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Electrochemical functionalization of polypyrrole through amine oxidation of poly(amidoamine) dendrimers: Application to DNA biosensor

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ABSTRACT

Electrochemical patterning method has been developed to fabricate composite based on polypyrrole (PPy) film and poly(amidoamine) dendrimers of fourth generation (PAMAM G4). PPy layer was generated using electrochemical polymerization of pyrrole on a gold electrode. PPy film was then modified with PAMAM G4 using amines electro-oxidation method. Covalent bonding of PAMAM G4 and the formation of PPy-PAMAM composite was characterized using Fourier Transform Infrared Spectroscopy (FT-IR) and X-ray Photoelectron Spectroscopy (XPS). Ferrocenyl groups were then attached to such surface as a redox marker. Electrochemical properties of the modified nanomaterial (PPy-PAMAM-Fc) were studied using both amperometric and impedimetric methods to demonstrate the efficiency of electron transfer through the modified PPy layer. The obtained electrical and electrochemical properties were compared to a composite where PPy bearing carboxylic acid functions was chemically modified with PAMAM G4 by covalent attachment through formation of amid bond (PPy-CONH-PAMAM). The above mentioned studies showed that electrochemical patterning does not disturb the electronic properties of PPy. The effect of the number of functional groups introduced by the electrochemical patterning was demonstrated through the association of various compounds (ethylenediamine, PAMAM G2 and PAMAM G6). We demonstrated that such compounds could be applied in the biosensors technology. The modified PPy-PAMAM-Fc was evaluated as a platform for DNA sensing. High performance in the DNA detection by variation of the electrochemical signal of ferrocene was obtained with detection limit of 0.4 fM. Furthermore, such approach of electrochemical patterning by oxidation of amines could be applied for chemical modification of PPy and open a new way in various biosensing application involving functionalized PPy.

1. Introduction

Polypyrrole (PPy) is one of the conducting polymers extensively applied to the design of bioanalytical sensors [1,2] including systems for DNA detection [3,4]. Immobilization of bioreceptors on PPy film is still an important issue in the use of this polymer for biosensors construction. Literature has reported different strategies, for example DNA probe can be immobilized via entrapment method during electropolymerization process [5]. However, this method can cause unfavourable orientation of molecules in the polymer layer. One of the powerful alternative for stable biomolecules attachment, is the chemical modifications of pyrrole monomer on N-nitrogen or 3-substituted C-carbon position followed by electrochemical polymerization [6]. This method consists in the modification of pyrrole with functional groups such as carboxylic acids [7], amines [8] or activated esters [9,10] which gives the flexibility for covalent attachment of biomolecules. Nevertheless, construction of these biosensors requires the synthesis of pyrrole modified with functional groups, which is time consuming and laborious.

Association of PPy with structured, three-dimensional materials such as poly(amidoamine) dendrimers (PAMAM) can greatly increase the quantity of attached biomolecules and consequently improve sensitivity of detection [11]. PAMAM are the spherical, hyper-branched, macromolecular polymers, that possess a large number of amine groups on the surface depending on the generation [12]. These molecules have been successfully applied in the fabrication of DNA biosensors using amperometric [13] as well as impedimetric techniques [14,15] showing improved quantity of attached DNA probes to the surface. It was also demonstrated that

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their association with others conductive nanomaterials such as gold nanoparticles or graphene can additionally amplify sensitivity of electrochemical signal and enable a low detection limit [16,17]. Recently, our group has showed the immobilization of PAMAM dendrimers of fourth generation (PAMAM G4) on PPy film modified with activated carboxylic groups using a chemical functionalization of pyrrole monomers. We proved that PAMAM G4 led to high surface coverage of DNA aptamers as bioreceptors improving sensitivity of protein detection [18].

In the current work, we demonstrate that electrochemical oxidation of amines could be applied for the chemical functionalization of conducting materials such as PPy. The advantage of this method is its simplicity and short time, without prior chemical synthesis of the functionalized pyrrole monomer. Such approach of surface functionalization with aliphatic molecules has been previously demonstrated in the case of carbon based materials such as glassy carbon [19–21], carbon nanotubes (CNTs) [22] and polyparaphenylene [23] modified electrode. We recently demonstrated that PAMAM G4 dendrimers could be attached to CNTs-PPy composite via electrochemical method [24] and such material was successfully used for DNA detection. In this work the modification of un-functionalized PPy film by electrochemical deposition of PAMAM G4 is demonstrated. To our knowledge, this is the first time that non-functionalized PPy film was modified with amine terminated macromolecules such as PAMAM G4 through electrochemical oxidation of its amine groups.

The PPy-PAMAM composite was characterized with various techniques and its properties were compared with material obtained following chemical binding of PAMAM G4 to the PPy layer modified with carboxylic acid groups (PPy-CONH-PAMAM). The obtained material was then applied for DNA sensing where sensitivity and selectivity were investigated through ferrocene signal variation attached as a redox marker to the modified surface.

Fabrication of PPy-PAMAM material as well as construction of biosensor is presented on Scheme 1. Steps a and b show electropolymerization of PPy following covalent grafting of PAMAM G4 by its amine moieties. Then ferrocene chemically functionalized with two phthalimidyls was attached to such surface by formation of peptidic bonds with PAMAM G4 (step c). ssDNA probe modified with amine group on 5′ position was anchored to unreacted phthalimidyl of ferrocene (step d) and interaction DNA probe/DNA target (step e) was followed by variation in ferrocenyl signal using square wave voltammetry (SWV) technique.

2. Materials and methods

2.1. Reagents

All DNA sequences were provided by Eurogentec company. The single-stranded DNA probe (ssDNA) was an amine terminated 15-bases sequence: NH_2-$(CH_2)_6$-5′GAT-ACT-TCT-ATC-ACC3′. The sequence of 15-bases target specific for DNA probe (DNA_C) was 5′ GGT-GAT-AGA-AGT-ATC3′. The non-complementary 14-bases oligonucleotide (DNA_{NC}) sequence was 5′CAT-TCC-CTC-TTA-GG3′.

1,1′-(phthalimide butanoate) ferrocene and pyrrole monomer bearing activated ester were synthetized according to the methods described previously [25,26]. Pyrrole, poly(amidoamine) dendrimers of fourth generation (PAMAM G4) and lithium perchlorate were purchased from Sigma-Aldrich. Prior to polymerization, pyrrole was distilled under argon and PAMAM G4 were purified by 0.22 μm membrane filters (Corning). All the electrochemical analysis were performed in phosphate buffer saline (PBS) pH 7.4 containing 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl and 137 mM NaCl, filtered through 0.22 μm pore size membrane and stored at 4 °C until use.

2.2. Instrumentation

Electrochemical polymerization was performed using a potentiostat-galvanostat Autolab PGSTAT 12 controlled by Nova software. The three-electrode cell was purchased from Basi and consists of a platinum mesh as a counter electrode, gold disc (surface 2.01×10^{-2} cm^2) as a working electrode and Ag/AgCl as a reference electrode. After polymerization and in between each step of biosensor construction, the modified surface was analyzed by cyclic voltammetry (CV) and square wave voltammetry (SWV) methods in 10 mM PBS buffer pH 7.4. CV was performed in the potential range from −0.6 to 0.4 V with the scan rate of 50 mV s^{-1}. SWVs were performed in the potential range from −0.6 to 0.4 V with conditioning time of 120 s, modulation amplitude of 20 mV and frequency of 50 Hz.

Electrochemical impedance measurements (EIS) were carried out in 10 mM PBS buffer pH 7.4. All impedance measurements were obtained at 0.10 V vs. Ag/AgCl corresponding to the steady sate potential with signal amplitude and a sinusoidal potential excitation corresponding to 10 mV is applied in a frequency range from 100 kHz to 0.1 Hz.

Chronoamperometric measurements were also performed in 10 mM PBS containing 5 mM/5 mM $[Fe(CN)_6]^{4-}$/$[Fe(CN)_6]^{3-}$ at the potential of 0.2 V vs. Ag/AgCl. The current was measured for 90 s.

Fourier Transform Infrared spectra (FT-IR) were measured using a Bruker IFS66 FT-IR spectrometer (Bruker, Germany) equipped with a Mercury-cadmium-telluride (MCT) detector and an attenuated total reflectance (ATR) germanium crystal. The analyses were performed on screen printed electrodes (DropSens).

X-ray Photoelectron Spectroscopy (XPS) measurements were performed with a K Alpha spectrometer from Thermo-Fisher, using a monochromated X-ray Source (Al Kα, 1486.6 eV). For all measurements, a spot size of 400 μm was employed. The hemispherical analyser was operated in CAE (Constant Analyser Energy) mode, with a pass energy of 200 eV and a step of 1 eV for the acquisition of surveys spectra, and a pass energy of 50 eV and a step of 0.1 eV for the acquisition of high resolution spectra. The spectra obtained were processed using the “Avantage” software provided by Thermo-Fisher. A Shirley-type background subtraction was used.

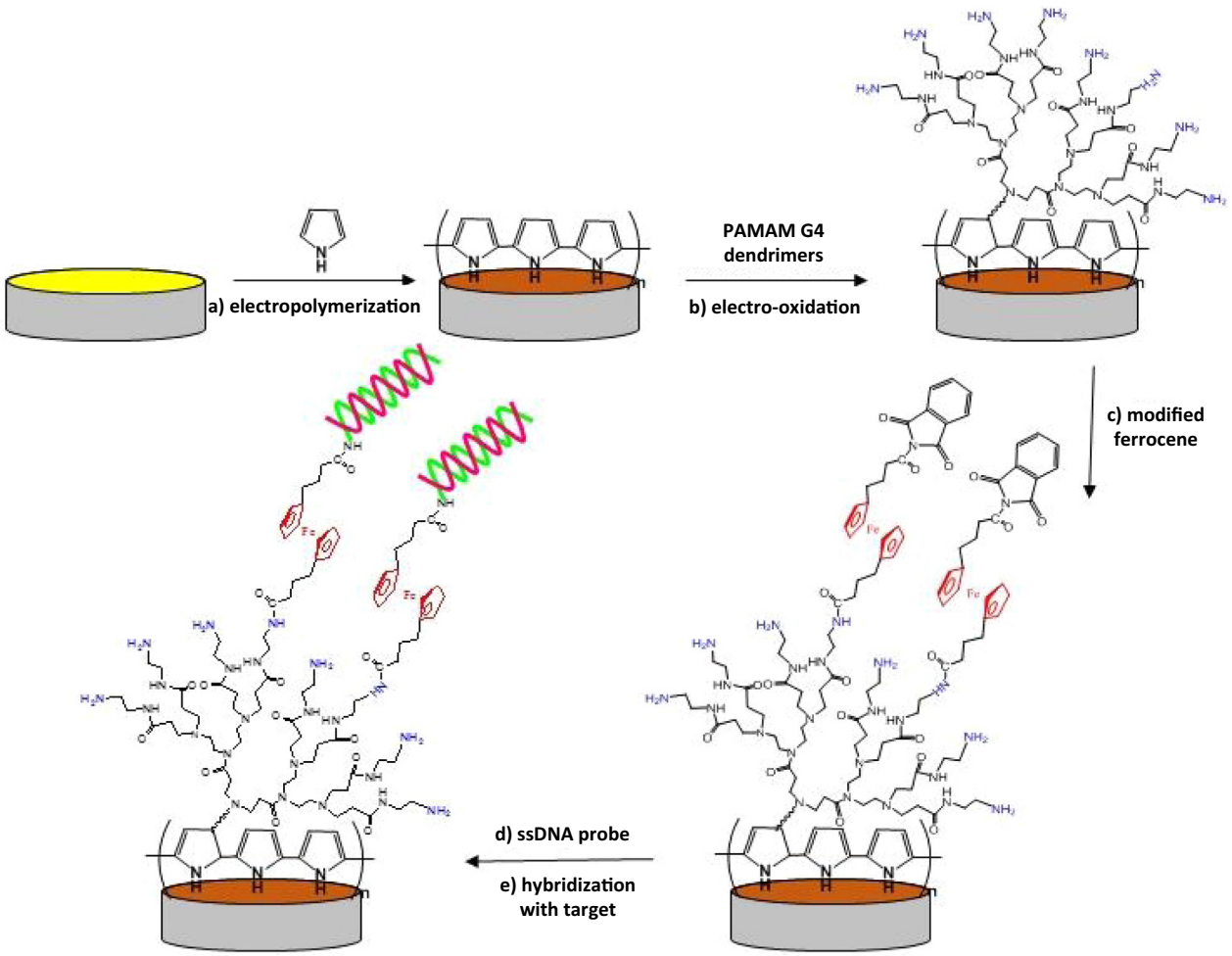
2.3. Electrochemical pattering of PPy and PAMAM G4 dendrimers

Polypyrrole film (PPy) was deposited on the gold electrode surface, by cycling the potential from −0.4 to 0.9 V vs. Ag/AgCl, with a scan rate of 100 mV s^{-1} for 2 cycles in H_2O solution containing 0.5 M $LiClO_4$. During electro-polymerization, the working and counter electrodes were separated in small volume cell (Basi) containing the solution of 100 mM pyrrole monomer. After reaction, the electrode was rinsed several times with double distilled water.

PAMAM G4 dendrimers were immobilized on the PPy layer by covalent bonding during electro-oxidation of amine groups of PAMAM G4. Electrodeposition was performed in H_2O containing 50 μM PAMAM G4 and 0.5 M $LiClO_4$ by scanning the potential from 0.0 to 1.1 V vs. Ag/AgCl as a reference electrode during 3 cycles with the scan rate of 50 mV s^{-1}. During the reaction the working and counter electrodes were separated in small volume cell (Basi) similar to electropolymerization method.

2.4. Association of ferrocene and ssDNA probe

Covalent bonding of ferrocene modified with two phtalymidyls was performed by immersing the modified PPy-PAMAM electrode in solution of 1 mM ferrocene prepared in acetonitrile for 1 h at room temperature. The time and concentration of ferrocene were



Scheme 1. Schematic presentation of DNA sensor based on modified PPy: (a) electropolymerization of PPy film; (b) electrodeposition of PAMAM G4; (c) covalent grafting of ferrocene; (d) anchoring of ssDNA probe; (e) detection of specific target.

optimized in order to obtain not only an adequate amount of ferrocenyl groups attached to the surface, but also to obtain sensitive variation of the redox signal during detection of complementary target. Later, non-bonded ferrocene residues were washed with acetonitrile and double distilled water. Thereafter, the electrode was immersed in 10 μM solution of single-stranded DNA probe (ssDNA) and incubated for 1 h at room temperature. Following the washing of the electrode with distilled water and PBS buffer, to saturate all non-bonded phthalimidyl esters of ferrocene and to avoid non-specific interactions of DNA target with PAMAM G4, the electrode was immersed in 1 mM of ethanolamine (EtOH) solution in PBS buffer and incubated for 30 min at room temperature. The biosensor was then carefully washed with distilled water and PBS solution and stored in PBS at 4 °C overnight for stabilization.

2.5. Hybridization with complementary target

The hybridization of complementary single-stranded DNA target (DNA_C) of 15-bases with the ssDNA probe immobilized on the surface, was performed by incubation of the modified electrode in solution of DNA_C target for 1 h at 37 °C. Hybridization with the following concentrations of DNA_C target was studied: 1, 10, 100 fM, 1, 10, 100 pM, 1, 10, 100 nM. After incubation, the electrode was carefully washed with distilled water and PBS buffer. The test for non-specific interactions was also performed by immersing the electrode in 1 μM of non-complementary target (DNA_{NC}) in 10 mM PBS buffer and incubated under the same conditions as for DNA_C.

3. Results and discussion

3.1. PPy modification and characterization

Gold electrode was modified with polypyrrole film (PPy) by electrochemical deposition of non-functionalized pyrrole monomer. It was performed by scanning the potential from −0.4 to 0.9 V vs. Ag/AgCl, with a scan rate of 100 mV s^{-1} in H_2O solution containing 0.5 M $LiClO_4$. Morphology of PPy film generated in aqueous solution reminds of a characteristic “cauliflower” structure that is strictly dependent on the number of CV cycles during polymerization and thus the thickness of polymer (Fig. 1). In this work polymers of various thickness were studied (obtained by one to five CV cycles) and among different conditions scanning the potential for two cycles was chosen as optimal.

In order to functionalize PPy with chemical groups, electrochemical pattering of PAMAM dendrimers of fourth generation (PAMAM G4) was performed. The modification was carried out by oxidizing the amine groups of PAMAM G4 by scanning the potential between 0 V and 1.1 V for 3 cycles. The oxidation of amine groups of PAMAM G4 leads to the formation of radical cations that attach to the PPy layer. In order to confirm such a reaction, the

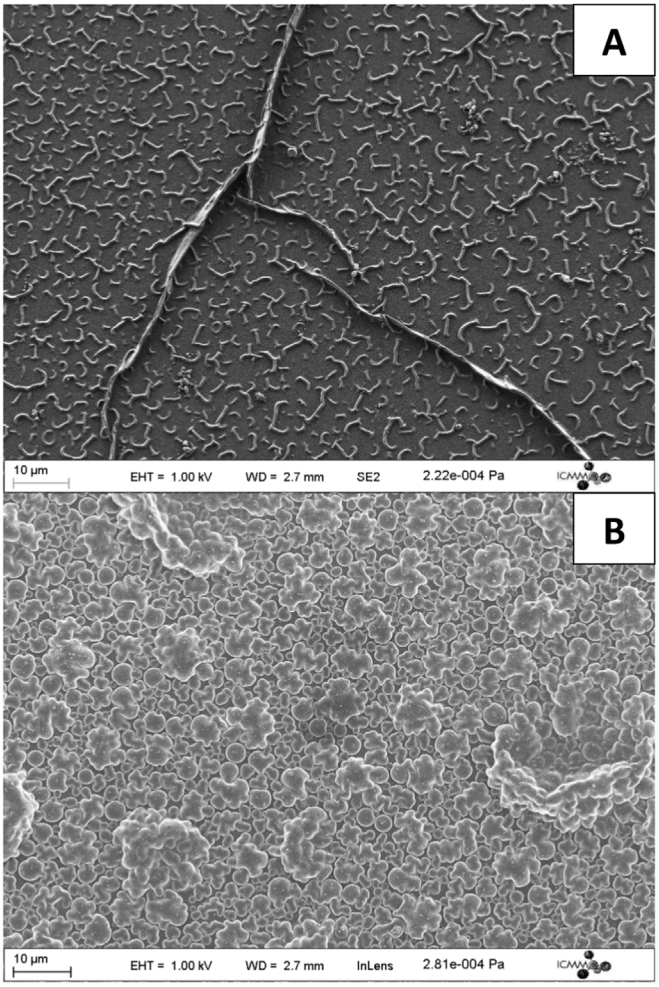


Fig. 1. Morphology of PPy layer electropolymerized in aqueous solution containing 0.5 M $LiClO_4$ using cyclic voltammetry in the potential range −0.4 to 0.9 V for (A) two and (B) five cycles.

composites PPy and PPy-PAMAM was characterized with FT-IR and XPS techniques. FT-IR spectra of PPy before and after functionalization with PAMAM G4 are shown in Fig. 2. Modification of PPy layer resulted in the appearance of new characteristic stretching vibration of PAMAM G4 at 1701 cm^{-1} assigned to the amide bond (C=O) as well as a vibration at 3250 cm^{-1} corresponding to amine groups (NH_2).

XPS spectra also confirmed the modifications of the PPy surface with PAMAM G4. Fig. 3 shows the results of XPS analysis in carbon and nitrogen regions for PPy and PPy-PAMAM layers. Functionalization of PPy with PAMAM G4 caused significant changes in spectrum of both regions. The results of deconvolution and peak separation as well as attribution of these peaks to chemical bonds are described in Table 1.

Functionalization based on electrochemical patterning has been demonstrated in the case of aliphatic amines where their electro-oxidation led to attachment to the surface such as glassy carbon electrode [19–21] or metal surfaces [27]. Other's work demonstrated that this method could be used for attachment of aliphatic amines to the CNTs [21]. In fact, the electrochemical oxidation mostly involves production of radical cations formed on nitrogen atoms that could covalently attach to the nucleophilic groups. The explanation of this reaction mechanism assumes that radical cations are very reactive and can link to nucleophilic groups such aromatic ring resulting in the rupture of the double bond C=C and formation of C–N bonds [21,22].

It has already been demonstrated that PPy can be modified with photochemical reaction [28]. However, this is the first time that functionalization of PPy film with chemical groups occurs following electrochemical patterning through amine oxidation. The advantage of electro-oxidation is demonstrated by the simplicity of this reaction taking place in a very short time without need of further chemical modification of pyrrole monomer prior polymerization.

PPy-PAMAM film was also studied using electrochemical impedance spectroscopy (EIS) in order to analyse the electrical properties and electron transport of such layer. The analysis have been performed in 10 mM PBS buffer free from redox probe (Fig. 4A). The Nyquist plot of PPy-PAMAM layer was compared with those assigned to non-modified PPy and PPy-PAMAM conjugated through chemical reaction (PPy-CONH-PAMAM) [18]. In this last case, pyrrole monomer prior polymerization was chemically functionalized with activated carboxylic groups allowing attachment of PAMAM G4 via a peptidic link. Nyquist plot of PPy showed a straight line characteristic for a conducting surfaces. PPy-PAMAM layers conjugated either chemically or following electrochemical patterning showed the same electrical behaviour where the semi-circle is obtained. EIS data were fitted with equivalent circuit model shown on Fig. 4B, where R_s is the electrolyte resistance and R_{ct} is the electron transfer resistance which is in serial with CPE; is corresponding to the behaviour of the electron transfer occurring in the PPy. Another CPE_2 is corresponding to ionic transfer in the PPy. CPE behaviour is obtained instead of capacitance when the distribution of time constant is

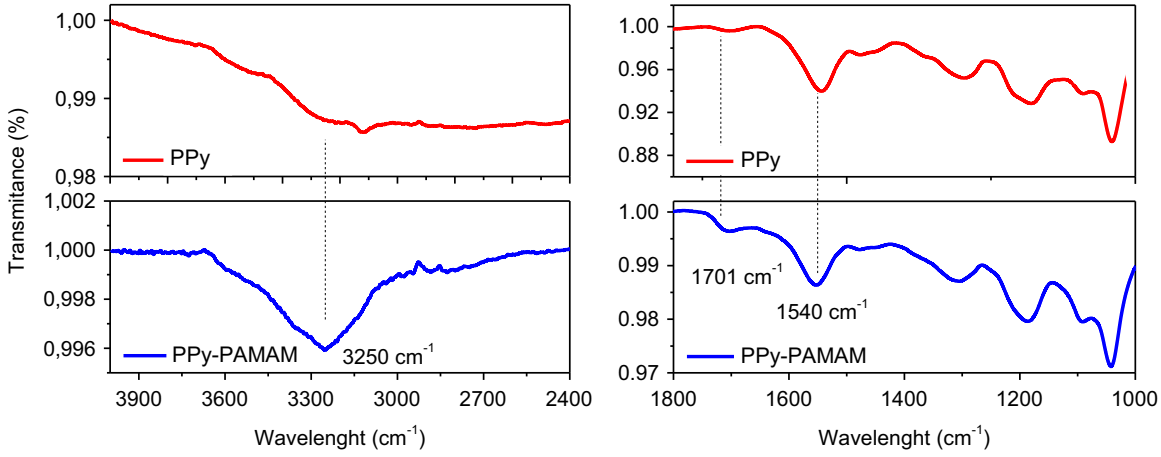


Fig. 2. FT-IR analysis of PPy layer before and after modification with PAMAM G4 dendrimers.

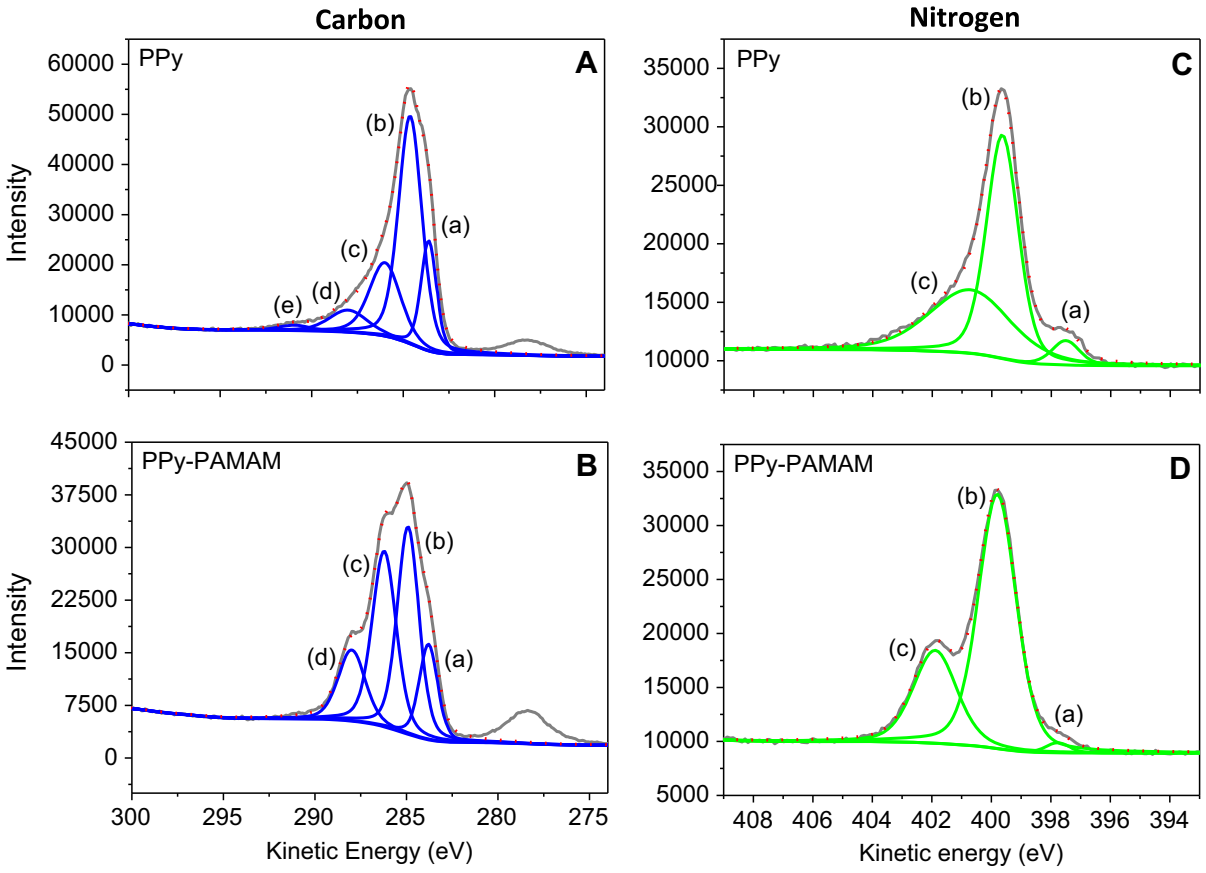


Fig. 3. XPS spectra of PPy layer in (A,B) carbon and (C,D) nitrogen region before and after electro-oxidation of PAMAM G4 dendrimers.

Table 1
Summary of XPS results for PPy and PPy modified with PAMAM G4 dendrimers.

	Carbon				Nitrogen			
	Nr	Bond	eV	FWHM	Nr	Bond	eV	FWHM
PPy	(a)	C–C α	283.60	1.00				
	(b)	C–C	284.62	1.50	(a)	Aromatic	397.51	1.10
	(c)	C–N	286.0	2.00	(b)	N–C, N–H	399.64	1.26
	(d)	Oxide	288.00	2.44	(c)	polaron/biopolaron	400.71	3.25
	(e)	π	290.94	2.02				
PPy-PAMAM	(a)	C–C α	283.80	1.19	(a)	Aromatic	397.76	0.90
	(b)	C–C	284.90	1.40	(b)	N–C, N–H	399.80	1.50
	(c)	C–N	286.21	1.55	(c)	Amide	401.89	1.72
	(d)	C=O, C=N	287.99	1.74				

not linear on the entire surface. The impedance of CPE could be written as $Z_{CPE}=\frac{1}{Q(j\omega)^n}$, where Q and n are independent of the frequency, ω is the radial frequency and n is a dimensional parameter with the value $-1<n<1$; for $n=1$ a pure capacitance is obtained; if $n=0.5$ Warburg impedance is obtained, and when $0<n<1$ the impedance is CPE. The analytical treatment of impedance data allows the demonstration of CPE behaviour. This could be obtained by plotting the logarithm of real part of impedance (log Z′) vs. logarithm of frequency (log f); the value of n determines the CPE behaviour. In this study, n was around 0.8 suggesting that CPE could replace the capacitance. Based on the EIS data fitting, R_{ct} value increased after PAMAM G4 attachment to PPy layer in the two approaches: after conjugation of PAMAM G4 by chemical and electrochemical methods. As the measurement was performed in label-free solution, without redox probe the measured charge transfer is corresponding to the electrical properties of PPy layer and its conductivity. In both case, modification of PPy by chemical or electrochemical methods led to decrease in the conductivity. This could be due to the large size of PAMAM G4 macromolecules that affects the kinetic of charge transfer of conducting PPy layer.

3.2. Kinetic characterization of PPy-PAMAM composite after association with redox marker

Association of a ferrocene as redox marker to the PPy-PAMAM layer was carried out to enhance the kinetics of charge transfer within the PPy layer and to follow electrochemical behaviour of

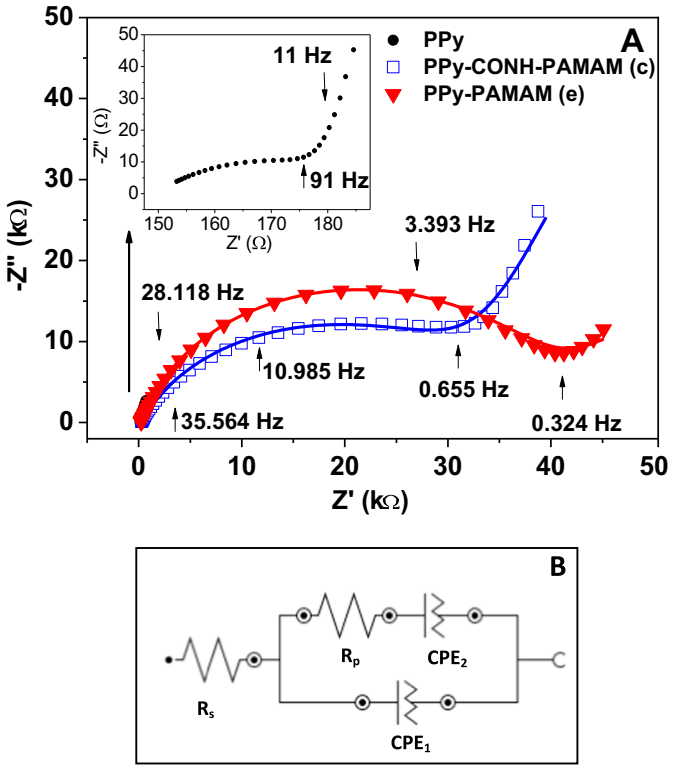


Fig. 4. (A) Nyquist plot obtained in 10 mM of PBS, pH 7.4 for PPy layer (•) and after modification with PAMAM G4 by chemical grafting (c, □) and electrochemical pattering (e, ▾). The impedance measurements were obtained in frequency range from 0.1 Hz to 100 kHz at 0.10 V vs. Ag/AgCl by applying DC potential of 10 mV. The symbols are the experimental data and the solids are the fitted curves using the equivalent circuit shown in (B).

material after such modification. Ferrocene was chemically modified with two carboxylic groups activated with phthalimide that allowed attachment to amines of PAMAM G4 present on the PPy-PAMAM surface forming PPy-PAMAM-Fc material. Covalent attachment of ferrocenyl groups to PAMAM G4 was studied by CV. Fig. 5A shows characteristic reversible redox signal of ferrocene obtained after attachment on PPy-PAMAM composite, with oxidation and reduction peaks at 0.15 V and 0.05 V (vs. Ag/AgCl), respectively. The surface coverage of ferrocenyl groups, bonded to the PPy-PAMAM can be then calculated by the charge exchanged during redox process, following the equation:

$$\Gamma = \frac{Q}{nFA} \quad (1)$$

where Q is the charge under the cathodic or anodic waves, n is number of electrons involved in the redox process, F is the Faraday constant, and A is the area of the electrode. Based on Eq. (1) we calculated the average coverage of the surface as 18.9 ± 1.14 nmole cm^{-2}. Comparing to the composite where PAMAM G4 were covalently linked to the PPy layer [18], the surface coverage increased 6 times (see Table 2).

To underline the influence of dendrimers size on the increasing active surface and quantity of attached ferrocene, additional experiment was performed. PPy was modified via electrochemical patterning with ethylenediamine (EDA) or PAMAM dendrimers of different generation: 2nd, 4th or 6th (PAMAM G2, PAMAM G4, PAMAM G6), which possess 16, 64 and 256 amine groups respectively. Then ferrocene was covalently linked to such surfaces and electrochemical response was studied using CV technique. PPy-EDA composite gave the lowest signal of ferrocene, which is due to the presence of fewer free amine groups on the surface that

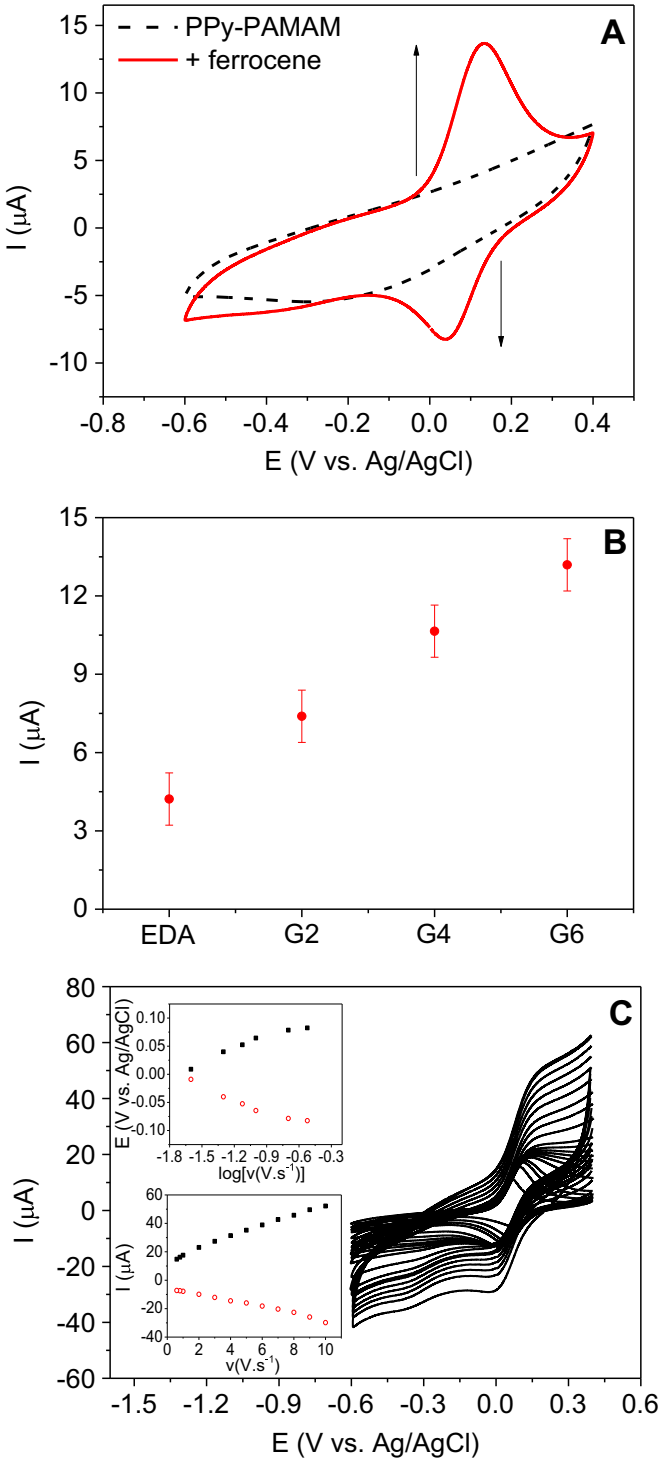


Fig. 5. (A) Cyclic voltammogram of electrochemical signal of ferrocenyl group linked to PPy modified with PAMAM G4 dendrimers, PPy-PAMAM-Fc (solid red line) compared to PPy-PAMAM (dotted black line) before modification obtained in PBS buffer, pH 7.4 with scan rate 50 mV s^{-1}; (B) comparison of the oxidation current corresponding to ferrocene when PPy was modified with: EDA, PAMAM G2, PAMAM G4, PAMAM G6; (C) CVs of a gold electrode covered by PPy-PAMAM-Fc at various scan rate in the range 0.005–1 V s^{-1}. The lower inset shows the variation of anodic and cathodic current peaks vs. scan rate and upper inset shows the variation of anodic ($E_{p,a} - E°$) and cathodic potentials ($E_{p,c} - E°$) vs. logarithm of scan rate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

can be attached with ferrocene. Modification of PPy with various size of dendrimers greatly increased the coverage of the surface with the redox marker which was the highest for PAMAM G6.

Table 2
Comparison of parameters of PPy-PAMAM-Fc layer obtained by electrochemical pattering (e) or chemical reaction with PPy modified with carboxylic groups (c).

	Γ (nmole $cm^{-2)}$	k_s (s^{-1})	k_{et} (s^{-1})	$10 \times {}^{6}D$ ($cm^2 s^{-1}$)	Ref.
PPy-PA-MAM-Fc (e)	18.87 ± 1.14	1.6 ± 0.01	0.16 ± 0.01	5.27 ± 0.01	This work
PPy-CONH-PAMAM-Fc (c)	3.00 ± 0.04	5.8 ± 0.3	0.50 ± 0.03	5.6 ± 0.3	[18]

Dependence of the oxidation current corresponding to redox signal of ferrocene and the generation of PAMAM attached to the PPy layer was found (Fig. 5B). In fact, the numerous chemical groups of dendrimers and its globular structure allowed to improve the density of functional groups and then the quantity of attached molecules on the surface.

Kinetic parameters of PPy-PAMAM nanocomposite was then studied. The variation of the redox signal with the scan rate was analyzed by varying the potential from 0.005 V s^{-1} to 10 V s^{-1}. The CV curves show an increase in current with increasing scan rate (Fig. 5C). The increase in the anodic and cathodic currents with the scan rate (Fig. 5C, lower inset) was linear from 0.005 V s^{-1} to 10 V s^{-1}. This demonstrates the surface-controlled process of charge transfer [29]. At relatively low scan rate as 5 mV s^{-1}, there was no difference between potentials of anodic and cathode peaks, demonstrating the reversible redox process. At high scan rate of 10 V s^{-1}, the anodic and cathodic peaks were still well defined and the difference between potentials of anodic peaks at high scan rates (ΔE_p) was less than 0.2 V, indicating a significant rate of electron transfer.

In order to study the electrochemical properties of the layer, the parameters of PPy-PAMAM-Fc material were analyzed by means of electron transfer kinetics of ferrocene. The permeability of the layer to ions diffusion as well as redox process was studied by measuring diffusion coefficient and rate constant of redox species from solution to modified electrode.

The rate of heterogeneous electron transfer (k_s) of PPy-PA-MAM-Fc layer was determined following the Laviron model, by measuring the variation of redox peak potential within scan rate (Fig. 5B). This can be obtained following the equation developed by Laviron (1979) with the Butler-Volmer model [30]:

$$m=\left(\frac{RT}{F}\right)\frac{k_s}{nv} \tag{2}$$

$$k_s=m\left(\frac{F}{RT}\right)nv \tag{3}$$

The value m was determined by adjustment of the curve $\Delta Ep=f(m^{-1})$ for the coefficient of electron transfer $\alpha=0.5$. The coefficient α was determined based on the variation in anodic potential ($E_{p,a}-E^{\circ}$) and cathodic potential ($E_{p,c}-E^{\circ}$) vs. logarithm of the scan rate (Fig. 5B, upper inset).

The k_s value of PPy-PAMAM-Fc was calculated as $(1.67 \pm 0.01)\ s^{-1}$. This value is lower comparing to k_s $[(5.8 \pm 0.3)\ s^{-1}]$ of PPy-CONH-PAMAM-Fc layer obtained by covalent binding of PAMAM G4 to modified PPy film [19]. It can be related to larger modification of the surface with the ferrocenyl groups leading to important properties of redox polymers where the electron transfer occurs through electron hopping process. This could be also explained by the decrease of the conductivity of the PPy after such attachment where some conjugation could be broken after electrochemical patterning method.

To confirm this parameter, the heterogeneous electron transfer through layers was determined using the impedance data. EIS measurement provides details in kinetics of charge transfer through the biolayer occurring at the electrodes. The apparent heterogeneous electron transfer rate constant (k_{et}) can be calculated as follows:

$$k_{et}=\frac{1}{2R_{ct}CPE_2}; \tag{4}$$

where R_{ct} is the value of charge transfer resistance and CPE_2 is the value of constant phase element obtained from the fitted parameters of the Nyquist plot (data not shown). The k_{et} values (Table 2) were lower comparing to k_s, when PPy-PAMAM material was obtained by electrochemical as well as chemical methods.

Another parameter that controls the redox process is the permeability of the layer to ion diffusion. The permeability of the layer could be determined by measuring the diffusion coefficient of redox species from buffered solution to the electrode. This parameter could be obtained from the Cottrell law (Eq. (5)) by measuring the current vs. time during chronoamperometric reaction performed in solution containing 5 mM/5 mM $[Fe(CN)_6]^{4-}/[Fe(CN)_6]^{3-}$:

$$i(t) = nFAC^*\sqrt{\frac{D}{\pi t}} \tag{5}$$

where n is number of electrons involved in the redox process, F is the Faraday constant, A is the area of the electrode and C^* is the bulk concentration of Fe^{2+}/Fe^{3+}. Under diffusion control, $i=f(t^{-1/2})$ is linear and D value can be calculated based on the slope of this curve. The diffusion coefficient of $[Fe(CN)_6]^{4-}/[Fe(CN)_6]^{3-}$ ions on the PPy-PAMAM-Fc layer was determined as $(5.27 \pm 0.01) \times 10^{-6}\ cm^2 s^{-1}$. These results are similar with those obtained for PPy-CONH-PAMAM-Fc, where PPy and PAMAM G4 were conjugated via chemical binding (Table 2). This demonstrates that both PPy layers have the same behaviour regarding the diffusion of electroactive species to the film.

The obtained kinetic parameters for PPy-PAMAM-Fc composite suggest that modification of PPy film by electro-oxidation process does not affect the electrochemical properties of polymer and can be used as an efficient method for polymer functionalization.

3.3. Application of PPy-PAMAM-Fc as a transducer for DNA detection

The generated PPy-PAMAM-Fc material was applied as transducer in construction of DNA sensor. Preparation of biosensor consisted of covalent attachment of single-stranded DNA probe (ssDNA) of 15 bases modified with amine group on 5′ end to PPy-PAMAM-Fc. Ferrocene bearing two activated esters acts as a linker: one of these groups was attached to amines of PAMAM G4 present on the PPy-PAMAM surface; the second ester covalently bonded the ssDNA probe forming an amide link, following the procedure described in Section 2. Then, ethanolamine (EtOH) was used to saturate all phtalymidyl esters of ferrocene not bonded with ssDNA probe. The construction of electrochemical biosensor was monitored by square wave voltammetry (SWV) method in 10 mM of PBS buffer pH 7.4. Fig. 6A shows the voltammograms performed after each step of biosensor construction which demonstrates a decrease of current after ssDNA attachment and saturation with EtOH. This behaviour could be related to the low electron transfer of the ferrocenyl groups or to the slow diffusion of electrolyte to the surface during redox process due to the attachment of the large molecules such as DNA as reported in the previous work [4].

The hybridization of complementary target (DNA_C) of 15 bases was performed by immersing the modified electrode in target solution and incubation for 1 h at 37 °C. Wide range of DNA_C concentrations was tested; the reaction was performed in

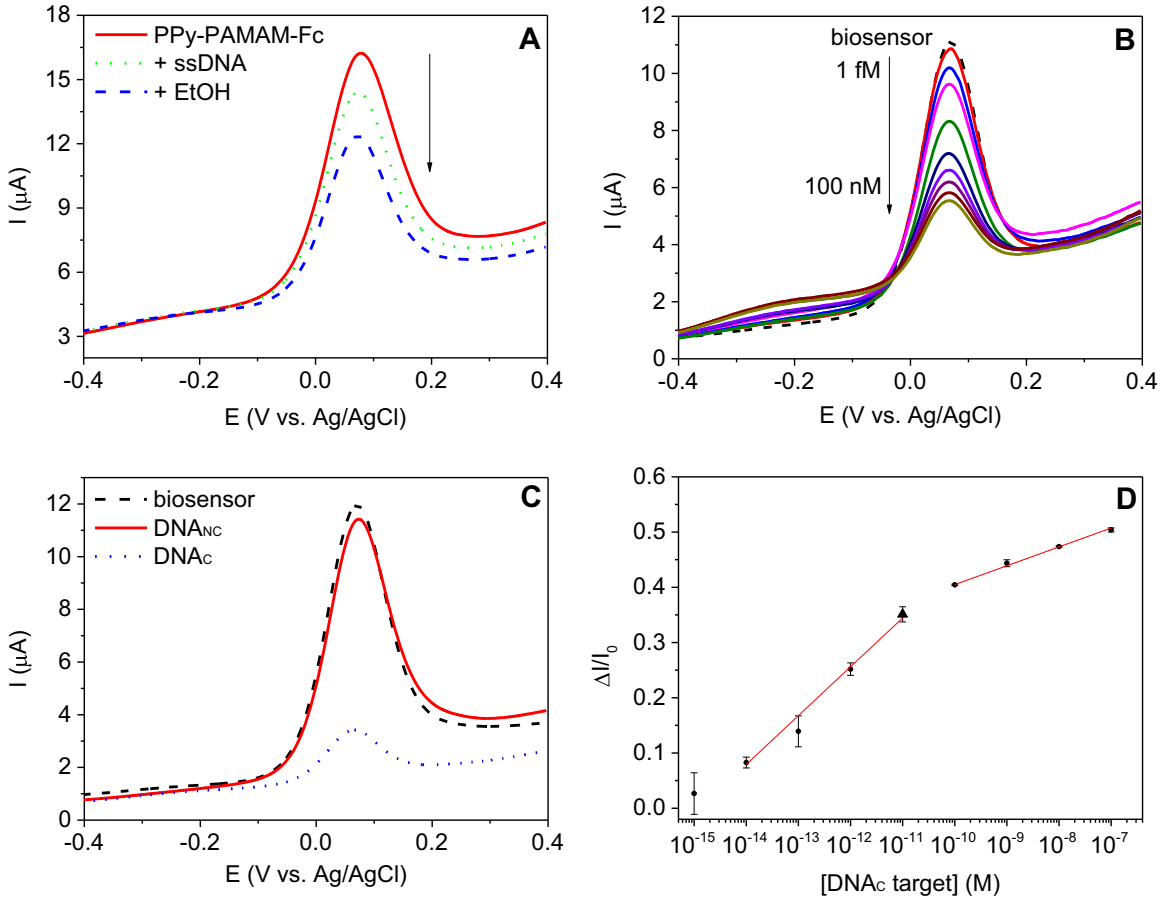


Fig. 6. (A) SWV data obtained after each step of biosensor formation: PPy-PAMAM-Fc (solid red line), PPy-PAMAM-Fc-ssDNA (doted green line), PPy-PAMAM-Fc-ssDNA-EtOH (dashed blue line). (B) biosensor's response after incubation of various DNA_C concentrations (100 fM–100 nM). (C) the results obtained for non-specific interactions compared to specific one. Black curves show SWV of biosensor after formation, red and blue curves show respectively response of biosensor after incubation in solution of 1 μM DNA_{NC} target and 1 μM of DNA_C target. All SWVs analysis were recorded in 10 mM PBS solution at pH 7.4 with modulation amplitude 20 mV, frequency 50 Hz and conditioning time 120 s. (D) The plot of the relative changes of the current peak vs. concentration of DNA target for the biosensor. $\Delta I=(I_0-I)$, where I_0 is the peak current prior addition of DNA target and I after incubation of the sensor with certain concentration of target. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

solutions containing from 1 fM to 100 nM of specific target. The electrochemical detection of DNA_C was directly monitored by redox signal of ferrocene. Fig. 6B exhibits the SWV plots of biosensor before reaction and after incubation with DNA_C of various concentrations. The current corresponding to ferrocene decreased with the increase of DNA_C concentrations. This decrease in the current is proportional to the amount of hybridized DNA_C with ssDNA probe immobilized on the surface and starts from low concentration of 10 fM which suggests high sensitivity of this biosensor. However, incubation of biosensor with highly concentrated solution (1 μM) of non-complementary DNA_{NC} target of 14 bases resulted in very week variation in electrochemical signal suggesting none non-specific interactions (Fig. 6C).

Based on the changes of current corresponding to redox signal of ferrocene, obtained after incubation of biosensor with each DNA_C concentration, the calibration curve was plotted (Fig. 6D). Calibration curve is represented in logarithmic scale in order to demonstrate the large dynamic range of detection and exhibits two linear parts: from 10 fM to 10 pM and from 100 pM to 100 nM. This behaviour can be explained by morphology of PPy-PAMAM composite. The structure of this nanomaterial is three-dimensional which results in different availability of attached DNA probes to DNA targets. DNA target first saturates the most accessible probes. However, with the increasing of DNA target concentration it interacts also with bioreceptors located far from the PPy-PAMAM surface, which changes the slope of calibration curve. Detection limit calculated based on signal-to-noise ratio of 3 was 0.4 fM. These measurements were highly reproducible; relative standard deviation of 0.5–3.3% for 4 independents measurements was obtained.

4. Conclusions

In this work we report a novel approach of PPy functionalization with the macromolecules bearing amine groups based on electrochemical patterning. We demonstrates that the functionalization of PPy layer with PAMAM G4 on the polymer surface could be performed through electro-oxidation of the amine groups which leads to amine functionalities on PPy layer. Such functions could be employed for further modification of PPy. This was demonstrated through covalent attachment of modified ferrocene. We show the maintenance of electrical properties of PPy compared to the chemical functionalization of polymer. The advantage of electrochemical patterning method used for PAMAM G4 association is demonstrated by the simplicity of this reaction, without the need of previous chemical modifications of PPy. Electrochemical patterning can be generalized for functionalization of PPy with different molecules bearing amine groups. Additionally, this technique allowed obtaining a homogenous distribution of molecules on the surface and hence, resultant PPy-PAMAM-Fc composite exhibited efficient electron transfer with the reversible

redox system. The PPy-PAMAM-Fc nanomaterial was successfully used as a transducer for DNA detection by monitoring the current corresponding to ferrocene redox marker after incubation of biosensor in various complementary DNA_C target concentrations. We have shown that the biosensor is very sensitive to detection of DNA_C target and detection limit of 0.4 fM was obtained. Moreover, the developed electrochemical DNA sensor could be generalized for detection of various other analytes involving their specific bioreceptors on PPy-PAMAM-Fc layer.

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