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Electrografting of carboxyphenyl thin layer onto gold for DNA and enzyme immobilization

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ABSTRACT

The convenient functionalization of metal surfaces by carboxyphenyl groups in aprotic media is not possible for two reasons. First, carboxy derivatives of diazonium salts are very unstable and, second, the electroreduction product is soluble in the solvent. So, the optimization of the conditions of the electrografting of the metal surfaces by applying aqueous solutions is much needed. Compared to earlier cyclic voltammetry approaches we have shown that the chronoamperometric deposition is more convenient. The constant potential equal to the voltammetric peak potential and the molar ratio 1:1 for the substrates: 4-aminobenzoic acid and $NaNO_2$ as the diazotization agent, in 0.5 M HCl, appeared to be very satisfying conditions for the deposition of a thin layer of deposit of perpendicularly oriented carboxyphenyl groups at the Au surface and for maximal elimination of the influence of the side-reactions products. Under the determined conditions the immobilization of DNA strands was optimal and the deposited laccase layer was tightly packed and very efficient toward the electroreduction of oxygen. Electrochemical impedance spectroscopy, electrochemical quartz crystal microbalance, cyclic voltammetry, chronocoulometry, atomic force microscopy, contact angle measurements and UV–Vis spectroscopy of the solution were used to characterize the electrografted carboxyphenyl layers and subsequent oligonucleotide and enzyme immobilization process.

1. Introduction

A derivatization of the surface is often required to improve the usefulness and properties of materials. It is also often critical for some particular applications. Many molecules and biomolecules, due to the direct contact with conducting surfaces, especially with metals, including gold, can undergo the deactivation or even denaturation process. Therefore, the modification of the metallic surface with an organic layer that provides specific functional end groups for the covalent attachment of various molecules has received much attention. Such modification appeared to be beneficial for applications in bioelectronics, long-range electron tunneling studies [1,2], sensors [3–5], and corrosion protection [6,7]. The selected organic layer accomplishes its function well when it is characterized by a good stability and evenly covers the modified surface. Also, the electrode conductivity can be tuned by the nature of the layer, depending on the application. The above conditions are well fulfilled by the phenyl films [8–14]. In general, the surface modification with a phenyl film requires the existence of the aryl radicals which are formed either spontaneously, or electrochemically or in the presence of appropriate reducing agent [15,16]. Diazonium cations can be obtained from diazonium salt or freshly generated in solution though diazotation of a primary amine [12].

In the case of the aqueous solutions the diazonium salt is freshly generated in solution though diazotation of a primary amine, so there is no need of external synthesis and isolation of the product. However, the amine derivative can become an unwanted component in the modifying layer and the diazonium salt may transform into either diazohydroxyde or diazoate [1,17]. The aim of this work was to determine the conditions of generation of diazonium salts and the formation, at the gold surface, of a thin, uniform carboxyphenyl layer. The application of correct electroreduction potential, time of reaction and appropriate molar ratio of the substrates significantly limited the unwanted side reactions in order to get good stability and functionality of the formed layer. The accumulation done at different potentials and for various molar ratios of the substrates was investigated using an electrochemical quartz crystal microbalance (EQCM). The obtained gold electrodes modified by carboxyphenyl film were characterized using atomic force microscopy (AFM), contact angle measurements, and electrochemical impedance spectroscopy (EIS) in the presence of electroactive species in the solution ($Fe(CN)_6{}^{3-/4-}$). The nature of the molecules bonded to the gold surface was determined by analyzing the UV–Vis spectra. We have examined the usefulness of the attached phenyl layers for the immobilization of DNA and enzyme–laccase.

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2. Experimental

2.1. Materials

All chemicals were of the highest purity available. NaOH, KH_2PO_4, K_2HPO_4, HCl, NaCl, and KCl, all 99.5%, were purchased from POCH, Poland. 4–aminobenzoic acid (4AA, 99%), sodium nitrite (97%), *N*-hydroxysuccinimide (NHS, 98%) and 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide hydrochloride (EDC, 99%) were delivered by Sigma–Aldrich. All oligonucleotides were purchased from MWG-Biotech (Germany). Laccase from *Trametes versicolor* was purchased from Sigma–Aldrich ($C = 15\,mg/cm^3$ in 0.02 M phosphate buffer solution, pH 3.8). The primary oligonucleotide sequence was taken from a strain of lactic acid bacteria of the genus *Lactococcus lactis*. These bacteria cause lactic fermentation by which sugars are transformed into lactic acid [18]. Their natural environment is the human digestive system (esophagus). They can be found in such food as leaven, cheese and yoghurt. The following oligonucleotide sequences were used:

- probe DNA: (5′ → 3′): $H_2N(CH_2)_6$CGCCAACGTTTTCGCCAACG
- complementary target DNA (5′ → 3′): CGTTGGCGAAAACGTTGGCG (designed from the gene of *Lactococcus*, a common milk bacteria).

The procedure for the activation of carboxyl groups and DNA attachment was described in the literature [19,20]. DNA concentration used for immobilization and hybridization processes was $2 \cdot 10^{-6}$ M.

2.2. Electrochemical measurements

Cyclic voltammetry (CV), chronoamperometry (CA), electrochemical impedance spectroscopy (EIS) and electrochemical quartz crystal microbalance (EQCM) measurements were performed using an Autolab, model PGSTAT 12 potentiostat equipped with an ECD amplifier module and the electrochemical analysis system (Eco Chemie B. V., Utrecht, Netherlands). For electrochemical measurements the three-electrode system consisting of a gold Au-EQCM electrode (6-MHz Au/TiO_2) used as the working electrode, a Ag/AgCl/3 M KCl reference electrode and a platinum wire used as the auxiliary electrode. The piezoelectrically active (geometrical) surface area of the working Au electrode was $0.352\,cm^2$ and the real surface area $A = 0.514\,cm^2$. The real surface area of the Au-EQCM electrode was determined from the charge corresponding to the upd Pb voltammetric peaks. The deposition solution was 0.01 M lead(II) perchlorate in 0.1 M perchloric acid. Before experiments, the Au-EQCM electrode was electrochemically pretreated: first by cycling between 0 V and 1.8 V (hold 10 s at 1.8 V) in 0.5 M NaOH with scan rate 50 mV/s, and then by cycling between −0.3 and 1.5 V (vs. Ag/AgCl) in 0.1 M H_2SO_4 solution until stable voltammograms typical for a clean gold electrode were obtained [21]. Before the deposition of the phenyl groups at the electrode surface the gold-coated quartz crystals were exposed to the potential cycling between −0.65 V and 0.95 V at high scan rate (>1 V/s) in 0.1 M perchloric acid. The electrochemical cell was kept in a Faraday cage to minimize the electrical noise. The impedance spectra were obtained for the frequency range 0.05 Hz–10 kHz with the ac amplitude equal to 5 mV.

2.3. Atomic force microscopy (AFM), contact angle and UV–Vis measurements

The measurements of the contact angle were performed using a Hetta Lite optical tensiometer, model TL100. A Nanoscope V AFM microscope (Veeco Instruments, Inc., USA) was used to examine the surface of the samples. Silicon cantilevers for high resolution imaging (MPP-12120, Bruker), of resonant frequency ca. 116–164 kHz and spring constant $5\,N\,m^{-1}$ were used in the AFM monitoring in the tapping mode (TM-AFM) in air. The experimental operating frequency was readjusted after engaging the tip on the surface. Calibration of the microscope was achieved by imaging the calibration gratings supplied by the manufacturer. The images presented in this work are height type images. AFM and contact angle measurements were done before and after modification of the gold surface by the electroreduction of diazonium salt freshly generated in the solution. Absorption spectra were recorded with a PerkinElmer spectrometer, model Lambda 25, using mixtures of 4-aminobenzoic acid and sodium nitrite of different molar ratios and pure 4-chlorobenzoic acid, 4-hydroxybenzoic acid and 4-[(4′-aminobenzoyl)oxy]benzoic acid.

More experimental details are given in the *Supplementary Data*. All electrochemical and UV–Vis experiments were done in room temperature (21 °C).

3. Results and discussion

The diazonium cations can be generated either in the electrochemical cell or in a flask by the reaction of the appropriate amine and sodium nitrite-diazotization agent. The synthesis is fast enough; however, some problems appear. They are related to the unsatisfying stability of the formed diazonium cation and the presence of side reactions, especially in the case of carboxyl derivative [22–25]. As in our study we used 4-aminobenzoic acid and $NaNO_2$ in different molar ratio (4AA:$NaNO_2$), so the possible side reactions in 0.5 M HCl solution are those presented in Scheme 1

To optimize the conditions for the gold surface modification with a carboxyphenyl layer (of the thickness close to the monolayer), the electroreduction process of an appropriate diazonium salt freshly generated in the solution was monitored using the following techniques: EQCM, AFM, UV–Vis, EIS, CV and chronoamperometry.

3.1. UV–Vis results

Typical spectra of all possible species are presented in Fig. 1A. According to Fig. 1A the pure-possibly chemical species formed in acidic media from the basic substrate (amine derivative and sodium nitrate) have the characteristic bands at: 227; 243; 210 and 257; 204 and 251; and 204 and 265 nm, for 4-aminobenzoic acid, 4-chlorobenzoic acid, 4-hydroxybenzoic acid, 4-[(4′-aminobenzoyl)oxy]benzoic acid and 4-carboxybenzenodiazonium salt. Fig. 1B presents the UV–Vis spectra recorded for the solutions containing the substrates present at various molar ratios. In the case of sufficient deficit of the sodium nitrate the solution contained unused amine and the diazonium salt. To monitor the participation of the side reactions products (see Scheme 1) the UV–Vis spectra were recorded just after preparation of the solution and then every 15 min for the next 4 h. For the molar ratio higher than 1:0.5 (4AA:$NaNO_2$) the presence of the amine derivative was not observed. With an increase in time of the solution preparation a small decrease (circa 10%) in the absorbance for the characteristic diazonium band at 265 nm and a small shift (3 nm) toward smaller values of the wavelength were noted. These changes were rather small for the equimolar ratio of the substrates during the first hour, see Fig. 2. Such behavior suggests that in the composition of the phenyl layer at the gold surface, for the molar ratio smaller than 1:0.5 (4AA:$NaNO_2$), the unreacted amine is also involved. In the case of higher than 1:0.5 (4AA:$NaNO_2$) molar ratios the participation of the 4-hydroxybenzoic acid in the formed layer also took place. This participation increased with increasing the