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Interfacial electron transfer on cytochrome-*c* sensitised conformally coated mesoporous TiO_2 films

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ABSTRACT

Hybrid protein films incorporating Cyt-*c* immobilized on TiO_2 films were prepared and characterised optically with UV–visible spectroscopy and electrochemically with cyclic voltammetry, and their conductivity properties were studied in detail. In addition the effects of a thin overlayer coating of a second metal oxide such as SiO_2, Al_2O_3, ZrO_2 and MgO_2 were studied and the effects over the electrochemical properties of the hybrid working electrodes were discussed.

1. Introduction

Mesoporous nanocrystalline TiO_2 electrode is a wide-band-gap (~3 eV) semiconductor and therefore optically transparent for wavelengths ≤390 nm. The thin transparent films comprise a rigid, porous network of 10–20 nm nanocrystalline TiO_2 nanoparticles with pore sizes between 5 and 20 nm, sufficiently large for proteins to diffuse throughout the porous structure. The surface area of such films is greatly enhanced over flat electrode surfaces (up to 1000 fold for an 8-μm-thick film). In addition to their optical transparency and high surface area, these films exhibit good stability, and electrochemical activity at potentials above the conduction band edge.

We have previously demonstrated that protein adsorption can be readily achieved on mesoporous TiO_2 electrodes from aqueous solutions at 4 °C with high binding stability and undetectable protein denaturation [1–5]. We have characterized the properties of such protein/TiO_2 electrodes by cyclic voltammetry and UV–visible spectroscopy and demonstrated that the immobilized proteins can be reduced by the application of an electrical potential to the film without the addition of any electron-transfer mediators [1–5]. Moreover, other groups have also shown the adsorption of a range of biomolecules on mesoporous metal oxide electrodes as working electrodes for sensing devices [6–15].

In this paper we aim to examine further the conductivity and electrochemical properties of these electrodes as a function of pH and scan rate by using a simple solution phase redox system such as $Fe(CN)_6$. Both cyclic voltammetry (CV) and spectroelectrochemistry will be used. In addition, the electrochemical behaviour of Cyt-*c* will be studied on TiO_2 films with a thin conformally deposited overlayer of a second metal oxide such as SiO_2, Al_2O_3, ZrO_2, and MgO_2. The effect on the interfacial electron-transfer process between the electrode and the biomolecules is studied.

2. Experimental section

2.1. Reagents

Horse-heart cytochrome-*c* was purchased from Sigma Chemical Co and Carbowax 20,000 from Fluka. Silicon Methoxide (99.9%), Alumina tri-*sec*-butoxide (99.9%), Zirconia *iso*-butoxide (99.9%), Magnesium ethoxide (99.9%), and the remaining chemicals were purchased from Aldrich Chemical Co. and used as received. Sodium dihydrogen orthophosphate (0.01 M) was used to prepare the supporting electrolyte, and its pH was adjusted to 7 using NaOH. Distilled water was demineralised to a resistivity of 10 MW cm^{-1}. Fluorine-doped tin oxide-coated glass slides (conductivity of 15 W cm^{-2}) were purchased from Hartford Glass (Hartford City, Indiana, U.S.A.), cleaned with water, rinsed with ethanol, dried at 100 °C and heated at 450 °C prior to film deposition.

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2.2. Preparation of electrodes

The TiO_2 paste, consisting of 15 nm sized particles was prepared from a sol–gel colloidal suspension containing 12.5 wt.% TiO_2 particles and 6.2 wt.% Carbowax 20,000 as reported previously [16]. The TiO_2 suspension was then applied to the surface of the conducting glass using the "doctor blade" technique. Masking the glass slide with Scotch tape controlled the thickness and the width of the area spread, with one layer of tape being employed to yield a final film thickness of 4 μm. The spread suspension was then allowed to dry before being sintered for 20 min at 450 °C. The thickness of the nanocrystalline TiO_2 films was measured with a DEKTAK profilometer. The TiO_2 films were cut in 1 cm^2 pieces. Immediately prior to Cyt-*c* immobilization, the films were heated to 450 °C for 15 min and then allowed to cool down to room temperature before being immersed in the protein solution.

2.3. Coating procedure

To achieve uniform coating of the preformed nanocrystalline mesoporous TiO_2 thin film we have used a previously described method [17]. In brief, the conformally coating of the different metal oxides were obtained by in-situ hydrolysis of the alkoxide precursors over the nanoparticles. The highly hydroxilated surface of the mesoporous thin films induces the formation of nanoscopic thin uniform layers of the corresponding metal oxide on the surface of the nanocrystalline particle. The precursor solutions concentrations used were 0.15 M for aluminium tri-*sec*-butoxide in dry 2-propanol, 0.15 M for silicon methoxide in dry methanol, 0.15 M for zirconium *iso*-butoxide in dry methanol and 0.15 M for magnesium ethoxide in dry methanol. These non-scattering precursor solutions were prepared under anaerobic conditions in a glovebox; however, once prepared the solutions were not air sensitive, allowing dipping to be conducted in ambient conditions.

After their initial sintering, the TiO_2 films were coated with metal oxides overlayers by dipping each film in a solution of the suitable precursor pre-heated to 60–70 °C, for 20 min, followed by heating at 435 °C for 20 min [17]. To keep similar conditions between the standard and coated electrodes, the mesoporous TiO_2 films without coatings were sintered at the same time as coated ones.

Experiments were conducted as a function of precursor concentration, dipping time and temperature and number of dipping/sintering cycles. The overlayer growth was found to be insensitive to dipping time or temperature, but dependent upon precursor concentration and the number of repeat cycles, consistent with previous observations [17]. For convenience, the precursor concentration was maintained at 0.15 M for all studies reported in this paper, with the overlayer thickness being controlled only by repeating the dipping/sintering cycle up to 4 times.

2.4. Protein immobilization

The protein immobilization was achieved by the immersion of 1 cm^2 pieces of TiO_2 (coated or uncoated) films in 2 ml of the protein solution (20 μM Cyt-*c* in a 10 mM phosphate buffer) at 4 °C for at least 1 to 2 days. Cyt-*c* adsorption onto the films was monitored by recording the UV–Vis absorption spectra of the films at room temperature.

2.5. Optical measurements

All absorption spectra were measured using a Shimadzu UV-2401 spectrophotometer with a sampling interval of 1 nm. Protein solutions were measured in the appropriate buffered solution, that was also used for the blank spectra, using plastic rather than quartz cuvettes in order to prevent the binding of protein molecules during the acquisition. During the Cyt-*c*/TiO_2 film analysis, the sensitised films were submerged in the buffer solution. Prior to all spectroscopic measurements, the films were removed from the immobilization solution and rinsed in a buffer solution to remove non-immobilized protein. Contributions to the spectra from scatter and absorption by the TiO_2 film were subtracted by using protein-free reference films.

2.6. Electrochemical measurements

The electrochemistry and spectroelectrochemical experiments were carried out using an Autolab PGStat12 potentiostat and a three-electrode cell with quartz windows, a platinum mesh flag as the counter electrode, a Ag/AgCl in 3.5 M KCl reference electrode, and the TiO_2 or Cyt-*c*/TiO_2 film on conducting glass as the working electrode. The electrolyte, an aqueous solution of 10 mM sodium phosphate (pH 7), was thoroughly degassed by bubbling argon prior to the experiments. For the spectroelectrochemical experiments, the above cell was incorporated as a sample in the Shimadzu UV-1601 spectrophotometer, and the absorption changes were monitored as a function of the applied potential. All potentials are reported against the Ag/AgCl electrode and an argon blanket was maintained during all measurements.

3. Results

3.1. Electrochemical studies

We have previously demonstrated that CV can be used to study the electrochemical behaviour of immobilized proteins such as Cyt-*c* on the mesoporous TiO_2 films [1–5]. CV is a useful technique to examine the redox electrochemistry between the TiO_2 electrode and the immobilized Cyt-*c*. From these studies, we concluded that the negative shift between the observed reduction peaks from the CVs of the immobilized Cyt-*c* on mesoporous TiO_2 films and the ones observed in solution can be attributed to the low conductivity of TiO_2 films [18] at moderate potentials or due to a shift in the redox potential of Cyt-*c* induced by its immobilization [3–5].

To lift this uncertainty, a number of control experiments have been conducted in this paper to further understand the interfacial electron-transfer reactions between the mesoporous semiconductor film and the Cyt-*c*. Using a simple solution phase redox couple such as potassium ferricyanide, $K_3[Fe(CN)_6]$ and an unmodified mesoporous TiO_2 film as a working electrode we obtained the CV shown in Fig. 1A. Although potassium ferricyanide is not immobilized on the TiO_2 film, a negative shift in its reduction peak, identical to the one observed with the Cyt-*c*, occurs. This was also confirmed by a control experiment in which a TiO_2 film is dipped into a $K_3[Fe(CN)_6]$ solution, taken out and washed thoroughly, and then subjected to CV in pure phosphate buffer solution and no characteristic Fe redox peak was obtained (results not shown here). Thus we can conclude that the voltammetry is dominated by the conductive properties of the TiO_2 film.

We did also examine the effect of the scan rate on the CV. Slower scan rates were applied to the Cyt-*c*/TiO_2 electrode in order to try to obtain a reversible peak shaped CV. Fig. 2 illustrates that even at slow scan rates no simple reversible behaviour for the Cyt-*c*/TiO_2 film was observed, consistent with the currents being limited by the low TiO_2 conductivity at moderate potentials.

It is known that the pH of the electrolyte solution affects the conductivity of the TiO_2 films. Indeed, the change in pH, by simply altering the $HPO_4^{2-}/H_2PO_4^-$ ratio but keeping the ionic strength constant, has a significant effect on the potential of the reduction peak for the immobilized Cyt-*c* as can be seen in Fig. 3. The CVs of this figure show that by lowering the pH by 1 unit, the potential of the reduction peak of Cyt-*c* changes by 64.5 mV due to the change in the conductivity of the TiO_2 films. These results are in agreement with the fact that a blank nanocrystalline TiO_2 film in an aqueous electrolyte shows an expected Nernstian shift of ~59.1 mV on increasing the pH of