copolymer was then recovered by using a roto evaporator and dried in a vacuum oven overnight.

The P(MMA-co-QDMA) copolymer (shown in Scheme 1) was soluble in some organic solvents, such as in THF and DMF but insoluble in water even with its cationic nature. Thus, it was used for modification of the disposable graphite electrode surface in order to immobilize more DNA by increasing the surface area.

2.3.2. Preparation of P(MMA-co-QDMA) solution and P(MMA-co-QDMA) modified PGEs

The required amount of P(MMA-*co*-QDMA) was suspended in the organic solvent N,N-dimethylformamide (DMF), and this mixture was then sonicated for 1 h at room temperature.

Pencil graphite electrodes (PGEs) were pretreated by applying +1.40 V for 30 s in ABS. Each pretreated PGE was immersed for 1 h into the vials containing 110 μL of 9000 μg/mL P(MMA-*co*-QDMA) solution for passive adsorption procedure [23,24].

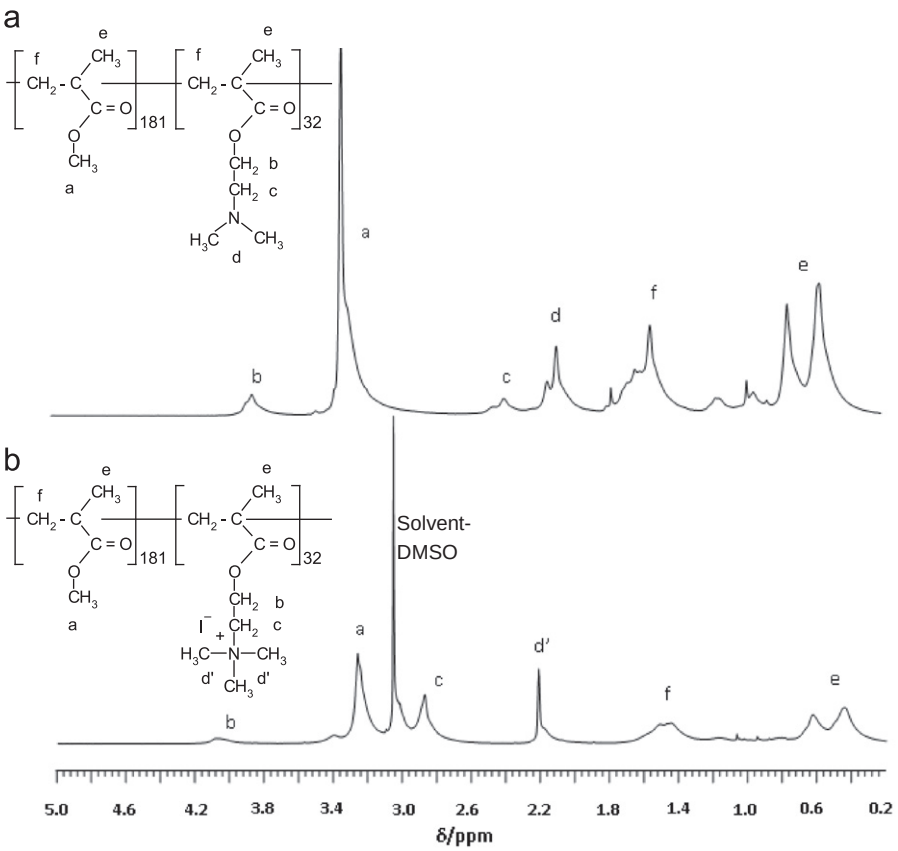
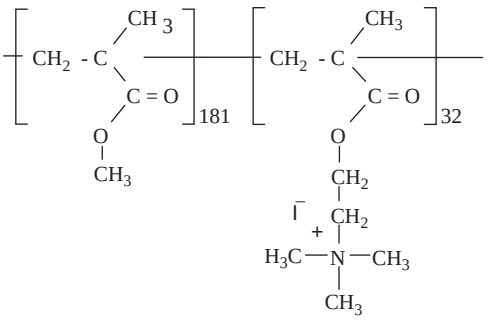


Fig. 2. ^{1}H NMR spectra of P(MMA-*co*-DMA) statistical copolymer: (a) before quaternization of DMA residues ($CDCl_3$) and (b) after quaternization of DMA residues (in d_6-DMSO). Note the disappearance or the shift of both peak "d" and peak "c" signals due to quaternization of tertiary amine residues of DMA segments.



Scheme 1. Molecular structure of P(MMA-*co*-QDMA) copolymer.

Each of the P(MMA-*co*-QDMA) modified PGEs (q-PGEs) was then allowed to dry for 5 min with upside down configuration.

The microscopic characterization of electrodes was obtained a by Quanta 400 FEI, field emission scanning electron microscope (FE-SEM) (Tokyo, Japan) with required acceleration voltage 5.0 kV in different resolutions (5 and 10 μm). The surface morphologies of unmodified PGE and P(MMA-*co*-QDMA) modified PGE (q-PGE) were investigated and are shown in various magnifications [5 μm (Fig. 1S-A, B) and 10 μm (Fig. 1S-C, D)]. After the modification of PGE surface with P(MMA-*co*-QDMA), it could be clearly seen that there was some invisible aggregation of polymer molecules on the graphite surface compared to ones obtained using unmodified PGE.

In the presence of P(MMA-*co*-QDMA), a decrease was observed in the height of the anodic peak of $[Fe(CN)_6]^{3-/4-}$ compared to that of unmodified PGE. The cyclic voltamograms were taken in 2.5 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ (1:1) containing 0.1 M KCl before and after immobilization of P(MMA-*co*-QDMA) or its copolymer P(MMA-*co*-DMA) onto the surfaces of PGEs in order to explore their electrochemical behaviors. The CV parameters were followed by the step potential, 25 mV s^{-1}; scan rate, 100 mV s^{-1}; forward scan, −0.40 to+1.20 V; and reverse scan, +1.20 to −0.40 V. The relative charge Q (C) is shown in Table S1.

Increase was observed in the height of the anodic peak and positive shift (approximately 25 mV) in the E_{pa} of $[Fe(CN)_6]^{3-/4-}$ using P(MMA-*co*-QDMA) modified PGE (q-PGE) compared to unmodified PGE due to the fact that polymer could provide an enhanced immobilization area for biomolecules by providing a higher surface area [6–8] (shown in Fig. S2).

It was also found that the relative charge (Q) decreased for [P(MMA-*co*-DMA)] modified PGE and q-PGE after their immobilization onto electrode surfaces (shown in Table S1).

2.3.3. *Immobilization of dsDNA onto the surfaces of P(MMA-co-QDMA) modified disposable graphite electrode*

Each P(MMA-*co*-QDMA) modified PGE (q-PGE) was immersed into the vials containing 110 μL of 120 μg/mL of dsDNA solution in ABS for an hour. Each of the electrodes was then rinsed with ABS for 10 s before voltammetric transduction.

2.4. *Voltammetric transduction*

After DNA immobilization onto the surfaces of bare PGEs/q-PGEs, the DPV measurements were performed in a blank ABS to measure guanine oxidation signal by scanning from +0.40 to +1.45 V at the pulse amplitude 50 mV and the scan rate 50 mV/s.

2.5. Impedance measurements

The surfaces of unmodified and modified PGEs (PGE and q-PGE) were characterized by the EIS. Respectively, the EIS measurements were carried out by using pretreated PGEs, q-PGE and DNA immobilized q-PGE according to the procedure given below. EIS measurements were performed in the presence of 2.5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) mixture as a redox probe prepared in 0.1 M KCl. The impedance was measured in the frequency range from 10^5 Hz to 10^{-1} Hz in a potential of open-circuit value +0.23 V versus Ag/AgCl with a sinusoidal signal of 10 mV. The frequency interval was divided into 98 logarithmically equidistant measure points. The respective semicircle diameter corresponds to the charge-transfer resistance, R_{ct}, the values of which are calculated using the fitting program AUTOLAB 302 (FRA, version 4.9 Eco Chemie, The Netherlands).

3. Results and discussion

Initially, the effect of modification of PGE surface using 10 mg/mL P(MMA-*co*-QDMA) was explored based on biosensor response. After 100 μg/mL dsDNA immobilization onto the surface of q-PGE, the guanine oxidation signal was measured by comparing the response obtained by unmodified PGE. As seen in Fig. 3, DPV measurements indicated no oxidation signal between +0.40 V and +1.45 V in the absence of DNA by using PGE or q-PGE, but, a well defined guanine oxidation signal at about +1.00 V after DNA immobilization onto the surfaces of both electrodes. It was found that the magnitude of guanine signal increased approximately 68.50% in the presence of P(MMA-*co*-QDMA) modification. This increase at sensor response indicated that P(MMA-*co*-QDMA) may possibly improve the sensor surface area for bounding of nucleic acids similarly to the results presented in earlier studies based on carbon nanotubes (CNTs) or polymer composite, or nanoparticle-conductive polymer matrix modified sensors [7,8,25–27].

The histograms presenting the changes at guanine oxidation signal is shown in Fig. 4. Before 100 μg/mL dsDNA immobilization step, each PGE was modified by using P(MMA-*co*-QDMA) in different concentration ranges varying from 3000 to 12,000 μg/mL. It was found that sensor response increased up to 9000 μg/mL and then leveled off. Thus, 9000 μg/mL was chosen as the optimum concentration of P(MMA-*co*-QDMA) for full coverage of PGE surface resulting in a good reproducibility of 1.82% RSD ($n=3$).

The effect of sonication time of P(MMA-*co*-QDMA) suspended in DMF was also examined. 9000 μg/mL P(MMA-*co*-QDMA) was suspended in DMF and this mixture was sonicated for a time between 15 min and 60 min. Concerning each sonication time, the guanine signal was consequently measured as a sensor response after DNA immobilization onto these q-PGEs, and the histograms representing the guanine oxidation signal are shown in Fig. 5. The optimum sonication time was found to be 60 min, giving adequate responses by resulting in the most homogenous solution of P(MMA-*co*-QDMA) and also a good reproducibility of 6.38% RSD ($n=3$).

The changes of guanine oxidation signal were also monitored in different concentration ranges of dsDNA varying from 5 to 160 μg/mL after DNA immobilization onto the surface of q-PGEs for 1 h (see Fig. 6). It was found that a sharp increase was obtained at responses up to 100 μg/mL. Due to less polymer

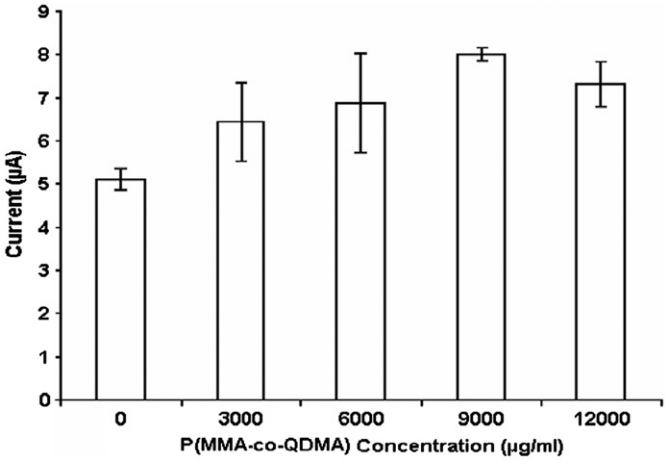


Fig. 4. Histograms presenting the changes of guanine oxidation signal measured in the presence of 100 μg/mL dsDNA on PGEs modified with P(MMA-*co*-QDMA) in various concentrations from 0 to 12,000 μg/mL. Other conditions are as in Fig. 3.

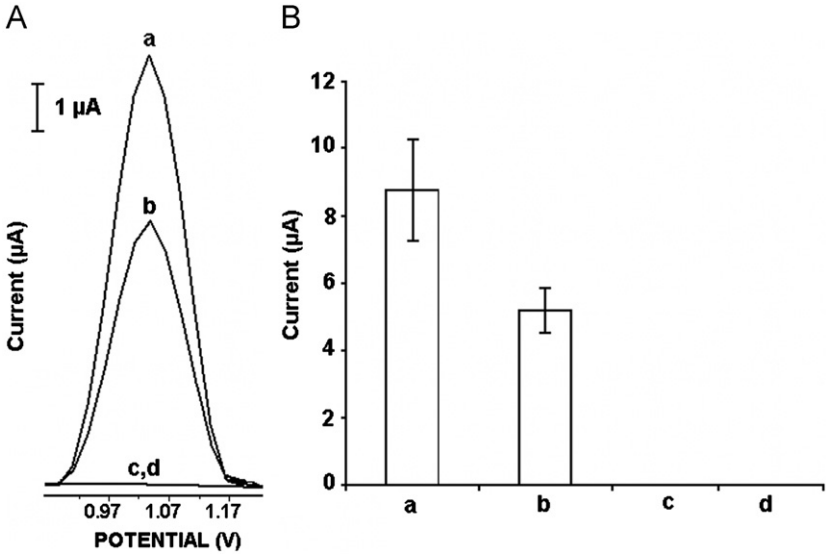


Fig. 3. . (A) DPVs and (B) histograms representing the guanine oxidation signals observed by using unmodified PGE and P(MMA-*co*-QDMA) modified PGE in the presence of 100 μg/mL dsDNA immobilized onto the surface of (a) 10 mg/mL P(MMA-*co*-QDMA) modified PGE, and (b) unmodified PGE and the results obtained in the absence of dsDNA using (c) 10 mg/mL P(MMA-*co*-QDMA) modified PGE, and (d) unmodified PGE. DPV measurement scanning between +0.40 V and +1.45 V at 50 mV pulse amplitude and 50 mV/s scan rate in ABS by using PGE.

binding capacity for more excess DNA onto the modified PGE surfaces, the guanine signal gradually decreased between 100 and 160 μg/mL DNA concentration range. Thus, 100 μg/mL was chosen as the optimum DNA concentration for development of P(MMA-*co*-QDMA) modified DNA biosensor. Based on three repetitive measurements on guanine signal, the RSD% ($n=4$) was calculated to be 8.03% using 100 μg/mL DNA modified q-PGEs.

A calibration plot for dsDNA is also given as an inset figure in Fig. S3. The limit of detection (LOD) was calculated as explained in the reference, with the regression equation and definition $y=y_B+3S_B$ (y_B is the signal of the blank solutions and S_B is the standard deviation of the blank solution) [28]. According to this procedure, the LOD was found to be 8.06 μg/mL dsDNA concentration, comparable to the ones obtained in earlier sensors developed by using materials of graphene oxide, or polymer composite [5,26].

EIS was used in order to characterize the successful modification of P(MMA-*co*-QDMA) onto the surface of PGE. Respectively, the R_{ct} values were measured before and after P(MMA-*co*-QDMA) modification onto surface of PGE, and also DNA immobilization (see Fig. 7).

After the modification of the PGE surface using P(MMA-*co*-QDMA), the average R_{ct} value was calculated as 580 Ω (in Fig. 7b), which is almost 3.5 times higher than the one obtained by unmodified PGE (i.e. recorded as 168 Ω). The change of the R_{ct} value after polymer modification is a strong proof that P(MMA-*co*-QDMA) is immobilized successfully on the PGE surface. The negatively charged phosphate backbone of double stranded DNA prevented redox couple $[Fe(CN)_6]^{3-/4-}$ from reaching the surface of q-PGE, leading to almost 1.8 fold increase in R_{ct} value (shown in Fig. 7c). These results clearly presented that the modification of

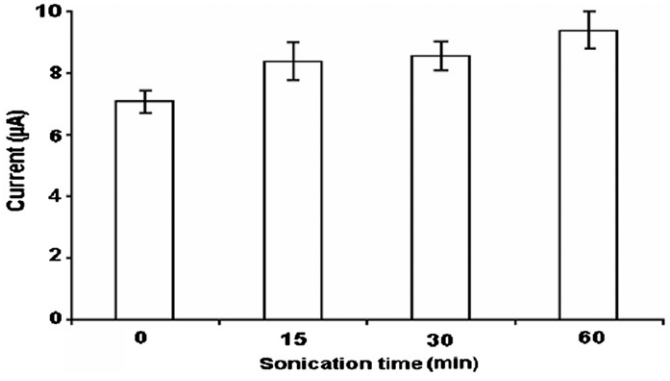


Fig. 5. Effect of sonication times varying from 0 to 60 min applied for the preparation of P(MMA-*co*-QDMA) solution before its immobilization onto PGE surface. Histograms representing the guanine oxidation signal observed by using unmodified PGE and 9000 μg/mL modified P(MMA-*co*-QDMA) PGE. Other conditions are as in Fig. 3.

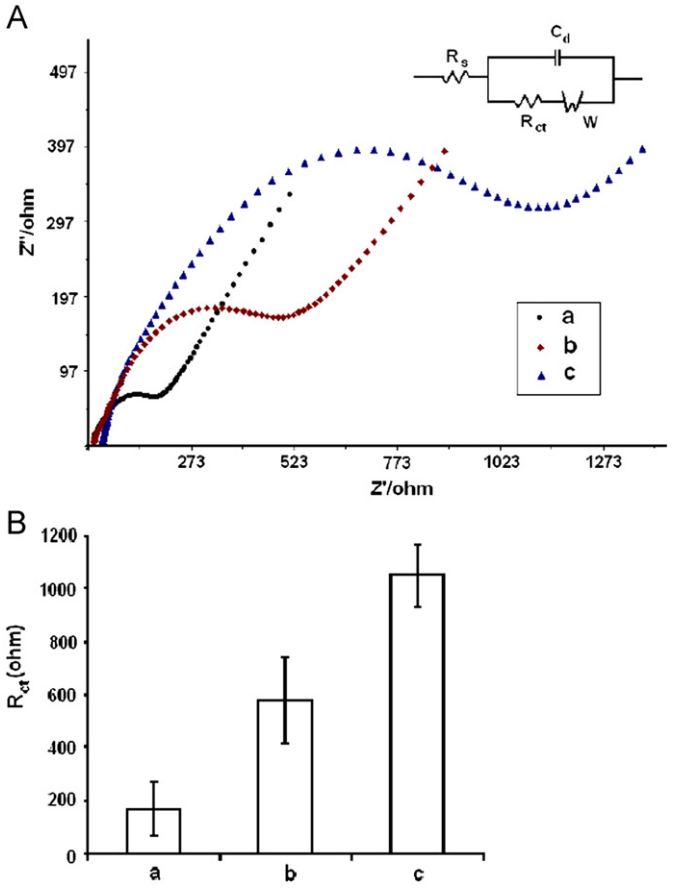


Fig. 7. . (A) Nyquist diagrams recorded (a) before and (b) after modification of P(MMA-*co*-QDMA) onto the PGE surface, and (c) dsDNA immobilization onto the q-PGE surface. Supporting electrolyte solution is 2.5 mmol L^{-1} $K_4[Fe(CN)_6]$/$K_3[Fe(CN)_6]$ (1:1) containing 0.1 mol L^{-1} KCl. Inset is the equivalent circuit model used to fit the impedance data, the parameters of which are listed in the text; R_S is the solution resistance. The constant phase element C_d is then related to the space charge capacitance at the DNA/electrolyte interface. R_{ct} is related to the charge transfer resistance at the DNA/electrolyte interface. The constant phase element W is the Warburg impedance due to mass transfer to the electrode surface (B) histograms representing the average R_{ct} values ($n=3$) measured at PGE surface (a) before and (b) after modification of P(MMA-*co*-QDMA) onto the PGE surface, and (c) dsDNA immobilization onto the q-PGE surface.

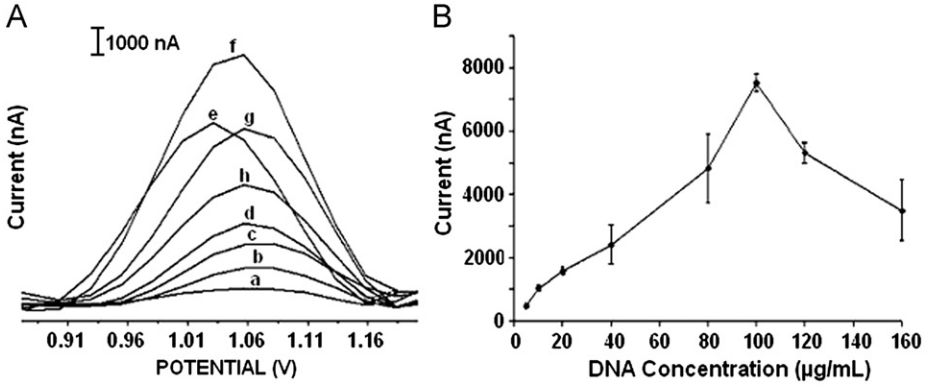


Fig. 6. . (A) Voltamograms and (B) line graph representing the guanine oxidation signals obtained by using DNA modified q-PGEs at different DNA concentrations: (a) 5, (b)10, (c) 20, (d) 40, (e) 80, (f) 100, (g) 120 and (h) 160 μg/mL. Other conditions are as in Fig. 3.

P(MMA-*co*-QDMA) onto the graphite surface improved the immobilization capacity of DNA similarly to the ones obtained by using a DNA immobilized dendrimer modified electrode [29] and DNA immobilized carbon nanotubes modified electrode [27].

4. Conclusion

In our study, a water-insoluble statistical copolymer having permanent cationic segments, P(MMA-*co*-QDMA), was successfully synthesized by copolymerization of methyl methacrylate (MMA) with 2-(dimethylamino)ethyl methacrylate (DMA) via group transfer polymerization (GTP) and then followed by quaternization of tertiary amine residues with methyl iodide. A single-use PGE was then modified with P(MMA-*co*-QDMA) copolymer by passive adsorption. In order to increase DNA adsorption capacity of PGE, for the first time, a cationic water-insoluble copolymer was used as a novel surface modifier for the modification of the disposable PGE surface. This novel sensor surface has provided some advantages in the development of DNA biosensor, such as, less time consumption, being inexpensive and easy preparation procedure resulting from an enhanced DNA detection protocol combined with the DPV technique and single-use sensor technology. The EIS results are compatible with the ones obtained by using the voltammetric method.

The most important and appreciable advantage of the P(MMA-co-QDMA) modification is signal enhancement effect on PGE electrode. An increase in the surface area of sensor due to modification with polymer resulted in more DNA response (68.50%) than that of the unmodified one. The increase in response can be comparatively closer to the increase obtained by using CNT modified surfaces [27]. As a conclusion, the results showed that P(MMA-co-QDMA) can offer an alternative route for development of advanced (bio)sensors for monitoring nucleic acids, proteins, and other molecules.

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Appendix. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.07.020.

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