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SECM characterization of immobilised enzymes by self-assembled monolayers on titanium dioxide surfaces

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Abstract

SECM in generator-collector mode was used to detect the presence of immobilised enzymes on titanium dioxide layers which were chemically or electrochemically generated with possible application as chemical sensors and biosensors. Glucose oxidase (GOx) and horseradish peroxidase (HRP) were immobilised by SAM generation using aminopropyltriethoxysilane (APTES) and ascorbic acid. The enzymes were successfully immobilised on two different TiO_2 surfaces. A simple test of durability of the system was made and a model of SAM organisation is presented.

Keywords: SECM; Titanium dioxide; Enzyme; Self-assembled monolayers

1. Introduction

The generation of self-assembled monolayers (SAM) is an elegant method to generate modified surfaces [1–3]. This method allows the immobilisation of biomolecules like enzymes, proteins, DNA etc. on different materials in order to obtain chemical sensors and biosensors [4–8] and to study the electrochemical behaviour and biocompatibility of metals used in medical implants [8,9]. SAMs were originally prepared on precious metals such as gold or platinum, nowadays they can also been generated on polymers, glass and non-precious metals [10,11]. However, there have been only a few attemps to use oxide surfaces and/or passivated metal surfaces. One of these was published by Hersh and Weetall [12] in 1970. These authors use aminopropyltriethoxisilane (APTES) SAM to fix glucose oxidase on thermal NiO. They wanted to find a chemical and biological stable surface to immobilise biologically active molecules. With the same goal, Jennissen et al. [8] treated oxidised titanium plates with APTES and immobilisied I-ubiquitin and bone morphogenetic protein (BMP-2) on these surfaces after an activation of the silanised plates with carbonyldiimidazole (CDI). Their aim was to obtain “chemotactic or juxtacrine surfaces” or both [8,13].

Titanium dioxide (TiO_2) is the most stable compound of the titanium oxides and has several important properties: optical transparency, semiconductor behaviour, chemical stability and a high surface area [6,14–16]. This material finds wide application in batteries and optical devices, electronic chips and as active surface for chemical reactions [17–24]. Beside these properties, TiO_2 is also biocompatible, i.e., its toxicity and its reactivity with biomolecules are very low which allows its use in the manufacture of biological devices and sensors [6,18–21] and as a component in biocompatible chemicals [6,12].

In the present work, the enzymes glucose oxidase (GOx) and horseradish peroxidase (HRP) were immobilised [5,25–30] on two different titanium dioxide samples: an anodically grown oxide film on a titanium metallic surface and a chemically generated titanium dioxide layer prepared with nanoparticles (12 nm diameter) sprayed on a glass plate. Our objective was the development of a stable biosensor surface.

2. Experimental

2.1. Sample preparation

Anodically grown titanium dioxide films were generated on the surface of a 99.99% titanium rod (Aldrich) with a 0.8 cm outer diameter, sealed in Teflon®; the exposited surface was polished to a mirror by using successively finer grades (down to 0.3 microns)

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of alumina slurry before each potentiodynamic experiment. The potential scan was programmed between −0.8 V and +1.3 V. Only one cycle was needed to obtain a thin film of approx. 30–50 nm thickness [15,16]. A classical three-electrode electrochemical configuration was used, connected to a home-made potentiostat, with a solution of 1.0 M NaH_2PO_4 as electrolyte at room temperature; the reference electrode was an Ag/AgCl electrode and a 1 cm^2 platinum plate served as counter electrode.

Nanocrystalline titanium dioxide films were prepared by spraying a titanium dioxide paste (nanocrystals with 12 nm diameter in average) on a glass plate in order to obtain a film of approx. 0.5 mm thickness that was dried initially at 80 °C for 30 min and sintered at 450 °C for 30 min in a furnace. After thermal treatment, the samples were cut into 1 cm^2 discs and kept dry in a container [27,31,32].

SAM generation: the method used for SAM generation was developed by combining two different methods found in the literature [8,12,28,29,33]. Both, anodically and chemically prepared oxide layers, were treated in the same way. The samples were washed with deionised water and in the next step they were placed in a solution of aminopropyltriethoxysilane (APTES—Merck) in toluene (1.0 ml APTES in 10 ml of toluene) for 14 h at room temperature, washed with toluene, acetone and ethanol and treated for 30 min with a saturated solution of ascorbic acid (Vitamin C — Fluka) in 90% ethanol (Merck) + 10% methanol (Merck). Berlin et al. were successful by using this substance to fix enzymes on NH_2-polymers [28]. After this treatment the samples were again washed with ethanol and water and dried in air for at least 2 h.

For glucose oxidase immobilisation, the silanized samples were treated for 20 h in 1 ml of a approx. 60 units/ml aqueous solution of glucose oxidase (Sigma 49180) at 4–5 °C. After this, the samples were washed again several times with a water spray to remove any no-linked enzyme. For the horseradish peroxidase immobilisation, the samples were treated for 15 h in a solution of 1 mg of peroxidase (Sigma P6782) in 900 µL phosphate buffer (pH 7). After this treatment the samples were carefully washed with neutral pH phosphate buffer.

2.2. SECM experiments

The SECM experiments to detect the activity of the enzymes GOx and HRP were performed immediately after the sample preparation with exception of the stability experiments of the SAMs on the oxide surface which were performed after a 6 days treatment in deionised water at 4 °C. The generator-collector mode [1] was used to investigate the presence of immobilised enzymes (GOx and HRP) on the TiO_2 layers [1,3,7,20,24,25,30]. A home-made computer program was used to monitor the experiment. The sample was fixed on the bottom of a small glass cell that was placed below the tip. A 25 µm diameter platinum wire (Goodfellow) sealed in a glass capillary and mechanically polished with carbon paste (up to 0.3 µm) was used as working electrode (ultramicroelectrode — UME or "tip"). The potential was monitored vs. an Ag/AgCl reference electrode and a platinum wire was used as counter electrode. Scans were performed in different samples and in different regions of the same sample in areas of 500×500 μm^2 or 1000×1000 μm^2. Before the SECM experiment, cyclic voltammetry was performed to test the contact of the electrode and to choose the best potential to make the tip-down (approaching the ultramicroelectrode to the sample surface). Tip-down was performed in different regions of the sample to correct any slope variation in x–y axes.

In the GOx experiments [3,5,9,12] the electrolyte was 0.05 M NaH_2PO_4 (pH 7). The reduction reaction of oxygen in aqueous medium was used to perform the tip-down. While approaching the electrode to the surface the reduction current will decrease due to the negative feedback-effect [1,3,8,19,20]. The UME was positioned in a distance of 5 µm above the sample in its initial position ($x=0$, $y=0$) and polarised at +0.7 V (potential at which the hydrogen peroxide, the product of the enzymatic reaction, is being oxidized on the UME surface and can be detected). After waiting to obtain a constant current, an initial SECM scan was run at 10 $\mu m\ s^{-1}$. After this initial scan, the tip was returned to the starting position and 2.0 ml of a solution of 0.2 mM β-D^+ glucose (Fluka) was added to the cell which leads to a total glucose concentration of 0.08 mM in the solution and a new scan was performed to investigate the effect of the immobilised enzyme.

A 2.0 mM hydroxyferrocene (in neutral phosphate buffer) served as electrolyte in the horseradish peroxidase detection. The tip-down was determined by the electrochemical oxidation of the ferrocene (Fc) between 0.25 V and 0.3 V.

After the tip-down the UME was adjusted to 5 µm above the sample in the starting position ($x=0$, $y=0$) and polarised at 0.0 V (potential of reduction of the ferrocinium to ferrocene). Once the current was constant, an initial scan was carried out. The UME was again reset to its starting position and 1.0 ml of a 1.0 mM hydrogen peroxide (H_2O_2) solution was added to the electrolyte as enzyme reactant to get a final concentration of 0.2 mM. A new scan was performed to determine the enzyme [3,5,19].

3. Results and discussion

In the present work chemically and anodically obtained titanium dioxide samples were modified by silanisation with APTES [33–37] in anhydrous medium. The enzymes glucose oxidase and horseradish peroxidase were successfully immobilised on these surfaces [8,12,13,28–37] by using ascorbic acid (Vitamin C) as a connector [28].

A mechanism of the formation of 1,3,5,7-tetramethylcyclotetrasiloxane SAM on thin oxide layers with application to photocatalysis was suggested by Tada [29] in 1995. Recently, Fadeev and Helmy [31] published a methodical work in which they studied the mechanism of the SAM generation of octosilane compounds in detail on thermal titanium oxide (Titania). They found a SAM density between 4.5 and 5.0 groups/nm^2 for different octosilanes which suggests a formation of well packed SAM. The initial step is the adsorption and the covalent linkage of APTES on the oxide surface. One of the problems is to know whether the adhesion between the silane and the oxide surface is physical adsorption or chemical (covalent) bonding. It is well known that water (liquid or atmospheric) is adsorbed on the surface of many oxides. The water molecules can also react with the oxides on the surface

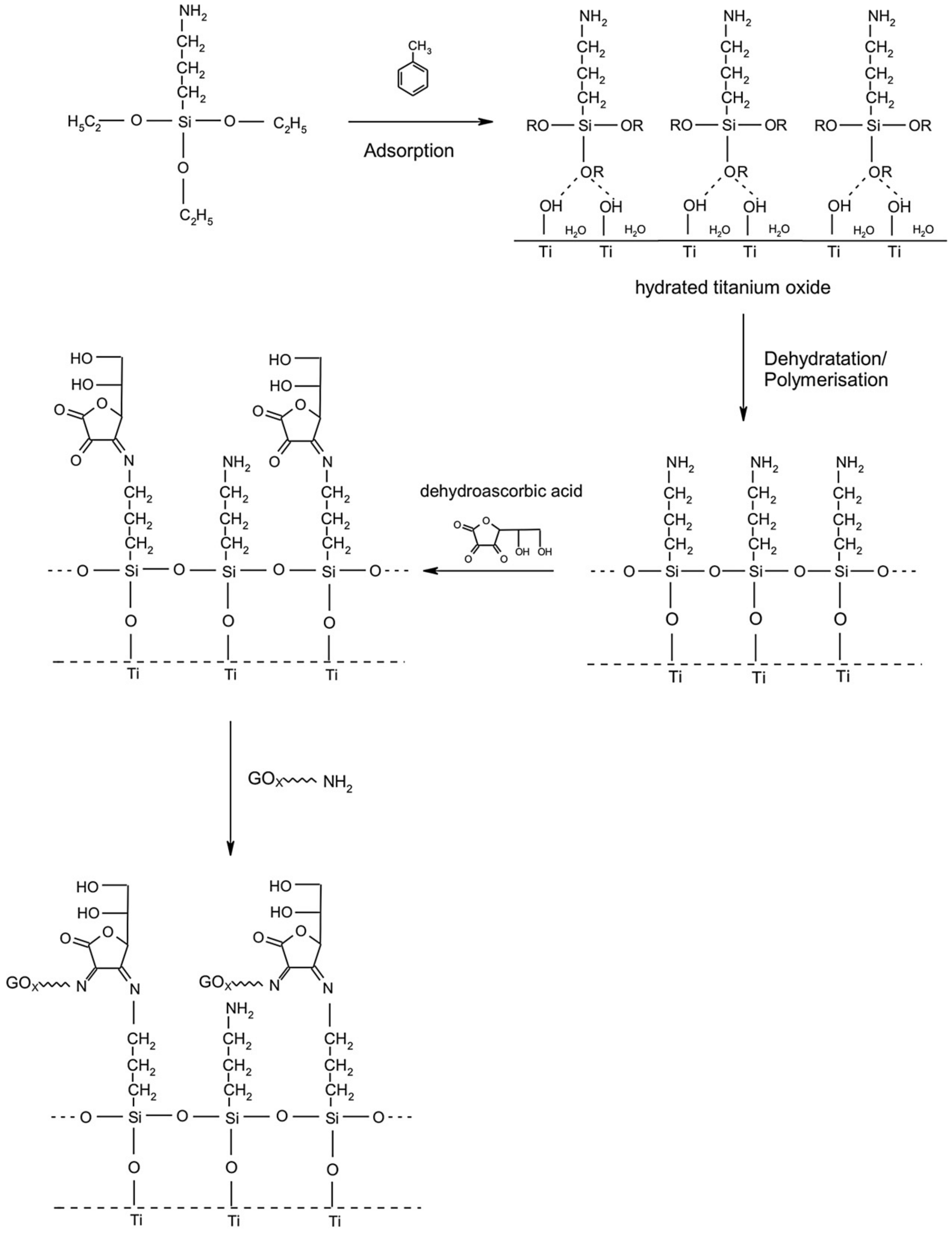

Fig. 1. Suggested mechanism to the formation of SAM of 3-aminopropyltriethoxysilane, dehydroascorbic acid and glucose-oxidase on titanium oxide surfaces.

forming hydroxyl groups to which silane molecules could be linked. This hydrolysis reaction is depending on the pH of the solution and on the zero point of charge (ZPC) of the oxide; the surface becomes charged by reacting with H^+ or OH^- ions. Below the ZPC hydroxide surfaces adsorb protons to produce positively charged surfaces. Above this point the hydroxyl groups loose protons to produce negative charges. This is the so called acid-base (amphoteric) behaviour of oxide surfaces. That

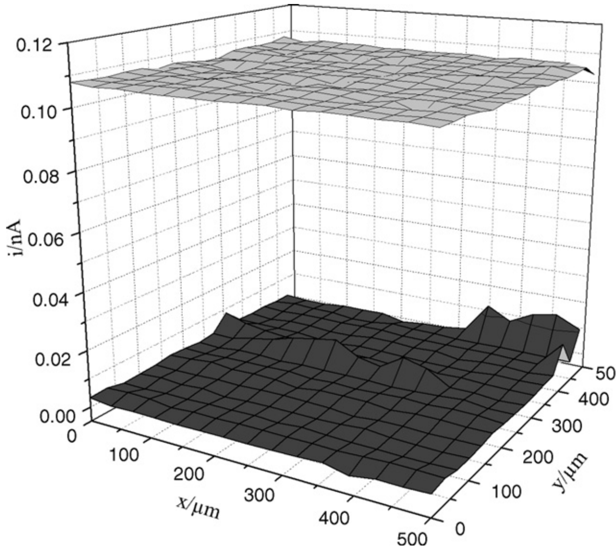

Fig. 2. SECM images of the system Ti–TiO_2–GOx in NaH_2PO_4 0.1 M (pH 7.0; 25 °C) before (low current) and after (high current) the glucose addition in an area of 500 × 500 μm^2. UME of Pt, 25 μm diameter polarised at +0.7 V. Scanning speed: 10 μm s^{-1}.

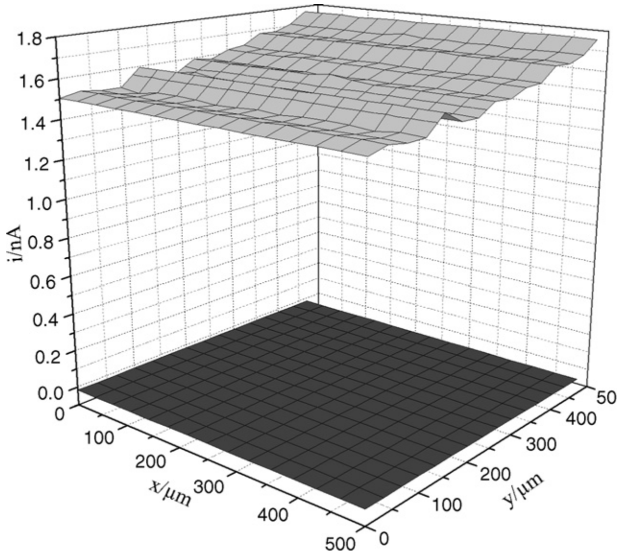

Fig. 4. SECM images of the system Ti–TiO_2–GOx in NaH_2PO_4 0.1 M (pH 7.0; 25 °C) before (low current) and after (high current) the glucose addition in an area of 500 × 500 μm^2. UME: Pt (25 μm diameter) polarised at +0.7 V. Scanning speed: 10 μm s^{-1}. In this case the sample was maintained 6 days in deionised water after the first SECM experiment to test the resistance of the enzyme bond.

has been often discussed in literature [15,16,34–36]. In neutral pH the titanium dioxide surface should be positively charged. The molecular structure of silane is also a function of the pH and for pHs close to neutral, amino silanes suffer hydrolysis into silanol and siloxane. The negative charges are concentrated on the silanol groups and the positive on the amino groups. The charge attraction explains the strong adhesion between the oxide surface and the silanol. The low water quantity adsorbed on the titanium oxide permits the polymerization of the aminosilane monomers on the surface by forming siloxane bonds [36].

The second step is the linkage of one keto group of the dehydroascorbic acid (product of oxidation of ascorbic acid in presence of alcohols) with the amino group (NH_2) of the APTES. Finally a NH_2-terminal group of the enzyme links to the remaining keto group of the connector dehydroascorbic acid. The method was suggested by Berlin et al. [28] and it is based on the diketo group present on the structure of the dehydroascorbic acid. The cited authors have used n-butylamine as a model molecule to test this method. They were successful in linking several of biomolecules.

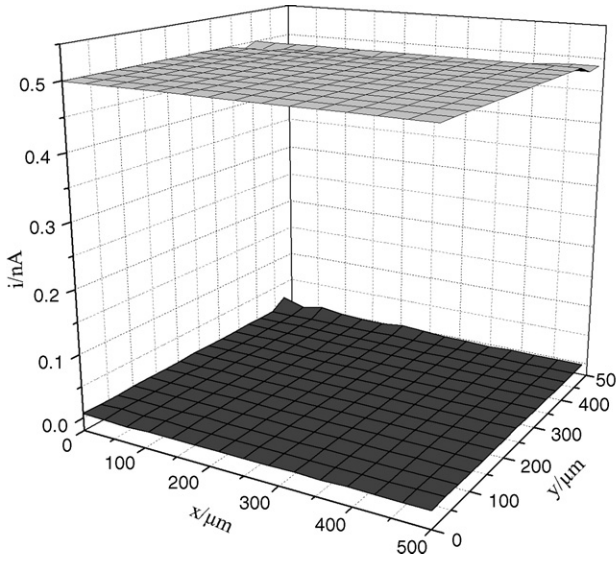

Fig. 3. SECM images of the system glass–TiO_2–GOx in NaH_2PO_4 0.1 M (pH 7.0; 25 °C) before (low currents) and after (high currents) the addition of glucose in an area of 500 × 500 μm^2. UME of Pt, 25 μm diameter polarised at +0.7 V. Scanning speed: 10 μm s^{-1}.

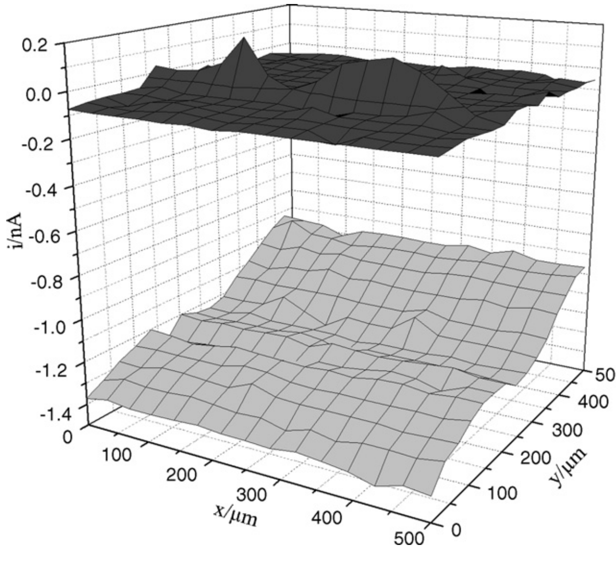

Fig. 5. SECM images of the system glass–TiO_2–HRP in solution of ferrocene 2.0 mM in buffer (pH 7.0; 25 °C) before (low current) and after (high current) the addition of H_2O_2 in an area of 500 μm ^{2}. UME of Pt, 25 μm diameter polarised at 0.0 V. Scanning speed: 10 μm s^{-1}.

A schematic representation of this plausible SAM organisation on the TiO_2 substrate is shown on Fig. 1.

3.1. Glucose oxidase

SECM experiments were carried out to investigate the presence of enzyme on the surface of the samples. The generator–collector mode was used to detect products of the reaction between the enzyme and substrate at the UME.

The following reaction was used to detect the GOx:

$$\text{Glucose} + O_2 + \text{GOx} \rightarrow \text{Gluconolactone} + H_2O_2 + \text{GOx} \quad (1)$$

The hydrogen peroxide generated by the reaction above is oxidised at the UME surface and the current signal is detected. Though the specific enzyme activity can not be directly quantified, its action on the substrate can be clearly observed by the difference between the SECM image before and after the addition of glucose. Typical SECM images on titanium dioxide surfaces (thin films anodically grown and thicker nanoparticle chemical films) are shown on Fig. 2 and on the Fig. 3, respectively. Both oxides were able to link the SAMs but the electrochemically obtained oxides gave more experimental problems: hampering a good tip-down, probably because of its smaller thickness (between 30 and 50 nm) and higher concentration of defects [14–16,37].

The currents measured at the UME differed significantly. In experiments made immediately after the immobilisation step, the enzyme reaction on an anodically grown TiO_2 layer caused currents between 0.05 nA and 0.15 nA while this reaction with samples of chemically prepared TiO_2 layers showed currents between 0.50 nA and 1.50 nA. Obviously, the surface density of the SAMs and the coupled enzymes is much higher at the chemically prepared TiO_2 layers on the glass plate. Experiments made in different regions of the same sample showed a small variation of the current, that may happen because of a non-complete homogeneous distribution of enzymes on the surface. The concentration of linked enzyme depends on the distribution of the SAMs on the oxide surface which, in turn, depends on the density of the oxide and the topography of the surface [6,12,14,24,27].

In order to test the stability of the linked enzyme on these modified surfaces (an important factor for a future application as a biosensor) immediately after the SECM experiment some samples of the electrochemically prepared oxides were washed and treated in deionised water for 6 days [9,16,36]. After this period the SECM experiment was repeated and the enzyme effect on the glucose was studied again. Fig. 4 shows the SECM image obtained for one of these samples. The magnitude of the current after this treatment was always larger – sometimes even up to 10 times – as compared to the first SECM experiment.

The positive enzymatic effect after this treatment proves the strong linkage of the enzyme on the oxide surface and the larger currents can be explained by an optimal configuration of the enzyme molecule: enzymes are big molecules with a 3D structure and are programmed to work in aqueous medium. The water adsorption opens the structure of the enzyme and permits a better exposition of the active sites [38].

3.2. Horseradish peroxidase

The presence of immobilised horseradish peroxidase on the oxide surfaces was investigated by SECM experiments using the enzymatic oxidation of ferrocene in accordance with the following chemical equation:

$$H_2O_2 + 2\text{Fc} + \text{HRP} \rightarrow 2\text{OH}^- + 2\text{Fc}^+ + \text{HRP} \quad (2)$$

An initial scan was made without the presence of hydrogen peroxide at 0.00 V (potential of reduction of the ferrocinium) and, after the addition of 1.0 ml of a 1.0 mM solution of hydrogen peroxide, a new scan was performed to analyse the effect of the enzyme. The ferrocinium ions generated by enzymatic catalysis are reduced to ferrocen at the UME surface and the current response can be observed [35].

To the anodically prepared oxides the SECM images were not reproducible. The chemically prepared oxides gave better results (Fig. 5). Current values here were, in average, larger than those observed in the experiments with glucose oxidase (between −0.8 nA and −3.0 nA). These larger currents may be an effect of the oxide thickness in combination with a better packing of the enzyme molecules because HRP has a lower molecular mass compared to GOx.

4. Conclusions

Composed aminopropyltrietoxysilane and dehydroascorbic acid self-assembled monolayers were generated on titanium dioxide surfaces to which the enzymes glucose oxidase and horseradish peroxidase were fixed. The enzyme pseudo-activity is dependent on the thickness of the oxide layers. It is smaller on thin anodically prepared films than on thicker nanocrystalline TiO_2 layers. The mechanical resistance, the determination of the specific enzyme activity and its distribution on the sample are problems that we need to solve before any potential application of this modified surface as a biosensor. Theoretical models [26] and microscopy techniques (like fluorescence and chemiluminescence microscopy) may be used to obtain this information. After a durability test of 6 days water treatment, the enzyme activity was even greater compared to the original experiment. The effect can be explained by an optimised configuration of the enzyme due to water absorption.

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