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Electropolymerized biotinylated poly (pyrrole–viologen) film as platform for the development of reagentless impedimetric immunosensors

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ABSTRACT

We describe herein the synthesis and electrochemical polymerization of a viologen monomer functionalized by a biotin group and the use of its redox properties for the impedimetric sensing of protein binding. The electrochemical oxidation of the pyrrole group at 0.85 V in CH_3CN led to a biotinylated polypyrrole film allowing the successive anchoring of an avidin layer and a biotinylated cholera toxin layer. EIS performed at −0.45 V/SCE without redox probe in solution showed the specific immobilization of the anti-cholera toxin antibody.

1. Introduction

Immunosensors based on the highly sensitive and selective nature of the recognition between antigen (Ag) and antibody (Ab), are the focus of intensive research due to the widespread potential applications such as medical detection, processing quality control, and environmental monitoring [1,2]. A key requirement in the immunosensor development is the reproducible and accurate immobilization of biomolecules onto the transducer surface without loss of their recognition properties. To ensure this, an attractive immobilization procedure consists in the use of electropolymerized films that present specific functional groups for biomolecules coupling [3]. In particular, biotinylated polymers, because of their ability of anchoring biotinylated biomolecules through the formation of an avidin bridge by affinity interactions between biotin and avidin, have emerged as promising materials for the development of immunosensors [4–6]. This immobilization strategy involves solely a single attachment point preserving an excellent accessibility to each immobilized biomolecule. A second key step concerns the direct transduction of the immunoreaction without additional labeling of the target. The impedimetric immunosensors have thus attracted extensive interest in the sensing formation of Ag–Ab [7,8]. An advantage of Electrochemical Impedance Spectroscopy (EIS) is that labels are no longer necessary, leading to an easier and faster detection procedure. The interaction between an antibody and a surface immobilized antigen, however, is often inadequate to generate a highly sensitive signal for the direct impedance measurement. Consequently, a redox probe, such as hexacyano-ferrate anions, was conventionally added in the aqueous phase.

With the aim of developing reagentless electrochemical impedance transduction method and improving their sensitivity, we report here the electrogeneration of a biotinylated redox polymer and its application to the direct impedimetric detection of an anti-cholera toxin Ab, without redox probe in the aqueous phase.

2. Materials and methods

2.1. Reagents

CH_3CN (Rathburn, HPLC grade), nBu_4NClO_4 (Fluka) and hydroquinone (Sigma) were used as received.

3-Bromopropyl pyrrole was prepared as follows: a stoichiometric amount of 3-bromopropylamine hydrobromide (10.943 g, 50.0 mmol) and dimethoxytetrahydrofurane (6.5 mL, 50.0 mmol) was stirred at 70 °C for 2.5 h with 1.1 equivalent of sodium acetate (7.538 g, 55.4 mmol) in 100 mL of a H_2O/acetic acid mixture (3/2, v/v). The aqueous layer was separated and extracted with Et_2O. The organic layers were collected, washed with water and brine and dried over sodium sulfate. After evaporation to dryness, the crude residue was purified by filtration on silica gel using hexane as eluant. After evaporation of hexane, the pure bromo-pyrrole (5.9648 g, 31.7 mmol) was obtained in 63% yield.

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^{1}H NMR (250 MHz, $CDCl_3$): δ ppm = 6.73 (2H, s); 6.21 (2H, s); 4.12 (2H, *t*, *J* = 6.3 Hz); 3.35 (2H, *t*, *J* = 6.3 Hz); 2.30 (2H, *quint*, *J* = 6.3 Hz).

^{13}C NMR (62.5 MHz, $CDCl_3$): δ ppm = 120.69 (CH); 108.49 (CH); 47.10 (CH_2); 34.24 (CH_2); 30.41 (CH_2).

N-(3-pyrrol-1-ylpropyl)-4-(4′-pyridyl) pyridinium was synthesized as follows: a mixture of bromo-pyrrole (0.4715 g, 2.5 mmol) and 2 equivalents of 4,4′-bipyridine (0.7819 g, 5.0 mmol) was stirred at 85 °C for 20 h in DMF (5 mL). After solvent evaporation, H_20 was added and the aqueous layer was extracted three times with CH_2Cl_2. After evaporation of water, the mono-alkylated product was dried under vacuum and thus obtained in 87% yield (0.7536 g, 2.19 mmol).

^{1}H NMR(250 MHz, DMSO-d_6): δ ppm = 9.15 (2H, *d*, *J* = 6.8 Hz); 8.87 (2H, *d*, *J* = 4.3 Hz); 8.60 (2H, *d*, *J* = 6.8 Hz); 8.03 (2H, *d*, *J* = 4.3 Hz); 6.75 (2H, *s*); 5.96 (2H, *s*); 4.65 (2H, *t*, *J* = 6.8 Hz); 4.03 (2H, *t*, *J* = 6.8 Hz); ~2.50 (2H).

^{13}C NMR (62.5 MHz, CD_3OD): δ ppm = 154.41; 151.70; 146.37; 143.32; 126.91; 123.42; 121.59; 109.50; 60.53; 47.28; 33.25.

3-Bromopropyl biotin was prepared as follows: a mixture of biotin (0.4877 g, 2.0 mmol), 3-bromopropan-1-ol (1.3880 g, 10.0 mmol) and *p*-toluene sulfonic acid (0.0380 g, 0.2 mmol) was stirred and refluxed at 120 °C in toluene (12 mL) for 72 h under nitrogen atmosphere. After toluene evaporation, the residue was dissolved in CH_2Cl_2, precipitated by adding Et_2O, filtered off and purified by silica gel column chromatography (CH_2Cl_2/MeOH, 9/1) providing a white solid (81% yield).

^{1}H NMR(250 MHz, $CDCl_3$): δ ppm = 5.27 (1H, *s*); 4.92 (1H, *s*); 4.52 (1H, *m*); 4.33 (1H, *m*); 4.21 (2H, *t*, *J* = 6.3 Hz); 3.47 (2H, *t*, *J* = 6.7 Hz); 3.17 (1H, *m*); 2.93 (1H, *dd*); 2.74 (1H, *d*, *J* = 12.7 Hz); 2.34 (2H, *t*, *J* = 7.1 Hz); 2.18 (2H, *quint*, *J* = 6.3 Hz); 1.71–1.44 (6H, *m*).

The biotinylated monomer (**1**) was synthesized as follows: a stoichiometric amount of 3-bromopropyl biotin (0.1434 g, 0.39 mmol) and N-(3-pyrrol-1-ylpropyl)-4-(4′-pyridyl) pyridinium (0.1248 g, 0.39 mmol) was stirred at 60 °C in EtOH (5 mL) for 12 days. The reaction mixture was cooled at room temperature and the ethanol was evaporated under vacuum. The residue was dissolved in MeOH (1 mL), precipitated by adding CH_2Cl_2 and filtered off. After anionic exchange, **1** with BF_4^- counter anions was thus obtained in 45% yield.

^{1}H NMR(250 MHz, CD_3OD): 9.34 (2H, *d*); 9.11 (2H, *d*); 8.70 (2H, *d*); 8.59 (2H, *d*); 6.69 (2H, *s*); 6.00 (2H, *s*); 4.76 (2H, *t*); 4.49 (1H, *m*); 4.32–4.16 (4H, *m*); 3.20 (1H, *m*); 2.92 (1H, *m*); 2.73–2.62 (4H, *m*); 2.49 (2H, *t*); 2.29 (2H, *t*); 1.64–1.29 (6H, *m*); 1.18 (2H, *t*). MS (ESI): *m/z* = 628.1 ($[M^{2+}+Br^-]^+$) and 274.8 (M^{2+}).

2.2. Electrochemical instrumentation and procedures

Electrochemical investigations were performed under an argon atmosphere in a conventional three-electrode cell with a potentiostat EG&G Princeton Applied Research 273 A in conjunction with a Kipp and Zonen BD90 XY/t recorder. A 10^{-2} mol L^{-1} Ag^+/Ag in CH_3CN electrode and a saturated calomel electrode (SCE) were used as reference electrode in CH_3CN and aqueous media, respectively. The working electrodes were platinum or glassy carbon disks (diameters 5 and 3 mm) polished with 2 μm diamond paste (MECAPREX Press PM).

Electrochemical Impedance Spectroscopy (EIS) was carried out with an Autolab potentiostat 100 (Eco Chemie, Utrecht, The Netherlands) using FRA software. A ZView software (Scribner Associates Inc.) was used to simulate the data thus obtained using an appropriate equivalent electrical circuit. All experimental impedance spectra were recorded by immersing the modified electrode into phosphate buffer (0.1 mol L^{-1}, pH 7). The frequency sweep was from 50 to 0.1 Hz at −0.45 V vs. SCE with an alternating voltage of 5 mV rms.

The permeability of the poly **1** was determined by using rotating-disc electrode (RDE) experiments carried out at different rotation rates in 0.1 mol L^{-1} phosphate buffer (pH 7) containing hydroquinone (2×10^{-3} mol L^{-1}). Stationary current was recorded between 0 and 0.5 V vs. SCE.

2.3. Avidin-alkaline phosphatase immobilization and characterization

UV–vis measurements were obtained using a Cary 1 absorption spectrophotometer. The poly**1**-Av-AP electrodes were prepared by incubation of an electrode modified by a film of poly (N-(3-pyrrol-1-ylpropyl)-N′-(3-propyl biotin)-4, 4′-bipyridinium) (poly**1**, $\Gamma = 1.58 \times 10^{-9}$ mol cm^{-2}) with Av-AP (20 μL, 0.5 mg mL^{-1}) for 30 min. The resulting enzyme electrodes were thoroughly washed in stirred Tris–HCl buffer for 30 min and soaked into 3 mL of Tris–HCl buffer (0.05 mol L^{-1}, pH 9.4) containing *p*-nitrophenylphosphate (10^{-2} mol L^{-1}) and $MgCl_2$ (2×10^{-3} mol L^{-1}). The Av-AP concentration on surface was measured by following at 410 nm the absorbance of *p*-nitrophenol produced from *p*-nitrophenyl phosphate as a function of time.

2.4. Immunoassay design

A drop (20 μL) of avidin (0.5 mg mL^{-1} in phosphate buffer) was incubated for 30 min onto the poly**1**. After rinsing with phosphate buffer, the resulting modified electrodes were incubated for 20 min with 20 μL of biotinylated Cholera Toxin B Subunit (CTB) (0.5 mg mL^{-1} in phosphate buffer). The resulting electrodes were rinsed and washed once with phosphate buffer for 10 min. The analyte, anti-Cholera Toxin B Subunit (anti-CTB) antibody (0.5 mg mL^{-1}), was then spread on the resulting electrode surface for 20 min.

3. Results and discussion

In this study a novel electropolymerizable biotin-labeled viologen functionalized by a pyrrole group (Fig. 1), was prepared by reaction of 3-bromoethyl biotin with N-(3-pyrrol-1-ylpropyl)-4-(4′-pyridyl) pyridinium and characterized by ^{1}H NMR and mass spectrometry. The 3-bromoethyl biotin was prepared by esterification of 3-bromopropanol and biotin. The electrochemical behaviour of biotinylated pyrrole–viologen **1** (2 mM) was investigated in CH_3CN + 0.1 mol L^{-1} nBu_4NClO_4 (Fig. 2A). Upon reductive scanning, the monomer exhibits two successive reversible peak systems at −0.70 V (ΔE_p = 0.06 V) and −1.12 V (ΔE_p = 0.06 V) corresponding to the successive one electron reduction of the viologen group (V^{2+}/V^{+} and V^{+}/V^{0}) [9,10]. Upon oxidative scanning, the cyclic voltammogram displays an irreversible peak at 1.0 V reflecting the oxidation of the pyrrole group. This potential value is similar to those previously reported for N-alkylpyrroles [11].

Electropolymerization of **1** was performed by controlled potential electrolysis (0.1 mC) at 0.85 V. Upon transfer into CH_3CN + 0.1 M nBu_4NClO_4 solution free of monomer, the cyclic vol-

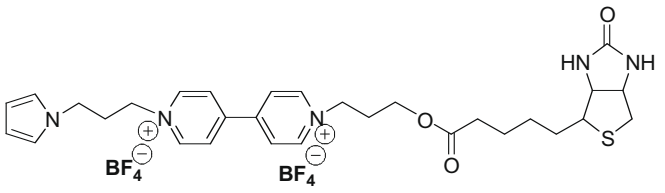

Fig. 1. Structure of biotinylated pyrrole–viologen: **1**.

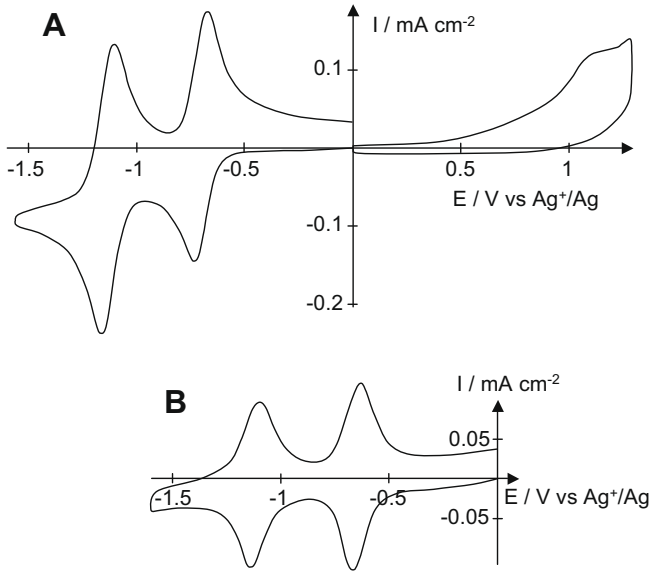

Fig. 2. Cyclic voltammograms recorded at a vitreous carbon electrode (diameter 3 mm) of (A) monomer **1** (2×10^{-3} mol L^{-1}) in CH_3CN + 0.1 mol L^{-1} nBu_4NClO_4; (B) poly **1** ($\Gamma = 1.58 \times 10^{-9}$ mol cm^{-2}) electrode in CH_3CN + 0.1 M nBu_4NClO_4. Scan rate 0.2 V s^{-1}.

tammogram of the resulting electrode exhibits two redox peak systems in the negative region at the same potential values (−0.67 V; ΔE_p = 0.04 V and −1.13 V; ΔE_p = 0.04 V) than those observed for the monomer (Fig. 2B). The apparent surface coverage of the electropolymerized **1** ($\Gamma = 1.58 \times 10^{-9}$ mol cm^{-2}) was determined from the charge recorded under the first reduction wave of the viologen groups leading to an electric yield of 24.8% for the polymerization process. As previously reported for the estimation of the thickness of poly(pyrrole-lactosyl) films [12], this value of poly **1** coverage (1.58×10^{-9} mol cm^{-2}) corresponds to a polymer thickness of 12.7 nm. The mass-transfer process through the poly**1** film was investigated by rotating disk electrode experiments with hydroquinone (2×10^{-3} mol L^{-1}) as a redox probe in phosphate buffer (pH 7). Thanks to the presence of hydrophilic V^{2+} groups, the poly**1** exhibits a higher permeability (8.5×10^{-2} cm^2 s^{-1}) in aqueous solution than those recorded for conventional biotinylated films [11,13].

The ability to immobilize avidin on poly**1** has been examined with an avidin-labeled alkaline phosphatase (Av-AP) as biomolecule model. After the specific anchoring of Av-AP, the phosphohydrolytic activity of alkaline phosphatase was determined by spectrophotometric measurements and correlated to a surface concentration of avidin, namely 6.5×10^{-12} mol cm^{-2}. Taking into account that the maximum coverage corresponding to a close-packed one molecular layer of avidin was estimated between 3.3 and 5×10^{12} molecules cm^{-2} ($5.5–8.3 \times 10^{-12}$ mol cm^{-2}) [11], this avidin immobilization corresponds to the formation of a compact avidin layer illustrating the efficiency of the polymerized biotin groups for avidin anchoring.

The possibility of using the redox properties of this biotinylated film for the impedimetric transduction of an immunoreaction without redox probe in solution, was investigated with a cholera toxin (Ag) and an anti-cholera toxin (Ab) as model system. EIS was thus performed at −0.45 V/SCE corresponding to the first reduction of the polymerized V^{2+} for characterizing the polymer and the successive binding of proteins (Fig. 3). An equivalent circuit was designed to fit the impedance spectra obtained. It appears that the high frequency semicircle of the Nyquist diagram corresponds to the charge transfer resistance, R_{CT}, related to the one

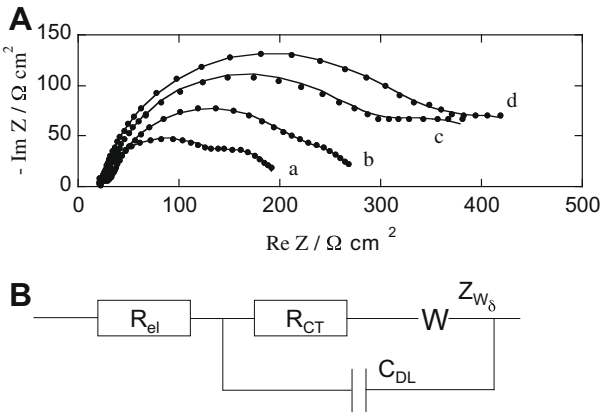

Fig. 3. (A) Nyquist plots of impedance of (a) a poly1 electrode and after successive incubation in 0.5 mg mL^{-1} of (b) avidin, (c) biotinylated cholera Toxin B and (d) anti-cholera toxin. RDE: 2000 rpm, CV: 5 mm, E_{app} = −0.45 V vs. SCE and ac signal = 5 mV rms from 50 kHz to 10^{-1} Hz; experimental data (symbols), fitting results (line). (B) Equivalent circuit for fitting the plots: R_{el}: electrolyte resistance; R_{CT}: charge transfer resistance; C_{DL}: double layer capacitance; Z_{Wd}: finite length Warburg impedance.

electron reduction of V^{2+} in parallel of double layer capacitance, C_{DL}. The low frequency loop was attributed to the finite length Warburg, W_δ, resulting from the diffusion of counter-ion caused by the change in the total charge of poly**1** during the reduction. The excellent concordance between the lines and experimental data reflects the validity of the electric model (Fig. 3). Thus the successive anchoring of avidin and biotinylated Ag on poly**1** was carried out by avidin–biotin interactions. The anti-cholera toxin was then bound by immunoreaction, on the immobilized Ag. As expected, the successive binding of avidin, biotinylated Ag and Ab induce a marked increase in the impedance response. Table 1 summarizes the parameters obtained from equivalent circuit fitting for the Nyquist diagrams. It appears that the charge transfer resistance increases with the number of biologic layers, which means that the number of electronic transfer due to the reduction of the polymerized V^{2+} decreases. As expected, the double layer capacitance decreases and therefore its length increases with the addition of biomolecules. A conventional value of about 20 μF cm^{-2} was determined in aqueous solution. The relaxation frequency decrease may be ascribed to the slowdown of electronic transfer due to the construction of the assembly on the poly**1** film. For the Warburg impedance, the diffusion resistance, R_δ, and the relaxation time, t, increase with the number of protein layers, which means that counter-ions have more difficulty penetrating the film. The marked difference in R_δ with (220 Ω cm^2) and without Ab (160 Ω cm^2) clearly illustrates the possibility to use the polymerized V^{2+} system for the reagentless detection of an antibody by EIS. Control experiments carried out with avidin-poly**1** film incubated with anti-cholera toxin have indicated no change in impedance. This demonstrates that the preceding immunosen-

Table 1
Values of equivalent circuit elements obtained for fitting of the experimental data in Fig. 3.

Spectrum in Fig. 3	R_{CT}/Ω cm^2	C_{DL}/μF cm^{-2}	F/Hz	R_δ/Ω cm^2	t/s
a	106 ± 4	26.5 ± 0.8	57	66 ± 2	0.18
b	183 ± 6	20.8 ± 0.7	42	71 ± 3	0.24
c	252 ± 8	19.2 ± 0.6	33	160 ± 5	0.69
d	285 ± 9	18.6 ± 0.6	0	220 ± 7	1.56

R_{CT}: charge transfer resistance; C_{DL}: double layer capacitance; F: relaxation frequency; R_δ: diffusion resistance; t: relaxation time of diffusion.

sor response was not due to non-specific binding of proteins on the avidin-polymer coating.

4. Conclusion

The results described herein demonstrate that the fact to confer electroactive properties to biotinylated films constitutes an elegant way for the development of reagentless impedimetric immunosensors.

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