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Electrogeneration of a biotinylated poly(pyrrole–ruthenium(II)) film for the construction of photoelectrochemical immunosensor†

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Received (in Cambridge, UK) 15th July 2004, Accepted 6th August 2004
First published as an Advance Article on the web 17th September 2004

A biotinylated photosensitive polymer was electrogenerated from on a ruthenium complex bearing biotin and pyrrole groups; the resulting polypyrrolic film allowed the bioaffine immobilisation of avidin and biotinylated cholera toxin and the photoelectrochemical detection of the corresponding antibody.

The development of non-manual immobilization methods of biomolecules on surface attracted a continuous substantial attention due to the exponential emergence of immunosensors and biochips as valuable tools in diagnostic laboratories and medical treatment. Among the conventional immobilization procedures, the strong affinity interactions between the glycoprotein avidin and four biotins, a vitamin (association constant $K_a = 10^{15}$ M^{-1})[1] have been extensively used for binding biological species to surfaces in various fields such as immunohistochemistry,[2] enzyme-linked immunoassay (ELISA)[3] and DNA hybridization.[4] The anchoring of the protein or oligonucleotide monolayer was performed by the formation of avidin–biotin bridges between biotinylated surfaces and avidin-conjugated enzymes, or biotinylated enzymes, antibodies, bacteria or oligonucleotides.[5] The avidin–biotin technique constitutes one of the few immobilization procedures involving solely a single attachment point of the biomolecule facilitating thus recognition phenomenon such as immunoreaction, hybridization or protein-ligand system. This affinity-driven immobilization method was mainly coupled with sophisticated fluorescent detection technologies.[6] These transductions, however, require an additional labeling step of the target.

With the aim of developing alternative transduction approach free of label, we report here, the electrogeneration of the first example of a biotinylated redox polypyrrole film allowing both the immobilization of biomolecules and the detection of their biological interactions *via* the change of its photoelectrochemical properties. For this purpose, to our best knowledge, the synthesis of the first electropolymerizable biotinylated metal complex possessing electrochemical and photochemical activities is described. The electrogeneration of polymer films indeed is one of the few procedures of surface functionalization with molecular reagents that allows the reproducible functionalization of conductive surfaces of complex geometry with a precise spatial resolution. In this study a novel biotin-labeled ruthenium (II) tris(bipyridyl) complex functionalized by four pyrrole groups (Fig. 1), was prepared by reaction of dichloro bis[4,4′-bis(4-pyrrole-1-butyl)2,2′-bipyridyl] ruthenium(II) with 4,4′-bis(biotin)2,2′-bipyridine and characterized by H^1H NMR and UV-visible absorption spectra as well as by EI mass spectrometry. The dichloro bis[4,4′-bis(4-pyrrole-1-butyl)2,2′-bipyridyl] ruthenium(II) was synthesized according to the published procedure[7] by reaction of the 4,4′-bis(4-pyrrole-1-butyl)2,2′-bipyridine with $Ru(III)Cl_3$ and characterized by ^1H NMR. As previously reported, the 4,4′-bis(biotin)-2,2′-bipyridine ligand was prepared by esterification of 4,4′-bis(hydroxymethyl)-2,2′-bipyridine with biotin, using the carbodiimide method and characterized by ^1H NMR and FAB mass spectrometry.[8]

The electrochemical behavior of the ruthenium complex (1 mM) was investigated in CH_3CN + 0.1 M nBu_4NClO_4. Upon reductive scanning, the monomer exhibits three successive reversible peak systems at −1.72 V (ΔE_p = 60 mV), −1.97 V (ΔE_p = 70 mV) and −2.24 V (ΔE_p = 70 mV) corresponding to the successive one-electron reduction of the three bipyridyl ligands. These values are similar to those previously reported for tris(bipyridyl)ruthenium(II) complex containing pyrrole groups.[9] Upon oxidative scanning, the cyclic voltammogram displays a weakly reversible anodic peak at 0.92 V on the one-electron oxidation wave of the complex. This behavior clearly indicates the irreversible oxidation of the pyrrole groups *via* the one-electron oxidation of the metal center (Ru^{II}/Ru^{III}) since *N*-alkylpyrroles are oxidized around 1.0 V (Fig. 2A).[10] Since the electrogeneration of the pyrrole cation radical is followed by its coupling while protons are released, the electropolymerisation properties of the ruthenium complex were investigated by repeated potential cycling over the range 0–1.1 V. The continuous growth of reversible peak systems due to the $Ru^{II/III}$ couple clearly

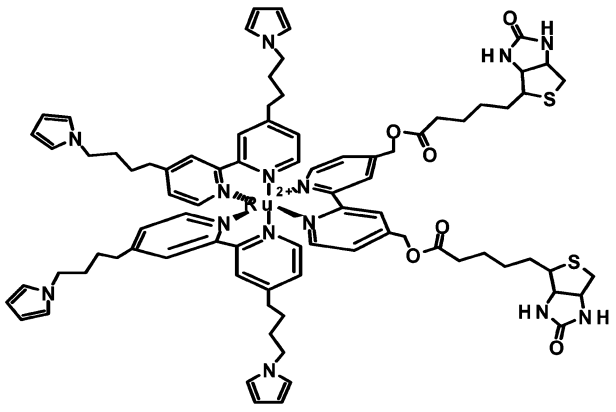

Fig. 1 Structure of tris(bipyridyl)ruthenium(II) complex.

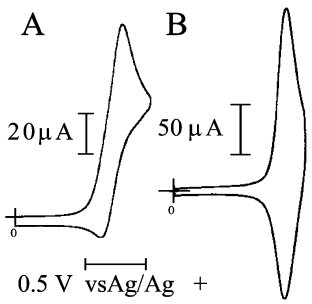

Fig. 2 A. Cyclic voltammogram recorded at a platinum electrode (diameter 5 mm) of the tris(bipyridyl)ruthenium(II) complex (1 mM) in CH_3CN + 0.1 M nBu_4NClO_4. Scan rate 0.1 V s^{-1}. B. Cyclic voltammograms of polypyrrole–ruthenium–platinum electrode ($\Gamma = 1.34 \times 10^{-9}$ mol cm^{-2}) in CH_3CN + 0.1 M $LiClO_4$. Scan rate 0.1 V s^{-1}.

† Electronic supplementary information (ESI) available: Synthetic procedures and characterization data. See http://www.rsc.org/suppdata/cc/b4/b410727f/

DOI: 10.1039/b410727f

indicates the formation of a polymeric redox film onto the electrode surface. Electropolymerization of the monomeric complex was also carried out by the controlled potential oxidation at 0.9 V. Upon transfer into CH_3CN + 0.1 M nBu_4NClO_4 solution free of monomer, the cyclic voltammogram of an electrode modified by controlled potential electrolysis (1 mC) exhibits a redox system corresponding to the Ru^{II}/Ru^{III} transition at the same potential value (0.9 V) than that observed for the monomer (Fig. 2B). As expected, redox peak systems appear in the negative region at the same potential values (−1.75 V, −1.99 V and −2.25 V) than those previously recorded for the three bipyridyl ligands.[9]

The ability to immobilize biotinylated biomolecules on the polypyrrole–ruthenium(II) film and hence the availability of the biotin groups attached to the polymerized tris bipyridyl complex for the binding of avidin was examined *via* the anchoring of a biotinylated biomolecule. A biotinylated glucose oxidase (B-GOX) was chosen as model for its ability to catalyze the production of electroactive H_2O_2 in the presence of glucose and oxygen. After successive incubation with avidin and B-GOX, the resulting electrode was potentiostated at 0.6 V and its amperometric response to glucose was recorded in 0.1 M phosphate buffer (pH 7). The current response increases as function of glucose concentration demonstrating the efficient immobilization of the enzyme on the polymerized film by formation of avidin–biotin bridges. This also indicates that biotin moieties can enter the binding site of avidin and were firmly immobilized although the latter were linked to the bipyridyl ligand. It should be noted that the resulting glucose sensitivity (1.45 mA M^{-1} cm^{-2}) is similar to those reported by Anzai *et al.* (1.4–3 mA M^{-1} cm^{-2}) for biosensors based on a monolayer of B-GOX immobilized through the avidin–biotin system.[11]

The possibility to exploit the photoelectrochemical properties of this biotinylated polymer film for the transduction of surface molecular recognition without labeling of the target was examined with the detection of anti-cholera toxin antibody as model system. For this purpose, the biotinylated film was applied to the conjugation of avidin and subsequent binding of cholera toxin B subunit biotin-labeled *via* avidin–biotin bridges. The analyte, anti-cholera toxin antibody, thereafter bound, by immunoreaction, the corresponding immobilized cholera toxin B subunit epitopes, the modified electrode thus constituting a potential immunosensor. Since Ru complexes were widely used as photosensitizer, the photoelectrochemical properties of the polypyrrole–ruthenium(II) film were investigated in 0.1 M acetate buffer solution (pH 4.5). The photoexcitation of the modified electrode in presence of an oxidative quencher, pentaaminechlorocobalt(III) chloride (1.5 10^{-2} M), induces the oxidation of the polymerized Ru(II) into Ru(III), which is subsequently reduced with an electron from the underlying platinum electrode held at 0.5 V generating thus a cathodic photocurrent. Fig. 3A shows the photocurrent response of the ruthenium film as the excitation light was turned on and off. The photocurrent was regenerated 20 times over 50 min without noticeable decrease in its intensity. This illustrates the repeatability of the photoelectrode response (RSD = 2%) and hence the mechanical and photophysical stability of the film. The specific build up of three protein layers (avidin and immunogenic material) was carried out on the polypyrrole–ruthenium(II) film. As expected, the successive binding of avidin and biotinylated antigen on the biotinylated film induces a marked decrease in the photocurrent response. The latter may be ascribed to the hindered diffusion of quencher molecules to the ruthenium film, lowering thus the amount of photogenerated Ru(III) center. Nevertheless, the final immunoreaction *via* the binding of the anti-cholera toxin leads to another strong photocurrent decrease (0.5 μA cm^{-2}) which corresponds to 35% of the preceding signal reflecting thus the antibody anchoring (Fig. 3B). Control experiments carried out with avidin-conjugated polypyrrole film incubated with anti-cholera toxin or successively incubated with non-biotinylated antigen and anti-cholera toxin indicated no change in photo-currrent intensity. This clearly demonstrates that the preceding immunosensor response was not due to non-specific binding of proteins on the avidin-polymer coating. It appears that the large magnitude of this photocurrent variation may be exploited for detecting immunoreactions involving non-labeled analyte. The necessary presence of an oxidative quencher, however, prevents the use of such approach for *in vivo* detection.

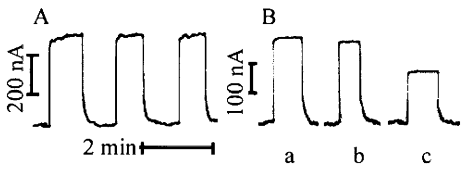

Fig. 3 A. Time-based photocurrent response of polypyrrole–ruthenium electrode ($\Gamma = 1.34 \times 10^{-9}$ mol cm^{-2}) in presence of $[Co(III)Cl(NH_3)_5]^{+2}$ 1.5 10^{-2} M in acetate buffer solution 0.1 M pH 4.5 as the excitation light turned on and off. B. Photocurrent response of polypyrrole–ruthenium electrode ($\Gamma = 1.34 \times 10^{-9}$ mol cm^{-2}) after incubation (a) in 0.5 mg mL^{-1} avidin solution, (b) and in 0.5 mg mL^{-1} biotinylated cholera toxin B solution, (c) and in 0.5 mg mL^{-1} anti-cholera toxin solution.

The results described herein demonstrate that the biotinylated ruthenium film constitutes an attractive electrogenerated coating for the construction of photoelectrochemical immunosensors and biochips, in particular, for protein chips due to the absence of protein labeling step.

The authors thank the ACI 031 "Nouvelles methodologies analytiques et capteurs 2002" for financial support.

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