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Design of a new electrogenerated polyquinone film substituted with glutathione. Towards direct electrochemical biosensors

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ABSTRACT

We developed a method to graft a tripeptide (glutathione) onto 5-hydroxy-1,4-naphthoquinone, an electropolymerizable molecule. The resulting thin conducting polymer presents a well-defined and stable electroactivity in neutral buffered solution, due to the embedded quinone group, and is able to covalently graft amino-modified DNA probe strands. It is shown that the bioelectrode presents positive current change following DNA hybridization. This makes a "signal-on" direct electrochemical DNA sensor. The results were obtained with low target concentration (50 nM) and the selectivity is excellent as a single-mismatch sequence can be discriminated from the full-complementary target.

1. Introduction

Biosensors are of great interest nowadays. These devices need immobilization of biomolecules (bioprobes) on the sensor surface. To achieve this, several procedures are currently described [1]. One can use adsorption on the surface [2], inclusion into an organic [3] or an inorganic [4] matrix, covalent binding [3,5], complexation (using biotin–avidin association for example) [6]. Adsorption is simple to achieve, but generally poorly reproducible, and unstable. Inclusion into polymer or inorganic matrixes usually makes bioprobes, in particular DNA, inaccessible for subsequent reactions. Complexation using antibody–antigen association is efficient and widely used, as this avoids any risk of bioprobe denaturation due to immobilization. However, in this configuration, the antibody–antigen system (which is heavy) is inserted between the biosensor surface and the bioprobe, then hinders some interactions between the surface and the probe. Therefore, the direct covalent binding of the biomolecule onto the biosensor surface seems more pertinent and presents the advantage to be irreversible, i.e. more stable during the biosensor lifetime, and 'keeps in contact' the biomolecule and the surface. Its eventual drawback is that it may denaturate the immobilized bioprobe due to a lack of control of the linking regions and the linker reactivity. That is why the choice of the linker (the molecule or the reactive group which belongs to the sensor surface and binds the bioprobe) is of great importance.

Glutathione (γ-L-glutamyl-L-cysteinyl-glycine) is a tripeptide, bearing one primary amine, two amide groups, two carboxylic groups and one thiol group (see Scheme 1). It contains an unusual peptide link between the amine group of cysteine and the carboxyl group of the glutamyl side chain. The thiol group carried by this molecule is one of the most stable thiol group in a biomolecule. For this reason, glutathione is well-known to have antioxidant properties. Glutathione is then currently associated with drugs, in order to protect them from oxidizing species (free radicals for example), and, in addition, make them more soluble in water. The p*K*a associated to the carboxylic acid, the thiol and the primary amine groups are, respectively, 2.1 (COOH on glutamyl), 3.5 (COOH on glycine), 9.6 (SH) and 8.7 (NH_2) [7]. Therefore, at neutral pH, the molecule carries the equivalent of one negative charge. As glutathione is an oligopeptide bearing reactive amine, carboxylic groups and considering properties described above, this molecule of interest has been described in the literature as a linker for biomolecules immobilization on surfaces [8–10]. However, very few works reported electrochemical biosensor using glutathione as the binding element [9].

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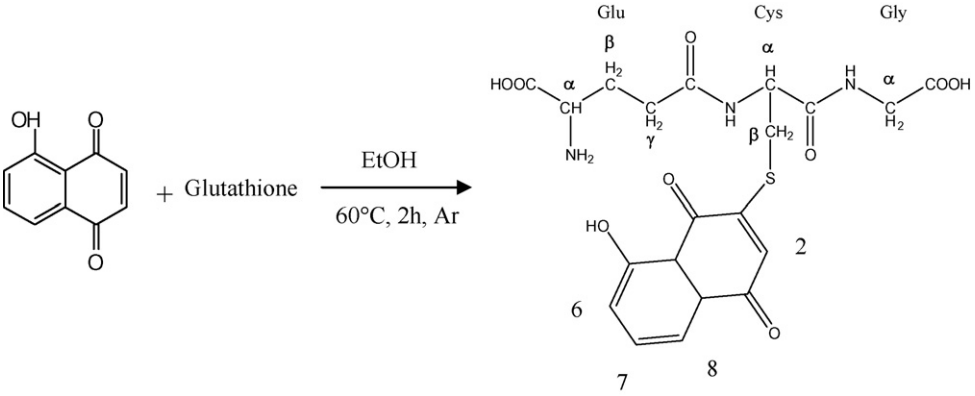


Scheme 1. Synthesis of 5-hydroxy-3-γ-L-glutamyl-L-cysteinyl-glycine-1,4-naphtoquinone (HNQ–Glu) from HNQ and glutathione, in one step. Proton's numbering corresponding to NMR attribution.

In this work, we developed a new electrogenerated polyquinone film substituted by glutathione. An original and direct procedure was used to chemically graft glutathione on 5-hydroxy-1,4-naphthoquinone. The resulting 3-(γ-L-glutamyl-L-cysteinyl-glycine)-5-hydroxy-1,4-naphthoquinone (HNQ–Glu) presents 3 functional groups: the hydroxyl group for electropolymerization (molecules of the hydroxynaphthoquinone family present the property to electropolymerize into a conducting polymer, via the hydroxyl group [11,12]), the quinone group for its redox and sensing properties, and the glutathione oligopeptide as a precursor for subsequent biomolecule linkage, via amine or carboxylic groups.

The synthesis and characterizations of the HNQ–Glu monomer are first described. After that, electrosynthesis of a conducting polymer is performed, by co-electropolymerization of a mixture of HNQ–Glu and unmodified HNQ, leading to poly[5-hydroxy-1,4-naphthoquinone-*co*-3-(γ-L-glutamyl-L-cysteinyl-glycine)-5-hydroxy-1,4-naphthoquinone], so-called poly(HNQ-*co*-HNQ–Glu) in the following. By this method, the quinone, carboxylic and amine functions are preserved in the polymer. The electroactivity of poly(HNQ-*co*-HNQ–Glu) film is investigated in neutral aqueous phosphate buffer (PBS), the modified electrode presents a well-defined, stable and reversible redox couple.

The quinone is well-known to be particularly sensitive to its chemical environment, in terms of pH or redox state. Therefore, the quinone group of poly(HNQ-*co*-HNQ–Glu) can be used as a redox reporter for chemical or electrochemical modifications of its vicinity. These modifications can be generated by change in ion concentration, for example. It has been shown in a previous work that in PBS the quinone groups of a poly(quinone) film exchange mainly sodium cations [13]. Therefore, the cyclic voltammograms of a poly(HNQ-*co*-HNQ–Glu) film, in the quinone redox domain, must change if sodium cation diffusion is modified. This can be generated by heavy molecules or charged molecules immobilized at the vicinity of the film surface, influencing the diffusion layer by steric hindrance or electrostatic effect. The transduction mechanism has been thoroughly investigated in some of our previous papers [14,15].

In this work, DNA probe strands (modified by an amine group at their 3′-end) are linked to the poly(HNQ-*co*-HNQ–Glu) copolymer via a free carboxylic group of the Glu moiety. After hybridization, the biosensor presents an increase in the redox current of the quinone group. As these current changes are mainly due to the faradic component instead of the capacitive one, Square Wave Voltammetry (SWV) is employed instead of classical voltammetry to characterize and emphasize the current variations. Addition of a probe strand which is perfectly complementary to the immobilized probe strand leads to a clear signal increase, whereas addition of a non-complementary sequence (random) or even a single-mismatch one does not generate significant change in current.

2. Materials and methods

2.1. Chemicals

N′-(3-Dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) and phosphate buffer saline (PBS, 0.137 M NaCl; 0.0027 M KCl; 0.0081 M Na_2HPO_4; 0.00147 M KH_2PO_4, pH 7.4) were provided by Sigma. Aqueous solutions were made with ultrapure (18 MΩ) water. 5-Hydroxy-1,4-naphthoquinone (HNQ) 1-naphthol (1-NAP) and L-glutathione were purchased from Fluka. Acetonitrile (ACN, HPLC grade) was supplied by Aldrich. All other reagents used were of analytical grade. Oligonucleotides were provided by Eurogentec. The probe strand (PROBE) is a 37-mer sequence bearing an amino group on its 3′-end (5′-TC-CCT CAT AGT CGC ACC CAT CTC TCT CCT TCT-AGCCT-3′C_7NH_2). Targets are 30-mer sequences: COMP (3′-GGA GTA TCA GCG TGG GTA GAG AGA GGA AGA-5′) is the complementary one. MISMATCH (3′-GGA GTA TCA GCG TGG GTA AAG AGA GGA AGA-3′) presents one mismatch base, and the random sequence RANDOM (3′-TTG AAC CAG TAT CCA TGC ATT CCG CCT AAG-5′) is non-complementary. As explained above, the transduction mechanism of this biosensor is closely related to the conformational changes of the ODN strands upon hybridization. For this reason, in this work, we preferred to have a total control of the spatial conformation of the probe-target hybrid (double strand). For this, we needed to use probe and targets of ca. the same length. However, our probe was chosen slightly longer (37b) than the target sequences (30b) to let some bases as spacers at both ends of the probe.

2.2. Electrochemical methods

For electrochemical experiments, a conventional one-compartment, three-electrode cell was employed. An *EG&G 263A* potentiostat was used with the *Echem* software (Ecochemie). The working electrodes were glassy carbon (GC) disks (Aldrich) of 0,07 cm^2 area or gold plates (2 cm^2 area) for FTIR and XPS characterizations. The auxiliary electrode was a platinum grid and the reference electrode a commercial Saturated Calomel Electrode (SCE, MetrOhm, France). Electrodes were polished prior to each use with alumina slurry (1 then 0.3 μm).

The electrochemical synthesis of this copolymer poly(HNQ-*co*-HNQ–Glu) was carried out by electrooxidation of 5×10^{-2} M HNQ + 5×10^{-2} M HNQ–Glu + 10^{-3} M 1-naphthol (1-NAP) + 0.1 M $LiClO_4$ in acetonitrile, on gold or GC electrodes, under a dried argon atmosphere, by potential scans at 50 mV s^{-1} from 0.4 to 1.05 V

versus SCE. Without 1-NAP, no polymerization occurs, while electrooxidation of a solution containing only 10^{-3} M 1-NAP does not lead to any film. This indicates that 1-NAP acts as an electropolymerization initiator.

Hybridization was detected by recording the modification of the redox process of the quinone group, using Square Wave Voltammetry (SWV). The following parameters were used: pulse height 50 mV, pulse width 50 ms, scan increment 2 mV, frequency 12.5 Hz. The medium was PBS, bubbled with argon for 20 min before and during SWV measurements. The SWV scans were repeated (usually two or three times) until complete stabilization of the signal (i.e. no difference observed between two successive responses). Differential currents (Δi) were calculated by subtracting the current obtained after probe grafting from the current obtained after the considered experiment.

All electrochemical experiments were conducted at room temperature (22 °C). After modifications, electrodes were kept in the dark, away from oxygen and humidity in a dry argon atmosphere. In these conditions, the electrodes are stable (no significant decrease of its electroactivity) for ca. 2 weeks. The modified electrodes were used only for one cycle of electropolymerization-probe grafting-hybridization, then polished prior to re-use.

2.3. X-ray photoelectron spectroscopy (XPS)

X-ray Photoelectron Spectroscopy (XPS) data were recorded on a Thermo VG Escalab 250 (Al anode). XPS signals were fitted with a sum of Gaussian (70%)/Lorenzian (30%) functions, and calibrated by assuming a 285 eV binding energy for aromatic and aliphatic carbons.

2.4. NMR

All NMR spectra were recorded on a Bruker Avance III 300 MHz spectrometer. D_2O was used as solvent for all NMR measurements. The temperature was set at 298 K. Water resonance was suppressed with a low-power presaturation [16]. Data were processed using TOPSPIN 2.1 software (Bruker). Proton chemical shifts are referenced using residual HDO as an internal standard (4.70 ppm).

2.5. Grafting and hybridization conditions

As described before, poly(HNQ-*co*-HNQ–Glu) films present two carboxylic functions which are free to react with a coupling agent. As γ-L-glutamyl-L-cysteinyl-glycine is a peptide, it was natural to use a binding reaction currently used in peptide chemistry, i.e. formation of an amide bond from a carboxylic group and a primary amine (amidation). Under normal conditions, amidation is slow. To increase reactivity, the carboxyl group can be transformed into an ester group with the coupling agent N′-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC). In aqueous solution, to obtain a better reactivity of the intermediate ester towards the primary amine carried on the DNA probe, another molecule must be used: N-hydroxysuccinimide (NHS). Indeed, water molecules act as nucleophilic molecules and induce hydrolysis of the intermediate ester. NHS competes with water and therefore increases the coupling yield.

Practically, poly(HNQ-*co*-HNQ–Glu) films were dipped into 1 mL of distilled water containing 0.1 μM probe (PROBE), 1.5×10^{-2} M EDC and 3×10^{-2} M NHS at 37 °C. After 20 h, the ODN immobilization reaction was stopped: the electrode was washed with distilled water (5 min) then with PBS (2 h at 37 °C) in order to remove non-covalently bound ODN.

At this stage, the electrodes were characterized by SWV, as explained in Section 2.2. Only those which present peak currents of 3 ± 0.5 μA were selected for the following steps.

For hybridization experiments, 1 mL of PBS solutions containing 50 nM of target (COMP, MISMATCH or RANDOM) were used. The electrode bearing the probe strand was dipped in the target solution then heated at a temperature above the melting temperature of the corresponding duplex (e.g. 72 °C for the PROBE/COMP duplex) during 2 h, and slowly cooled down to room temperature. After that, the electrode was washed in PBS for 1 h at 25 °C in order to remove the non-hybridized strands.

2.6. Fluorescence experiments

The apparatus used is an *Aminco-Bowman* Series 2 spectrofluorometer. Fluorescent targets (COMP*, MISMATCH* and RANDOM*) are modified with fluorescein (*Eurogentec*) on their 5′-end. These labeled targets were used following the same hybridization protocol than for the unlabelled targets, using 2 cm^2 GC plates as electrodes instead of 0.07 cm^2 GC disks. For quantification, dehybridization was performed in order to make spectrofluorometric analysis in solution and not directly onto the polymer surface.

The dehybridization protocol consists in a washing procedure made of 4 steps. To remove unspecific association, the three first steps consist in soaking the film in 2 mL of PBS, at 25 °C, under magnetic stirring, during 5 min (step 1), 10 more min (step 2), and 30 more min (step 3). After each step, the 2-mL solution is removed, stored for subsequent fluorescence experiments, and replaced by a fresh one. After these extensive washing steps, the fourth step consists in soaking the film in 2 mL of pure water at 80 °C during 10 min. Under these very stringent conditions, the hybrids which are present on the film are denatured and the target strands withdrawn towards the solution, which is stored for subsequent fluorescence experiments.

To determine the ODN concentration in these solutions, calibration curves were first obtained, for each fluorescent strand (in the range 0.25–10 nM). ODN concentrations were then converted into surface concentrations, which were found in the 0–15 pmol cm^{-2} range.

3. Results and discussion

3.1. Synthesis of HNQ–glutathione (HNQ–Glu)

3.1.1. Synthesis of 5-hydroxy-3-γ-L-glutamyl-L-cysteinyl-glycine-1,4-naphthoquinone (HNQ–Glu)

The reaction of thiols on various hydroxynaphthoquinone derivatives led, in one step, to substituted quinone rings, under mild conditions [17]. It is one of the most elegant substitution reactions on quinone rings, which are known to be quite unreactive in usual conditions. Therefore, the grafting reaction of glutathione onto hydroxynaphthoquinone derivatives appears highly interesting and original (to our knowledge, this reaction has not been described elsewhere).

5-Hydroxy-3-γ-L-glutamyl-L-cysteinyl-glycine-1,4-naphthoquinone (HNQ–Glu) was synthesized in one step from 1 equiv. of 5-hydroxy-1,4-naphthoquinone (HNQ) and 2 equiv. of γ-L-glutamyl-L-cysteinyl-glycine in 25 mL ethanol at 60 °C (Scheme 1). The solution was stirred for 15 min at this temperature and cooled at room temperature. The mixture was then allowed to stand for 2–3 days at 5 °C in the dark. The precipitate obtained was filtered and washed with ethanol. Evaporation of the solvent led to a brown/orange powder. Finally, a recrystallization step was made. The powder was dissolved in hot ethanol, filtered, then activated charcoal was added, and the mixture was filtered again. After one or 2 days at 4 °C, needles appeared at the bottom of the flask, which were separated by filtration, washed with cold ethanol (−18 °C) and kept for further analysis.

Table 1
NMR chemical shifts and multiplicity in D_2O at 298 K.

Proton	δ (ppm)	Multiplicity
Glu-H_α	3.76	t
Glu-$H_{\beta1}/H_{\beta2}$	2.13	m
Glu-$H_{\gamma1}/H_{\gamma2}$	2.52–2.51	m
Cys-H_α	4.53	d
Cys-$H_{\beta1}/H_{\beta2}$	2.92–2.90	m
Gly-H_α	3.92	s
H_2	–	–
H_8	7.72	d.d
H_7	7.78	t
H_6	7.40	d.d

The yield is ca. 40%. The purity of HNQ–Glu was verified by TLC and its structure was confirmed by FTIR (in KBr pellets, ν/cm^{-1}): 3028 [ν(N—H), weak]; 2975,2932 [asym. and sym. ν(C—H) of –CH_2–, strong], 1713 [ν(C—O), acid, strong]; 1661, 1559 [ν(C—C_{ar}), strong], 1630 [ν(C—O) of quinone, strong], 930 (δC—H_{ar}, 1H, weak), 759 (δC—H_{ar}, 3 adj. H, weak). A typical band of the ν(C—S) bond was found at 550 cm^{-1} (weak). The HNQ–Glu melting point is 250 °C versus 164 °C for HNQ.

NMR was performed in several deutered solvents, as MeOD, CD_2Cl_2 or D_2O. It was found that the whole HNQ–Glu molecule is only partially soluble in organic MeOD or CD_2Cl_2, whereas its solubility is acceptable in D_2O. This is due to the opposed behaviour between the glutathione part and the juglone part. If the second one is easily soluble in organic solvents, that is not the case for the glutathione moiety. The NMR identification was therefore performed in D_2O. L-Glutathione (Glu) and 5-hydroxy-1,4-naphthoquinone (Jug) were analyzed in D_2O and used as references. Their NMR spectra were compared to that of HNQ–Glu. Their profiles are identical. Proton's chemical shifts and attributions of HNQ–Glu are reported in Table 1 (see Scheme 1 for numbering). It can be noted that β protons of the glutamic acid present the same chemical shift (2.13 ppm) whereas the γ ones are slightly different (2.52–5.51 ppm), as well as the β protons of the cysteine moiety (2.92–2.90 ppm). The H_2 proton was expected but cannot be seen. This can be explained by interactions with the Glu moiety at close vicinity.

3.1.2. Electrosynthesis of poly(HNQ-co-HNQ–Glu)

Due to its relatively high molecular weight, the glutathione moiety induces a strong steric hindrance on the HNQ moiety. Moreover, the carboxylic acids (negatively charged) may change the ion-exchange properties of the polymer. This is why we chose to make a copolymer with an equivalent proportion (1:1) of HNQ and HNQ–Glu instead of poly(HNQ–Glu). Conditions are described in Section 2.2.

The voltammograms obtained during electrosynthesis of poly(HNQ-*co*-HNQ–Glu) films present during the first cycles an oxidation wave situated above 1 V. After a few cycles, a redox couple appears around 0.85/0.90 V. Currents increase gradually with potential scans (Fig. 1).

The FTIR spectrum of the resulting film (synthesized on a gold plate) is presented on Fig. 2. A peak at 3059 cm^{-1} of medium intensity can be attributed to free N—H stretch (asymmetric vib.). Bands situated between 3000 cm^{-1} and 2800 cm^{-1} concern CH_2 stretching vibrations. The presence of several peaks (asym. νCH_2, 2954 cm^{-1} and 2924 cm^{-1}; sym. νCH_2, 2860 cm^{-1} and 2870 cm^{-1}) shows that two different –CH_2– groups are present in the glutathione moiety: –CH_2– close to carboxyl groups and –CH_2– close to amine groups. The bands present in the 2000–700 cm^{-1} range are typical for the HNQ moiety, with a shoulder at 1737 cm^{-1} due to ν(C=0) of the carboxylic acid group, two strong bands at 1670

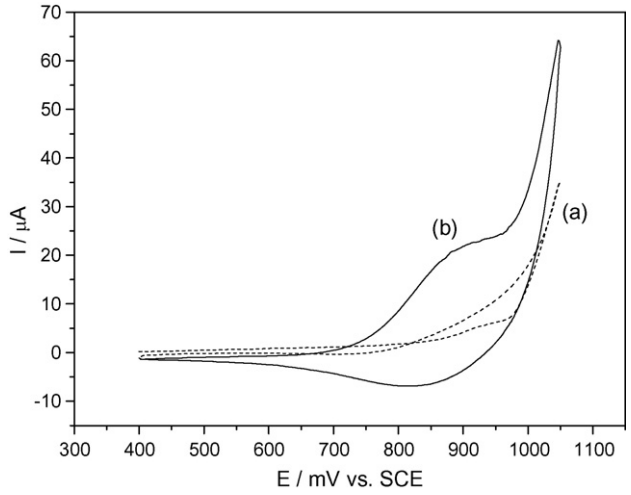


Fig. 1. Cyclic voltammograms recorded during electrosynthesis of a poly(HNQ-*co*-HNQ–Glu) film in ACN + 0.1 M $LiClO_4$ + 5×10^{-2} M HNQ + 5×10^{-2} M HNQ–Glu + 2×10^{-3} M 1-naphthol on a GC electrode under argon atmosphere. Scan rate: 50 mV s^{-1}. Electrode surface: 0.07 cm^2. (a, dashed line), fist cycle; (b, plain line) 50th cycle. Film thickness after 50 scans: ca. 100 nm (measured by SEM).

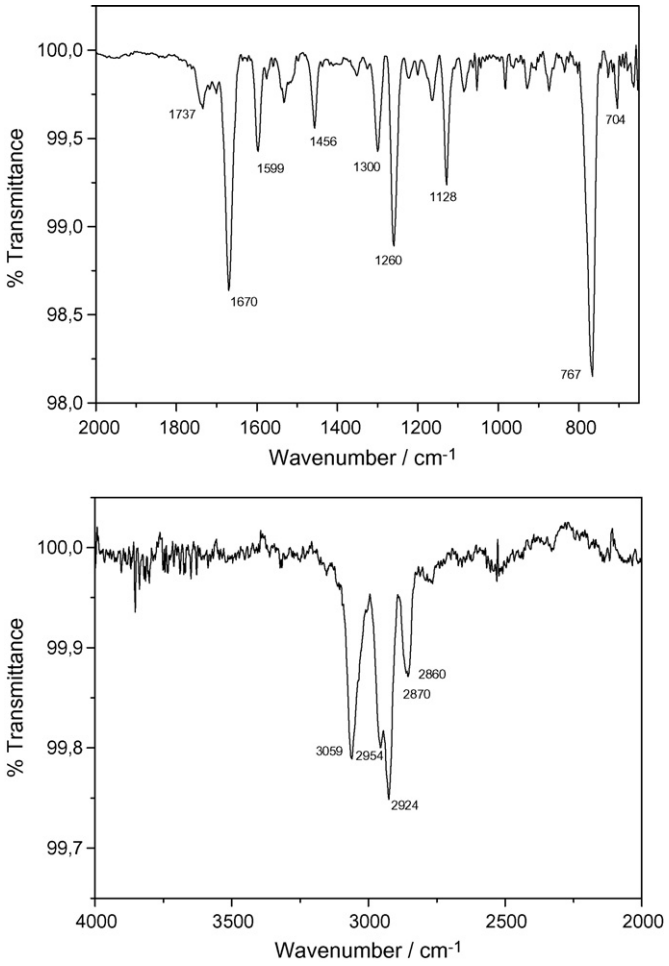


Fig. 2. FTIR spectra of the poly(HNQ-*co*-HNQ–Glu) film electrodeposited on a gold substrate, in the 650–2000 cm^{-1} and 2000–4000 cm^{-1}.

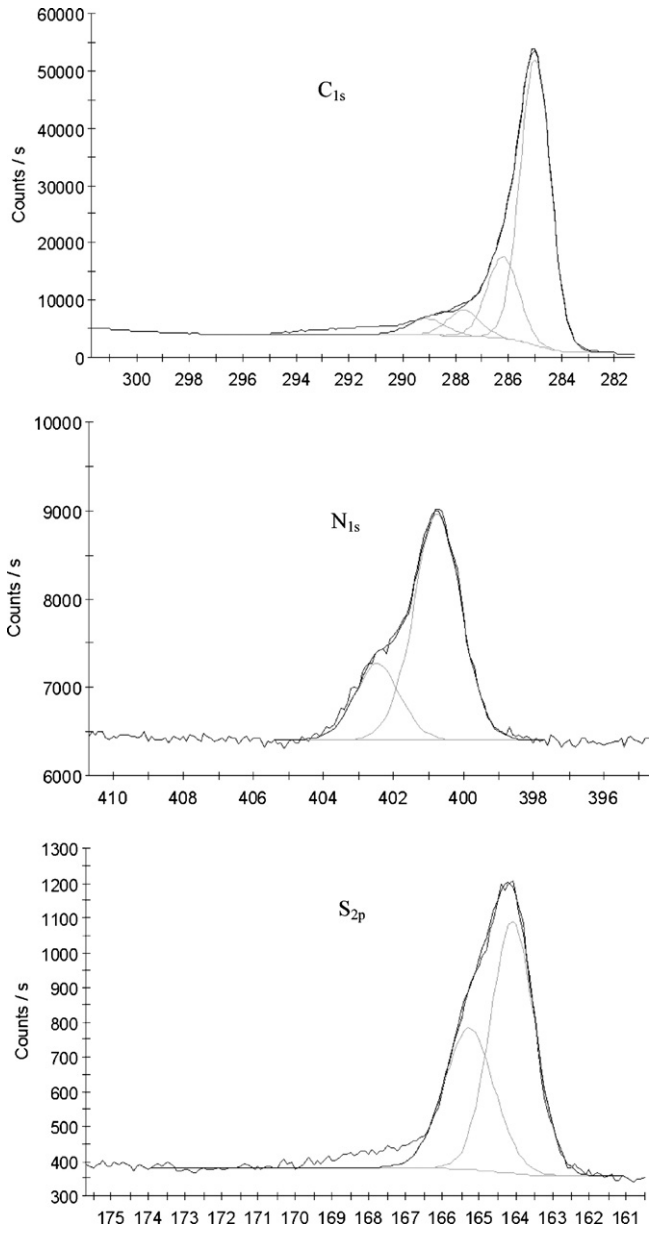


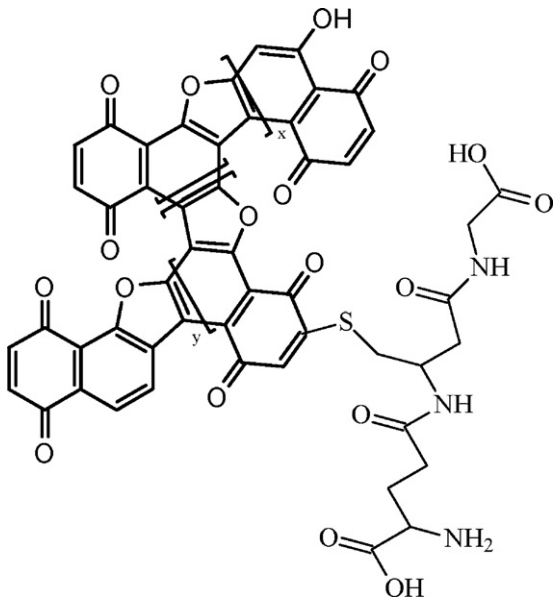
Fig. 3. XPS spectra of the poly(HNQ-*co*-HNQ–Glu) film for C, N and S elements.

and 1599 cm^{-1} are due to $\nu(C{=}C)_{ar}$ of the aromatic structure and of the quinone group, bands at 1260 and 1300 cm^{-1} are attributed to $\nu(C{-}O{-}C)_{as}$ of the furan cycle and a strong band at 767 cm^{-1} is typical for naphthalene structure.

3.2. XPS characterization of poly(HNQ-co-HNQ–Glu) films

The poly(HNQ-*co*-HNQ–Glu) films were analyzed by X-Ray Photoelectron Spectroscopy (XPS). Fig. 3 presents spectra of C, N and S. Carbon atoms are present in the polymer structure as well as in the Glu moiety whereas nitrogen and sulfur belong only to Glu.

The C1s spectrum displays four peaks, at 285 eV, 286.2 eV, 287.7 eV and 289.1 eV. They can be respectively attributed to C—C and C=C (present into the polymer backbone and the Glu moiety), C—O, C—S and C—N (polymer backbone and Glu), C=C (of the quinone group), and O—C=C or O=C—N (of the carboxylic and amide groups belonging to the Glu moiety).



Scheme 2. The proposed structure for poly(HNQ-*co*-HNQ–Glu).

The N1s spectrum displays 2 peaks, at 400.7 eV and 402.5 eV. These peaks can be attributed to the amide (–NH—C=C) and amine groups ($-NH_2$) respectively.

The S2p spectrum displays two peaks (as it is a 2p level) at 164.1 eV and 165.3 eV. It corresponds to the only sulfur present in the Glu moiety, which binds Glu and HNQ via a C—S bond.

The calculations from the peak surfaces, using the sensitivities for C, N and S which are respectively 1, 1.80 and 1.67, give an experimental atomic ratio N/S of 2.95. This ratio corroborates very well with the theoretical ratio (3:1) of the Glu structure. The experimental C/S ratio equals to 99. The theoretical one equals to 20:1 for the HNQ–Glu moiety, whereas no sulfur belongs to the HNQ molecule. This indicates that the ratio between the HNQ moieties and the HNQ–Glu moieties in the copolymer is around 4, that means 1 HNQ–Glu for 4 HNQ. Scheme 2 presents a proposed structure for poly(HNQ-*co*-HNQ–Glu) film, deduced from XPS and FTIR results.

3.3. Electroactivity of poly(HNQ-co-HNQ–Glu) films

The electroactivity of the copolymer film was investigated in phosphate buffer saline (PBS). In the cathodic potential domain of potential (between −0.9 V and 0.25 V), a redox couple develops at −0.51/−0.63 V (Fig. 4a).

The observed peaks are due to quinone electroactivity. By charge integration, 5×10^{-10} mol cm^{-2} of quinone are estimated to be electroactive for a 100-nm thick film (thickness determined by SEM at grazing angle). The redox systems present excellent stability upon cycling (several hundreds of cycles without significant current decrease, as shown in Fig. 4a). Of interest is the fact that the potential range is low enough to avoid interference with many electroactive species that can be present in biological media. Moreover, in applications for DNA grafting and hybridization detection, this behaviour avoids any risk of nucleobases oxidation, as they are oxidized at much higher potentials. At last, it is known that the redox system of the quinone group exchanges cation and not anion. More precisely, it has been shown by electrogravimetric experiments [13] that quinone exchanges mainly sodium cations in neutral PBS medium. This behaviour is a strong advantage of poly(HNQ-*co*-HNQ–Glu) film over other more classical polymer films. Indeed, DNA strands (which are negatively charged) are not able to play the role of counterions for this polymer which

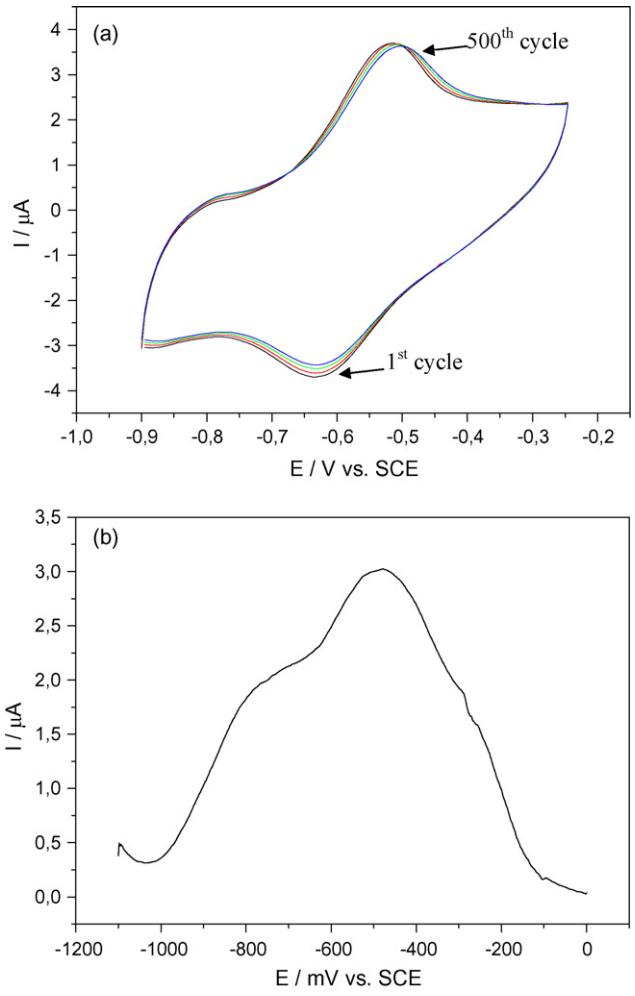


Fig. 4. (a) Cyclic voltammograms of a poly(HNQ-*co*-HNQ–Glu)-modified electrode recorded in PBS under argon atmosphere. Scan rate: 50 mV s^{-1}. Electrode surface: 0.07 cm^2. Film thickness: 100 nm. Several voltammograms were recorded between the first and the 500th without significant current decrease. (b) Square wave voltammogram (SWV) of a poly(HNQ-*co*-HNQ–Glu)-modified electrode recorded in PBS under argon atmosphere. Parameters: pulse height 50 mV, pulse width 50 ms, scan increment 2 mV, frequency 12.5 Hz, potential domain (−1.1 V; 0 V vs. SCE).

exchanges cations. As poly(HNQ-*co*-HNQ–Glu) polymer is neutral or negatively charged, it repels probe DNA strands (which still remain available for hybridization), and prevents non-specific adsorption of target strands. This property is interesting for the film to be used in biosensor matrix.

3.4. SWV of poly(HNQ-co-HNQ–Glu) films

As shown on Fig. 4a, the voltammograms present a strong capacitive component. We have shown in a previous work that DNA hybridization induces more change on the faradic current than on the capacitive current [13]. Therefore, square wave voltammetry (SWV), which is able to suppress much of the capacitive component, was chosen as the best analytical tool to study the influence of DNA grafting or hybridization on the film electroactivity. SWV was performed on the poly(HNQ-*co*-HNQ–Glu)-modified electrode before treatment with the ODN probe (Fig. 4b). The potential was swept from −1100 mV versus SCE to 0 V versus SCE (oxidation scan). Two oxidation peaks appear in this range. The main peak presents a maximum at around −500 mV/SCE, and the second one appears as a shoulder at around −800 mV/SCE. These peaks correspond to the redox process of the quinone group.

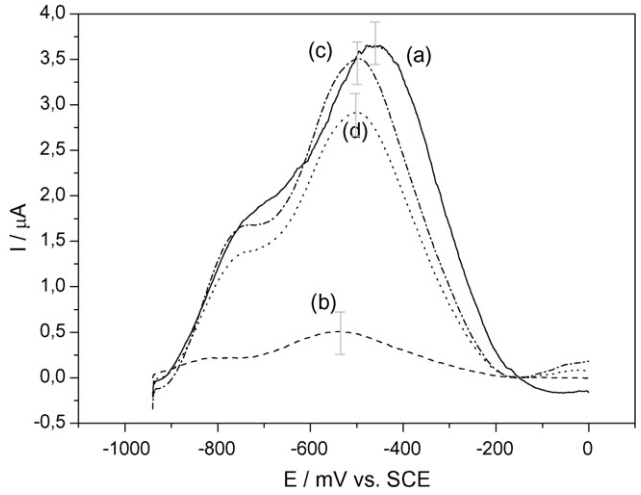


Fig. 5. SWV of a poly(HNQ-*co*-HNQ–Glu)-modified electrode. (a) Before PROBE grafting; (b) after PROBE grafting; (c) after incubation with PROBE without EDC nor NHS; (d) after incubation with EDC and NHS, without PROBE. Medium: PBS, under argon atmosphere. Parameters: pulse height 50 mV, pulse width 50 ms, scan increment 2 mV, frequency 12.5 Hz, potential domain −0.95/0 V versus SCE. Results obtained from 3 sets of independent experiments. Reproducibility is materialized by error bars.

This electroactivity involves ion exchange at the polymer/solution interface. Therefore, changes made on this interface will affect electroactivity. The presence of DNA at the interface strongly influences the ionic flux, therefore the switching rate of the polymer, and as a consequence the recorded current.

Two main explanations for the current change could be envisaged: electrostatic effect (due to the polyelectrolyte character of ODN strands) and/or steric effect (due to the fact that ODN are relatively large molecules). Due to the strong ionic force used in our experiments, the charges localized on the double-stranded ODN (dsODN) backbone are totally screened. The electrostatic effect can be neglected. The steric effect is therefore predominant.

An interesting particularity of ODN is that the double-stranded state (after hybridization) presents a conformation which is very different from the single-stranded state (before hybridization). One can assume that the single-stranded ODN (ssODN) behaves as a classical polymer chain. This means that it is folded as a statistical coil. On the contrary, the double-stranded ODN can be modeled as a rigid cylinder [18–20]. Therefore, steric hindrances generated by ssODN or dsODN are very different. This influences the ionic transport dynamics.

This model has been thoroughly developed in previous papers [14,15] and is the basic principle of the transduction mechanism which is concerned in this work. We have shown that, under certain conditions, dsODN generate a lower steric hindrance than ssODN. Considering that the peak current is controlled by ions transport through the polymer/solution interface, this allows to conclude that ssODN grafting onto the surface generates a current drop; Conversely, the hybridization event (i.e. formation of dsODN) will lead to a current increase.

3.5. Electrochemical detection

3.5.1. Probe grafting

The probe ODN (PROBE) was grafted as described in Section 2. SWV was used to characterize grafting. A SWV curve was recorded before PROBE grafting, and another one was recorded, in the same conditions, after PROBE grafting. As shown on Fig. 5, the current intensity is high for the unmodified film (before grafting, curve a, plain line), and strongly diminishes after grafting (curve b, dashed

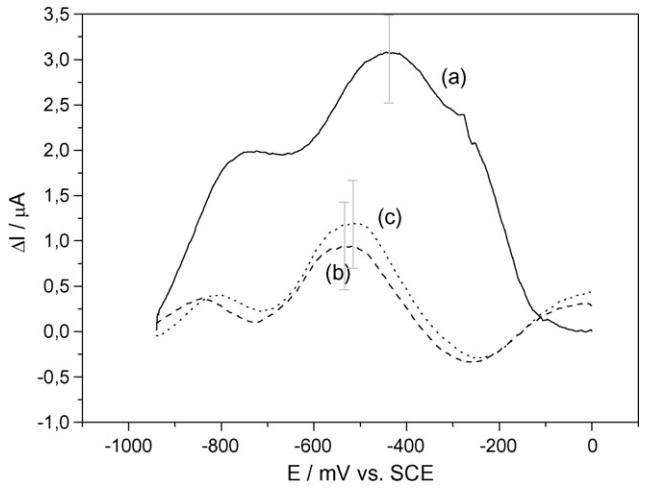


Fig. 6. Differential SWV (Δi) obtained after incubation of the probe-grafted poly(HNQ-*co*-HNQ–Glu) with complementary TARGET (curve a); incubation with RANDOM (curve b); incubation with MISMATCH (curve c). Medium: PBS, under argon atmosphere. Parameters: pulse height 50 mV, pulse width 50 ms, scan increment 2 mV, frequency 12.5 Hz, potential domain −0.95/0 V versus SCE. Differential currents (Δi) were obtained by subtraction of the current obtained after probe grafting. Results were obtained from 3 sets of independent experiments. Reproducibility is materialized by error bars.

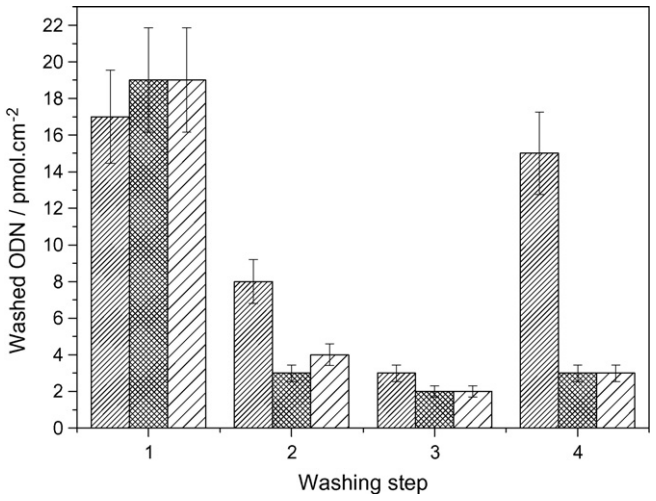


Fig. 7. ODN surface concentration deduced from fluorescence analysis. The fluorescence measurements were made as explained in Section 2, by 3 steps: (1) washing in PBS at 37 °C during 5 min; (2) washing in PBS at 37 °C during 10 additional min; (3) washing in PBS at 37 °C during 30 additional min; (4) washing in pure water at 80 °C during 15 min. The left-handed bars correspond to the COMP target; The central bars correspond to the RANDOM target; The right-handed bars correspond to the MISMATCH target. Results obtained from 3 sets of independent experiments. Reproducibility is materialized by error bars.

line). This can be attributed to the steric hindrance generated by the PROBE strands immobilized on the film surface. As shown, only 10% of the initial electroactivity remains after grafting.

During probe grafting, the electrodes were dipped into three different media: water containing reactants, pure water, and then PBS. To be sure that the current change is only due to probe grafting, blank experiments were performed. The first consisted of the same experiment as above, without NHS nor EDC. The second consisted of the same experiment as above, without ODN probe. The only case for which a significant current change occurs is for probe grafting. Probe alone in solution (curve c) does not lead to any change (this could indicate that there is no probe adsorption), whereas esterification with NHS and EDC (without ODN probe) leads only to a slight (20%) current drop (curve d).

We do no discuss the potential shift which appears between SWV (Figs. 5 and 6), because it cannot be interpreted as it would be for classical voltammetry. Indeed, SWV is a complex technique which subtracts what is called a reverse current (Ir, negative) from a forward current (If, positive).

3.5.2. Hybridization and selectivity

A similar experiment was performed to characterize target hybridization onto the grafted film. A SWV was recorded after PROBE grafting, before addition of the COMP target; another SWV experiment was recorded, in the same conditions, after COMP hybridization (target concentration: 50 nM). To make changes more visible, differential currents (Δi) were plotted. Δi corresponds to the current difference between two SWV recorded for two different electrode states. For example, the Δi which characterizes COMP hybridization corresponds to the difference between the SWV recorded after COMP hybridization and the one recorded before COMP addition.

As shown on Fig. 6 (curve a), a positive response ($\Delta i > 0$, meaning that the current increases upon hybridization) is obtained consecutively to addition of the full-complementary strand.

To be sure that the positive response is truly due to hybridization and not an artefact, a similar experiment was performed under the same conditions, with a non-complementary (RANDOM) strand. As previously, SWV was recorded before and after RANDOM addition. The resulting differential current is plotted on Fig. 6, curve b. As shown, current variations are weak. This result indicates that the current change observed after addition of the COMP sequence is truly due to hybridization.

In a last experiment, the selectivity was checked. A similar experiment than above was performed and a MISMATCH sequence was added (i.e. a sequence which differs from the COMP sequence by only one base, a G being replaced by a A). As shown on Fig. 6, curve c, the current change is weak. This means that the biosensor is selective and discriminates one mismatching base in a 30-bases sequence.

3.5.3. Sensitivity

The results concerning hybridization with a full-complementary target were obtained with a target concentration of 50 nM, in 1 mL PBS solution, at 72 °C for 2 h. As shown in Fig. 6, the current change upon hybridization is ca. 3 μA in these conditions, with a S/N ratio of around 3. This sensitivity is comparable to that obtained on another system developed by our group [15]. We have tested with various target concentrations, with 10 nM, the S/N ration is around 2 while with 1000 nM target concentration, the current change recorded is not more than 3 μA, but is reached faster, after 30 min instead of 2 h. These results illustrate the fact that the current change is limited by the surface concentration of hybrids, i.e. the probe surface concentration and not by the target concentration, as soon as the quantity of target is sufficient. For example, 1 mL at 1 nM contains 1 pmol of target, which corresponds approximately to the probe quantity on our electrodes.

3.5.4. Fluorescence experiments

Besides the electrochemical experiments, we have performed fluorescence experiments in order to demonstrate the stability of the probe grafting as well as the selectivity of the target hybridization.

A systematic procedure was followed, in four steps, as described in Section 2. Three different targets were studied: COMP*, MISMATCH* and RANDOM*. As shown in Fig. 7, the first wash removes quite high quantities (around 18 pmol cm^{-2}) for the three targets. This corresponds to elimination of unspecifically adsorbed ODN. Since the second wash (still under mild conditions, 37 °C), the

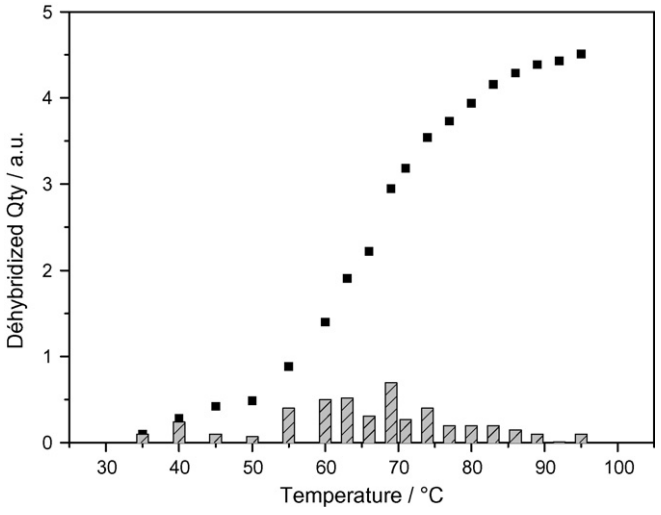


Fig. 8. Dehybridized COMP sequence as a function of the temperature. Nineteen washes were performed, from 35 °C up to 95 °C, in PBS. Bars indicate the dehybridized quantity (fluorescence intensity) corresponding to each wash at a given temperature. The curve (in black squares) is obtained by successive integration of dehybridized quantities.

results begin to be sequence-specific. Indeed, there are at least two times more desorbed COMP* sequences (equivalent to around 8 pmol cm^{-2}) than MISMATCH* or RANDOM* ones (equivalent to around 4 pmol cm^{-2}). We suppose that a part of hybridized COMP* targets may dehybridize even under these mild conditions. At the third wash, desorption is low for all (around 2 pmol cm^{-2}).

The results are different for the 4th step (dehybridization under stringent conditions, 80 °C in pure water. At this temperature, one can assume that all previously hybridized COMP* are denatured). Fluorescence remains low for MISMATCH* and RANDOM* targets (equivalent to around 3 pmol cm^{-2}), whereas it reaches five times more, i.e. 15 pmol cm^{-2} for COMP*. These data show that hybridization occurs efficiently on the modified electrode, and is highly sequence-specific.

In addition to these results, we performed an original and complementary experiment. This time, dehybridization was performed in PBS instead of pure H_2O, not only at 80 °C but at increasing temperatures from 35 °C up to 95 °C and fluorescence was followed stepwise, as a function if temperature. As shown (Fig. 8), fluorescence increases significantly for temperatures above 55 °C. The shape of the curve corresponds to the melting temperature of the PROBE-COMP* duplex (given at 72 °C in homogenous conditions). This result demonstrates without any doubt that dehybridization is responsible for this behaviour.

4. Conclusion

The modification of 5-hydroxy-1,4-naphthoquinone by γ-L-glutamyl-L-cysteinyl-glycine (glutathione) has been obtained by a direct and elegant method. The resulting 2-(γ-L-glutamyl-L-cysteinyl-glycine)-5-hydroxy-1,4-naphthoquinone (HNQ–Glu) presents 3 functional groups: the hydroxyl group for electropolymerization, the quinone group as the redox reporter, and the glutathione oligopeptide as a precursor for subsequent biomolecule linkage, via amine or carboxylic groups.

It is shown that the co-electrooxidation of HNQ–Glu and unmodified HNQ leads to poly(HNQ-*co*-HNQ–Glu), into which the quinone group remains electroactive, even in neutral buffered solution.

The poly(HNQ-*co*-HNQ–Glu)-modified electrode allows to detect directly DNA hybridization with a "signal-on" current (i.e. a current increase in Square Wave Voltammetry curve).

This DNA sensor is selective as a positive response is detected when a complementary DNA strand is added, while no significant signal change is observed consecutively to addition of a non-complementary or a mismatch sequence.

The molecular recognition is detected by changes in the electroactivity of the quinone group. This is due to the fact that DNA strands generate both steric hindrance and electrostatic effect on the electrode surface, which influences the quinone electroactivity.

The high selectivity could be explained by the fact that as the Glu moiety is negatively charged and poly(HNQ-*co*-HNQ–Glu) film is a cation (sodium) exchanger, non-specific adsorption of DNA strands are prevented.

The high selectivity as well as the "signal-on" response make this film original and efficient to be used in a direct DNA biosensor. This is, to our knowledge, the only work reported in the literature concerning an electrochemical DNA sensor using glutathione as the binding element. This approach can be generalized with other polypeptides, providing that a thiol group is available.

Acknowledgments

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References

[1] W.M. Albers, I. Vikholm, T. Viitala, J. Peltonen, Handbook Surface Interf. Mater. 5 (2001) 1–31.
[2] I. Palchetti, M. Mascini, Topics Curr. Chem. 261 (2005) 27–43.
[3] S. Cosnier, Anal. Lett. 40 (2007) 1260–1279.
[4] V.B. Kandimalla, V.S. Tripathi, H. Ju, Crit. Rev. Anal. Chem. 36 (2006) 73–106.
[5] S. Griveau, D. Mercier, C. Vautrin-Ul, A. Chausse, Electrochem. Commun. 9 (2007) 2768–2773.
[6] M.I. Pividori, A. Lermo, E. Zacco, S. Hernandez, S. Fabiano, S. Alegret, Thin Solid Films 516 (2007) 284–292.
[7] N.W. Pirie, K.G. Pinhey, J. Biol. Chem. 84 (1929) 321–333.
[8] H.H. Tai, S.O. Jung, J.M. Lee, K.Y. Lee, Y. Lee, J.S. Park, B.H. Chung, Anal. Chem. 79 (2007) 546–556.
[9] S. Akella, C.K. Mitra, Indian J. Biochem. Biophys. 44 (2007) 82–87.
[10] M. Musameh, N. Moezzi, L.M. Schauman, M.E. Meyerhoff, Electroanalysis 18 (2006) 2043–2048.
[11] M.C. Pham, J. Moslih, P.C. Lacaze, J. Electrochem. Soc. 138 (1991) 449–454.
[12] B. Piro, J. Haccoun, M.C. Pham, L.D. Tran, A. Rubin, H. Perrot, C. Gabrielli, J. Electronal. Chem. 577 (2005) 155–165.
[13] A. Rubin, H. Perrot, C. Gabrielli, M.C. Pham, B. Piro, Electrochimica Acta, submitted.
[14] B. Piro, S. Reisberg, V. Noel, M.C. Pham, Biosens. Bioelectron. 22 (2007) 3126–3131.
[15] S. Reisberg, B. Piro, V. Noel, M.C. Pham, Bioelectrochemistry 69 (2006) 172–179.
[16] M. Gueron, P. Plateau, M. Decorps, Prog. NMR Spectrosc. 23 (1991) 135–209.
[17] F.G. Rothman, Chem. Mater. 23 (1958) 1049–1051.
[18] S.B. Smith, Y. Cui, C. Bustamante, Science 271 (1996) 795–799.
[19] B. Tinland, A. Pluen, J. Sturm, G. Weill, Macromolecules 30 (1997) 5763–5765.
[20] M.D. Frank-Kamenetskii, Phys. Rep. 288 (1997) 13–60.