Development and study of anodic Ti/TiO₂ electrodes and their potential use as impedimetric immunosensors

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Abstract
Titanium dioxide films were anodically formed at various potentials up to 65 V in 1 M H₂SO₄. Oxide films were characterized by performing various techniques, including electrochemical impedance spectroscopy, scanning electron microscopy, Raman spectroscopy, ellipsometry and diffuse reflectance FT-IR spectroscopy. Low voltage anodization (up to 10 V) results to amorphous TiO₂, whereas at higher applied potentials (up to 65 V), anatase is the predominant form. Anatase films were further hydroxylated with an acidic agent and the effect of this treatment on the overall impedance of the electrodes was studied with impedance spectroscopy. The potential use of anodic (anatase) Ti/TiO₂ electrodes in the development of impedimetric immunosensors is also demonstrated by monitoring the immunoreaction of avidin/anti-avidin with different instrumental approaches based on a FRA analyzer, an LCR-meter and a home-built charge integrator (Multipulsar).

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1. Introduction
The stability of titanium in a variety of corrosive environments is due to the presence of a thin coherent oxide film on its surface. The spontaneously formed (native) oxide, which is rebuilt within milliseconds after any damaging, is amorphous at room temperature and has a thickness of 2–6 nm [1]. Titanium dioxide (TiO₂), hereafter titanium oxide or titania, is a wide band-gap oxide that is potentially of use in a number of applications, including transparent conducting oxides [2], chemical sensors [3,4], biosensors [5–7], medical implants [8], pulp and paper industry [9], optical components, aeronautics, decoration and art [10], etc. TiO₂ has also been investigated extensively as one of the most promising semiconductor photocatalysts [11] at a wide range of processes, including solid-state solar cells [12], organic synthesis [13] and mineralization and/or detoxification of organic compounds [14].

TiO₂ has three crystal modifications, rutile, anatase and brookite with dielectric constants (bulk oxides) 110, 48 and 78, respectively. Anatase and rutile both have a tetragonal structure with a disordered octahedral coordination of oxygen ions around the Ti⁴⁺ cation, but the number of edges shared by the octahedral increases from 2 (out of 12) in rutile to 4 in anatase. Although rutile has been assumed to be the most stable form because of its common occurrence, anatase is 8–12 kJ/mol more stable than rutile [15].

So far a wide variety of methods has been used for the development of TiO₂ films. These include, atomic layer deposition [16], metal-organic chemical vapour deposition [17], sputtering [18], sol–gel techniques [11,21], thermal evaporation [19], pulsed laser deposition [20], thermal oxidation [21], anodization [21], etc.

Regarding the anodic oxide films, numerous works have been published, which can be categorized in the following (main) modes: (i) the potentiostatic growth mode, according to which a constant potential is applied to the anode, (ii) the galvanostatic growth mode, according to which a constant current flows through the electrochemical cell, and (iii) the combined growth mode, according to which a constant current flows through the cell until the anode potential reaches a predefined value and then, this potential is maintained for a given period of time [22]. A potentiodynamic growth mode based on repetitive potential cycles within a potential window has also been proposed [23].
Electrochemical impedance spectroscopy (EIS) experiments were performed with an Autolab Analyzer (PGSTAT12/FRA2, EcoChemie) in a one-compartment three-electrode cell. Titanium (or titanium oxide) electrodes were used as working electrodes. The reference electrode was a Ag/AgCl/3 M KCl (BAS, IN) and a platinum coiled wire served as the auxiliary electrode. Impedance spectra were recorded over a frequency range of $10^{-1}$ to $10^5$ Hz using a single sinusoidal excitation signal, superimposed on 0.020 V. Excitation amplitude of 10 mV was used throughout. All potentials up to 10 V are referred to a Ag/AgCl/3 M KCl reference electrode.

Ellipsometry studies were conducted with a SOPRA ES4G spectroscopic ellipsometer equipped with a Xenon lamp and a phototube over the spectral range 250–900 nm. Functions $\tan(\psi)$ and $\cos(\Delta)$ were analyzed with Wvase32 software (J.A. Wool-lam Inc.). Raman spectra were conducted at 532 nm (2.5 mW laser power) using a Renishaw 1000 spectrometer equipped with a Leica microscope. SEM images were taken with a JEOL JSM-5600 scanning electron microscope. Diffuse reflectance FT-IR spectra were recorded using a Perkin-Elmer spectrometer.

2.2. Electrodes fabrication

Titanium electrodes of 2 mm diameter active surface were constructed by using the commercial kit EasyCon (EasyCon Hellas, provided by EcoChemie). Titanium disks (>99.6%, 0.5 mm thick, 2 mm diameter) were glued on a bronze rod (2 mm diameter) with the aid of a silver-based conductive adhesive. A two-component epoxy resin was used to ensure no electrical contact between the conductive adhesive and the bronze rod with the electrolyte. Titanium electrodes were further covered with 4 mm diameter heat-shrinkable tubing.

Before use, titanium electrodes were polished with a series of emery papers (400, 1200, 2000 and 4000 grit), sonicated for 3 min in double distilled water (DDW), polished with Al2O3 slurry (0.03 μm grain size), sonicated once more for 3 min in DDW and then etched in a HF/HNO₃/H₂O (1 + 3 + 4) mixture for 20 s.

2.3. Anodic oxidation of titanium substrates

Passive films of titanium oxide were anodically formed at 0.5–65 V in 1 M H₂SO₄ for 1 h under mild stirring. Low voltage (up to 10 V) anodization experiments were potentiostatically formed in a three-electrode mode using the Autolab analyzer (see Section 2.1). High voltage (up to 65 V) anodization experiments were carried out using a lab-build dc supplier (10–220 V; 1–30 A) working in a two-electrode mode with a large surface platinum electrode (1 cm × 2 cm) as the counter electrode.

After oxidation, electrodes were extensively cleaned with DDW, dried with Argon and kept in a dust-protected chamber.

2.4. Construction of immunosensors

Direct protein immobilization onto the oxide layer was made based on the procedure reported by Kennedy et al. [32]. Ti/TiO₂ electrodes were anodically formed at 30 V as described above, hydroxylated in an HF/HNO₃/H₂O (1 + 3 + 4) mixture for 1 min and then conditioned overnight in 0.9% NaCl solution [32]. Electrodes were then incubated overnight at +4°C in a 1 mg/ml avidin (Calbiochem) in 50 mM 4-(2-hydroxyethyl)piperazine-
1-ethanesulfonic acid (HEPES; Sigma) buffer solution, pH 8. Avidin-modified electrodes were thoroughly rinsed with copious amounts of the buffer solution, incubated for 2 h in room temperature with 1 mg/ml bovine serum albumin (BSA; Sigma) to block any non-specific binding sites, thoroughly rinsed with the buffer solution, and then incubated overnight in a sample of 10 μg/ml anti-avidin (Sigma) in the buffer solution.

3. Results and discussion

3.1. Development and characterization of anodic oxide films

The first visual observation of the anodically formed titania layers was the different colours of the oxidized surfaces. Depending on the applied voltage they appeared light gold, deep gold, light blue, dark blue and violet. In accordance with previous studies [21], this effect is attributable to the different thickness of the oxide layers.

Electrochemical properties for a series of Ti/TiO2 electrodes being anodized and tested under specific experimental conditions are given at Table 1. The stability of Ti/TiO2 electrodes in various buffering systems (50 mM, pH 7.4–8.0) including PBS, MOPS and HEPES was tested. Best results were obtained in HEPES solution, and thus further experiments were made in it [7]. All ac measurements were conducted at 1 kHz (except otherwise stated), as at this frequency biomolecules better resemble the behaviour of a capacitor [33]. Taking into account that even low potentials are likely to modify protein binding [34], measurements were carried-out at a 0.020 V bias voltage.

According to the equation $C = \varepsilon \varepsilon_0 A/d$ (terms have their usual meaning), the electric capacitance ($C$) between the working electrode and the electrolyte is determined by the thickness ($d$) of the oxide layer, considering constant both the area ($A$) and their dielectric constant ($\varepsilon$). For films developed up to 10 V ("low potential"), results are consistent with this assumption, as capacitance decreases from 14 to 1.4 μF/cm². The same trend is also observed for films developed at higher applied potentials (30–65 V), since capacitance also decreases from 3.6 to 1.4 μF/cm². The capacitance "gap" between films developed at "low" and "high" applied potentials is attributable, to a certain extent, to the different instrumentation used for their formation (Table 1).

The electrochemical behaviour, the morphology and the structure of the produced oxides were studied and results are discussed. Films developed at "low" potentials are relatively smooth (especially in the case of the oxide formed at 1 V), compact without any severe cracks, whereas films developed at >25 V bear characteristic superficial craters (images not shown).

The different electrochemical behaviour of the oxides formed at "low" and "high" voltages is clearly reflected at Bode plots illustrated in Fig. 1. As mentioned in Section 1, semiconductive properties of titania layers are likely attributable to oxygen vacancies, Ti3+ interstitials and solvent impurities, which are among the defects of the system [28]. This assumption seems valid for anodic films developed at >10 V applied voltage. As it can be seen in Fig. 1, up to 5 V, the pattern of Bode plots is indicative of a uniform, compact layer exhibiting a single time constant attributable to the interfacial capacitance ($C_{oxide}$) and the over-

**Table 1**

<table>
<thead>
<tr>
<th>Anodization voltage $a$ (V)</th>
<th>Capacitance/surface $b,c$ (μF/cm²)</th>
<th>Thickness $d$ (nm)</th>
<th>Raman peaks $e$ (cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>14.0–14.5</td>
<td>3.5</td>
<td>None (amorphous)</td>
</tr>
<tr>
<td>0.5</td>
<td>13.1–13.9</td>
<td></td>
<td>None (amorphous)</td>
</tr>
<tr>
<td>1.0</td>
<td>10.2–11.7</td>
<td>8.2 ± 1.0</td>
<td>None (amorphous)</td>
</tr>
<tr>
<td>2.0</td>
<td>3.9–4.4</td>
<td>3.0</td>
<td>None (amorphous)</td>
</tr>
<tr>
<td>5.0</td>
<td>2.4–2.8</td>
<td>18.5 ± 0.2</td>
<td>None (amorphous)</td>
</tr>
<tr>
<td>10</td>
<td>1.4–1.7</td>
<td>29.9 ± 0.2</td>
<td>None (amorphous)</td>
</tr>
<tr>
<td>25–30</td>
<td>3.6–3.7</td>
<td>86.6 ± 6.0</td>
<td>653, 514, 398, 146 (anatase)</td>
</tr>
<tr>
<td>40–45</td>
<td>2.9–3.1</td>
<td>93.6 ± 5.0</td>
<td>653, 514, 398, 146 (anatase)</td>
</tr>
<tr>
<td>60–65</td>
<td>1.4–1.8</td>
<td></td>
<td>653, 514, 398, 146 (anatase)</td>
</tr>
</tbody>
</table>

$^a$ At "high voltage" experiments values are given as a potential range indicating the potential drift observed throughout the anodization experiments.

$^b$ Average of five electrodes.

$^c$ Measurements were conducted at 1 kHz and 0.020 V bias voltage (rms 10 mV) in 50 mM HEPES buffer solution, pH 8. The geometrical surface of the electrodes is 0.0314 cm².

$^d$ Ellipsometry studies (except in case of 0V, Ref. [1]).

$^e$ Average of five different points.
all) resistance \( (R_{\text{oxide}}) \) of the oxide. From 10 V already, oxides tend to reveal a second time constant possibly attributable to the existence of donor gradient throughout the film, or to the different electrochemical behaviour of grain boundary/electrolyte and grain body/electrolyte interfaces, according to the model proposed by Zhu and Ding [35]. The first hypothesis is closely connected to a semiconductive behaviour, and it is in full agreement with the study of Ibris [36]. According to the latter, semiconductive properties of titania films were found to depend on the formation potential. The second hypothesis seems also to be valid, as the existence of different capacitances at the grain boundary/electrolyte and the grain body/electrolyte interfaces is closely connected with the different morphologies of the oxides as they occurred with respect to the forming potential (see Table 1). Indeed, this phenomenon is more pronounced at the oxide formed at 65 V.

Fig. 2. (A) Diffuse reflectance FT-IR spectra of titania films developed at: (a) 1 V, (b) 10 V, (c) 45 V and (d) 60 V. (B) Raman spectra of titania films developed at: (a) 10 V, (b) 45 V and (c) 60 V.

Diffuse reflectance FT-IR and Raman spectra illustrated in Fig. 2, show that “low voltage” films consisting of amorphous TiO\(_2\), where at higher applied voltages, anatase is the predominant form. Spectra in Fig. 2A show that a peak at 830 cm\(^{-1}\) can be observed at films developed at potentials higher than 10 V. At lower potentials no peak was observed, indicating the formation of amorphous titanium oxide. Based on the literature data [21,30,37], we failed to conclude the exact structure of the resulting oxides, since absorptions of anatase and rutile forms differ between previous studies (838 and 824 cm\(^{-1}\) [37], 830 and 870 cm\(^{-1}\) [21], respectively, whereas peaks at 653–676 cm\(^{-1}\) are generally attributed to Ti=O vibrations) [30]. Moreover, discrimination between anatase and rutile forms is not clear, e.g. 838 and 824 cm\(^{-1}\).

On the other hand, Raman spectra found to provide quite acute and reproducible spectra, in full agreement with previous ones [22,36,38]. Oxides developed at “low” potential gave a flat spectrum, which indicates the formation of amorphous oxide, whereas at “high voltage” films bands at 146, 398, 514 and 653 cm\(^{-1}\) are due to the anatase modification. These bands become more intense as the potential raised, presumably due to increasing film thickness.

Ellipsometric measurements were performed at 60° of incidence angle, in the vicinity of the pseudo-Brewster angle of the tested samples. Using a single-layer model it was not possible to reproduce the data or to fit the ellipsometric functions tan \( \Psi \) and cos \( \Delta \). The best fit was obtained using a model consisting of two-layers of different composition. According to this model, the first layer is 50% TiO\(_2\) and 50% voids and accounts for mesoscopic surface roughness. The second layer is a mixture of titanium oxide and substrate material (pure Ti) and accounts for the oxide/metal interface roughness and for impurities or non-stoichiometric oxide content in the film. An additional parameter accounting for the uniformity of the layer thickness found not to have particular importance and thus not considered further.

Film thickness derived from the ellipsometric measurements was plotted with respect to the formation voltage \( (V_f) \) and the growth factor of the anodic film over the specific potential region was calculated 2.41 nm/V, a reasonable estimate, given that other published values ranges from 1.9 nm/V at 84 V to 2.87 nm/V at 20 V [24 and references therein]. Intercept of 6.04 nm accounts for the spontaneously formed oxide considering at the same time any deviations owned to the graphical calculation method.

Plotting the (overall) reciprocal capacitance of the electrode, as it is calculated from the equation \( Z = 1/\omega C \), with respect to the forming potential (Fig. 3A), the (apparent) dielectric constant was calculated using the formula \( \varepsilon = \varepsilon_0 B \), where \( \varepsilon \) is the film growth factor \( (2.41 \text{ nm/V}) \), \( B \) the slope of the graph \( (1/C = \alpha V_f) \) and \( \varepsilon_0 \) is the permittivity of the free space \( 8.85 \times 10^{-12} \text{ F/m} \) [24]. Values of 41 and 49 were calculated taking the slopes over the potential ranges 0.5–5 V \( (\varepsilon = 41) \) and 0.5–10 V \( (\varepsilon = 49) \), respectively (Fig. 3B), and they are in a good agreement with previous published values [24]. Due to the lack of acute (forming) potential values, a similar study for the “high voltage” oxide films was not made.
3.2. Application to immunosensing

From the data given in Table 1, it is obvious that, an anatase-type oxide layer is preferable than an amorphous oxide layer of the almost half thickness in terms of both conductivity and mechanical strength. Thus, the first step in the construction of Ti/TiO₂-based immunosensors is the development of an anatase film at 30 V. Another step, which aims to increase the population of free –OH groups on the oxide surface, making thus the latter more active as a protein–host platform is the hydroxylation process [3–7]. A number of immobilization protocols including silanization, activation with cyanuric acid, or cyanogen bromide, have been successfully used so far for the immobilization of biomolecules onto hydroxyl-ended surfaces [39]. Treatment of oxides with various hydroxylation agents resulted in significant changes of EIS spectra, which varied with solution corrosivity and exposure time. As it can be seen in Fig. 4, hydroxylation of the tested electrode assembly with an acidic agent, HF/HNO₃/H₂O (1+3+4), resulted in decrease of the overall impedance. This behaviour cannot be solely attributed to the reduction of the thickness of the oxide film. It could also be the result of the formation of a more hydrated and porous oxide, which exhibits thereby a higher (observed) dielectric constant, and thus a higher (overall) capacitance [40]. The importance of this feature to the increase of both the sensitivity and the dynamic range of the resulted sensors has been well documented by Gebbert et al. [31].

Various hydroxylation procedures based on acidic and alkali media as well as various silanization protocols based on liquid and gas-phase anhydrous conditions have been tested and comparative results are described elsewhere [7]. In this work, we aim to demonstrate the performance of an impedimetric immunosensor based on the direct coupling of the biomolecule on an anodically formed Ti/TiO₂ electrode. Direct coupling of biomolecules to titanium oxide has been earlier reported and processed through hydroxyl groups of the transition metal, which act as new ligands replacing other ligands of biomolecules as free carboxy groups, phenolic hydroxyl groups and/or amino groups of any specific residues [32,39].

The performance of the present immunosensor was evaluated by performing three different instrumentation approaches, including measurement of impedance with a FRA analyzer (AUTOLAB/FRA2, EcoChemie), measurement of capacitance using an LCR-meter (model 821, Instek, USA), and measurement of the total charge using a home-built instrument, the so-called “Multipulser” [41]. The operation of Multipulser is based on the integration of the electric charge used for the repetitive charging of the electrochemical cell capacitance after the application of a predetermined number of short-duration, low-amplitude voltage pulses (perturbation pulses) [41]. As it can be seen in Fig. 5, binding of anti-avidin to avidin-modified Ti/TiO₂ electrode surface is associated with increase in the impedance and decrease in both the capacitive current and accumulated charge. This behaviour is attributable to the formation of the immuno-based complex, which is considered as an extra capacitor, connected in series with that represents the insulating (oxide) layer. As it can be seen in Bode plots, higher sensitivity was observed in frequencies lower than 1 kHz, and thus measurements at the LCR-meter were conducted at 20 Hz. This behaviour is not in full agreement with the theoretical model proposed by Pethig and Kell [33], however is inconsistent with previous published results [7,42]. Recorded changes in each of the measured signal (ΔZ/Z = +34%, ΔC/C = −22% and ΔQ/Q = −22%) for a concentration of 10 μg/ml anti-avidin indicate promise for further use, mostly in various diagnostics tests based on immunoreactions.
4. Conclusions

Titania films were anodically formed over titanium substrates of a small diameter of 2 mm, mounted in an electrode body with convenient geometry for sensor development.

Titania films were characterized with various techniques and results showed that for sensoring applications, an anatase-type film is preferable than an amorphous oxide layer of the same thickness in terms of both conductivity and mechanical strength.

Hydroxylation process, a necessary step to create efficient protein–host substrates, results to the decrease of the overall impedance.

Immunobinding reaction between avidin and anti-avidin was effectively monitored by three different instrumental approaches. Signal changes up to 34% show that the sensitivity of the sensor is optimal for many analytical uses.

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References
