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Highly ordered transparent mesoporous TiO_2 thin films: an attractive matrix for efficient immobilization and spectroelectrochemical characterization of cytochrome c†

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We demonstrate remarkably fast incorporation and high loading of cytochrome c within thin films of periodically ordered nanocrystalline TiO_2 deposited on transparent electrodes. The immobilized cytochrome c is not denaturated and it can be reversibly reduced without mediator over the time scale of a few seconds as evidenced by spectroelectrochemistry.

Over the last years, growing attention has been directed toward the immobilization of redox proteins or enzymes within optically transparent thin films of semiconductive mesoporous metal oxides deposited on electrode surfaces. These efforts have been motivated by the potential for developing unique spectroelectrochemical strategies for the characterization of redox proteins,[1,2] as well as by the possibilities of developing new electrochemical biosensors.[3,4] These novel protein host matrixes combine the attractive properties of transparency, high surface area, electrical semiconductivity, biocompatibility, ease of fabrication and high chemical, mechanical and thermal stability, with the possibility of direct electron transfer between the protein redox sites and the host mesoporous metal oxide.

Attention to date has been largely focused on the electrochemical investigations of heme proteins immobilized within semiconductive mesoporous metal oxide films (M_xO_y, with M = Ti, Sn, Zn) produced from randomly sintered nanosized particles deposited on an electrode surface (film thickness of a few micrometers).[1–6] The structure and porosity of these films is however relatively ill-defined since they are constituted by irregular aggregates of interconnected metal oxide nanoparticles (possibly with a binder) and, in the case of electrochemical applications, such heterogeneity can significantly affect and complicate mass transport within the film.[7,8] Moreover, despite a noteworthy increase of the electrical conductivity at potentials above the conduction band edge, the electron transport through such metal oxide nanoparticulate films was shown significantly to be altered by poor interparticle electron transfer,[9] leading thus to an electrical conductivity much lower than at compact polycrystalline or monocrystalline materials. For these reasons, we have chosen to examine the possibilities offered by highly ordered mesoporous thin films of metal oxide formed by a continuous crystalline inorganic phase of regular pore architecture. Our attention has been more specifically focused on ordered mesoporous titanium oxide films prepared from evaporation-induced self-assembly (EISA).[10,11] This method is based on the sol–gel dip-coating of an amorphous TiO_2 gel containing a self-organized organic template, which upon aging and thermal crystallisation leads to a regular 3D network of nanocrystalline anatase-TiO_2 with a well-opened pore structure (cubic mesostructure). The high permeability of these films was evidenced by electrochemistry using small diffusing redox probes.[12] Depending on the synthesis conditions, crack-free mesoporous TiO_2 films of controllable thickness (ranging from a few tens to several hundreds of nanometers), texture, and porosity (pore size up to 20 nm) could be obtained.[13] Therefore, it may be a suitable material for immobilization of small proteins with diameter of a few nanometers. Other attractive features of mesoporous TiO_2 films are their relatively high stability in aqueous media (much better than mesoporous SiO_2[14]) and their good optical transparency, allowing thus characterization of adsorbed biomolecules by spectroscopies.

Here, we report on the immobilization of a small globular hemoprotein into mesoporous nanocrystalline anatase-TiO_2 thin films built up onto microscope glass slides and also semi-transparent gold-conductive substrates for UV-visible spectroelectrochemical study (Fig. 1). The films were prepared in the presence of a pluronic triblock copolymer template as previously described.[11] The TiO_2 film thickness was 230 nm and the pore size 7.5 nm.

Immobilization of horse heart cytochrome c (cyt-c, see Fig. 1, 3.1-nm diameter, pI = 10.9) was achieved by immersing the mesoporous TiO_2-modified glass slides into 1–50 μM protein solution (Hepes 10 mM, pH 7.0, T = 20 °C) for 30 min. The slides were next rinsed and characterized by UV-visible spectroscopy in a buffer-free solution. The resulting spectrum of Fe^{III}-cyt-c/TiO_2 film showed the characteristic heme absorption bands at 409 nm (Soret) and 529 nm (Q-band) (spectrum c in Fig. 2), in good agreement with the solution spectrum of this protein (spectrum d in Fig. 2). It indicates that the native low-spin Fe^{III}-heme coordination of cyt-c is retained and that the hemoprotein is not denaturated upon incorporation within the porous

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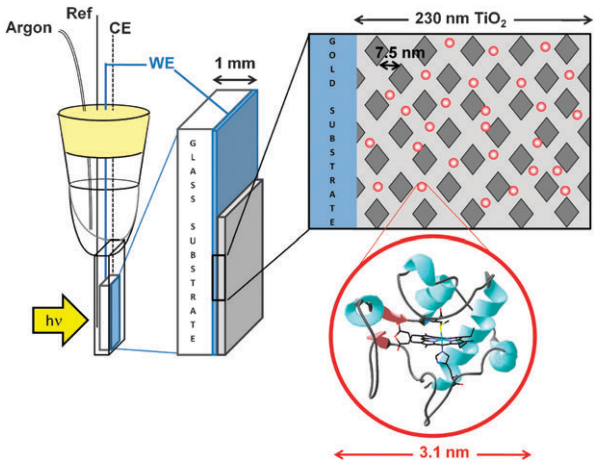

Fig. 1 Schematic representation of the spectroelectrochemical cell used for studying the hemoprotein (here cytochrome c) adsorbed within the open grid like architecture of the mesoporous TiO_2 thin film deposited on a semitransparent gold electrode (Ref, CE and WE are the reference, counter and working electrode, respectively).

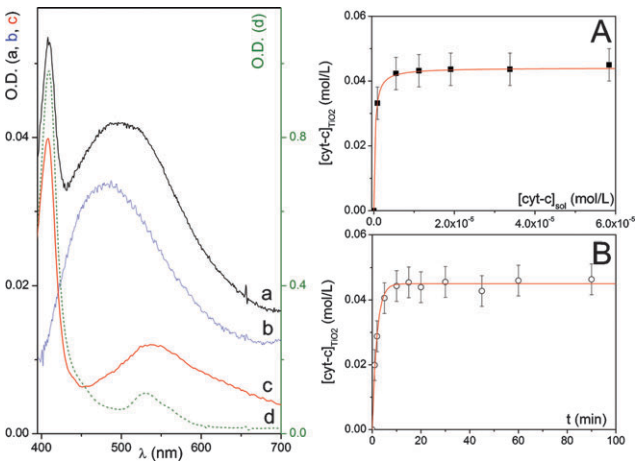

Fig. 2 Left: UV-visible spectra of Fe^{III}-cyt-c adsorbed within a mesoporous TiO_2 thin film (230-nm thick) on a glass substrate (1 h immersion in a 10 μM Fe^{III}-cyt-c solution) (a) before and (c) after subtraction of the (b) blank spectra. (d) Fe^{III}-cyt-c in homogeneous solution ($[cyt\text{-}c]_{sol}$ = 9 μM in a quartz cell of 1-cm path length). Right: (A) adsorption isotherm of Fe^{III}-cyt-c at a TiO_2-modified glass substrate and fit to a Langmuir isotherm (red line); (B) adsorption kinetics of Fe^{III}-cyt-c ($[cyt\text{-}c]_{sol}$ = 11 μM, Hepes 10 mM, pH 7.4, T = 20 °C) and fit to a first-order kinetic process (red line) using k_{ads} = 32 s^{-1}.

structure (denaturation of cyt-c causes a blue shift of the Soret peak to 406 nm[15]). Assuming a same $\varepsilon_{409\ nm}$ value of the immobilized ferricytochrome c as in homogeneous solution (*i.e.* 1.06 × 10^5 M^{-1} cm^{-1}) and considering a fractional void volume of 0.4 cm^3 per cm^3 of film, the concentration of hemoprotein inside the 230-nm-thick thin film could be estimated from OD. On this basis, the adsorption isotherm as a function of protein concentration in solution was obtained (Fig. 2A). The plot reveals that the saturation of the mesoporous TiO_2 film by cyt-c is almost achieved for a protein solution concentration >10 μM, leading to a maximal cyt-c film concentration of 45 ± 10 mM. This high protein loading, about 2-fold higher than that previously obtained in thick films of sintered TiO_2 nanoparticles,[5] could result from the high film porosity combined with its well-opened and highly organized structure. Considering cyt-c as a sphere of 15 nm^3 volume, it is indicative of a cyt-c packing factor of ~0.4 inside the void volume of the mesoporous structure (*ca.* 5 cyt-c/nanopore), a value close to the maximum of 0.74 for a close-packing of spheres. The experimental data of Fig. 2A were finally fitted to a Langmuir isotherm from which an affinity binding constant (K_b) of 3.5 ± 0.5 × 10^6 M^{-1} was obtained. This value is much higher than those determined for the binding of cyt-c in thick films of sintered TiO_2 nanoparticles (K_b = 10^5 M^{-1} in 10 mM phosphate buffer, pH 7.4).[16] The adsorption kinetic of cyt-c was also monitored by UV-visible spectroscopy (Fig. 2B). The resulting experimental data were fitted to a first-order kinetic rate law from which a rather high rate constant of k_{ads} = 32 ± 2 s^{-1} was obtained. This high value means that the diffusion–adsorption rate of cyt-c through the highly ordered film porosity is a relatively fast and easy process, almost achieved over 10 min. Such a fast kinetics is in contrast with those earlier observed for the incorporation of cyt-c in a thick film of sintered TiO_2 nanoparticles, usually requiring several days of soaking in the protein solution before reaching equilibrium binding.[5,16] To check the strength and reversibility of the binding, the desorption kinetics of cyt-c was also monitored spectroscopically after transferring the protein-loaded film in a protein-free buffer solution (10 mL of 10 mM Hepes buffer, pH 7.0). An only 30% decrease of the Soret band intensity was observed after 6 h immersion, which corroborates well the high affinity binding measured from the adsorption isotherm plot.

Having demonstrated the possibility to immobilized cyt-c on nanostructured TiO_2 electrodes, it was next interesting to undertake a spectroelectrochemical study of their properties. For such a purpose, TiO_2-modified semi-transparent gold-conductive electrodes were saturated with Fe^{III}-cyt c as described above and then introduced in a one-compartment spectroelectrochemical cell filled with 0.35 mL of a carefully deaerated buffer (Hepes 10 mM, pH 7.0) (Fig. 1). Under these conditions, the spectroelectrochemical experiments could be carried out during at least 1 h without significant protein desorption. Upon applying a sufficient cathodic potential (−0.8 V *vs.* Ag/AgCl), a clear and rapid shift of the Soret band from 409 to 416 nm with a concommitant increase of the α and β bands at 521 and 550 nm was observed. The final spectrum was in good agreement with the solution spectrum of the reduced form of cyt-c (Fig. S1, ESI†) and were indicative that >90% of Fe^{III}-heme moiety of cyt-c incorporated in the porous thin film was electrochemically reduced to Fe^{II} and this without the need of a soluble redox mediator. Moreover, by following the relative absorbance at 419 nm with time, an almost complete reduction of cyt-c was reached in less than 3 s (Fig. 3), which is one order of magnitude faster than at thick films of TiO_2 nanoparticles.[5] On stepping back the potential from −0.8 to +0.3 V, the protein could be completely reoxidized, as shown on the difference spectra of Fig. 3A. Moreover, the three negative maxima at 419, 521 and 550 nm

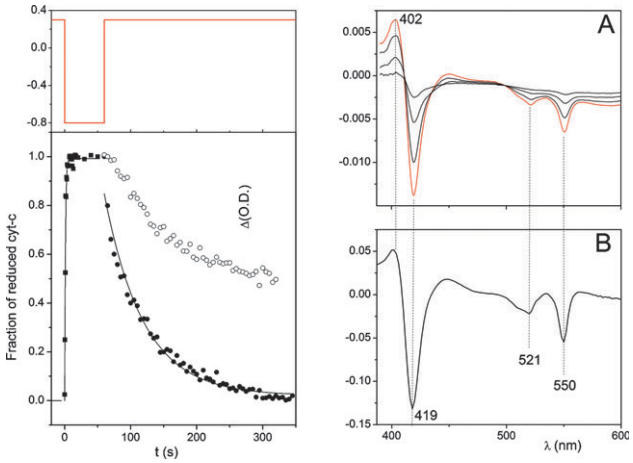

Fig. 3 Left: (bottom) fraction of reduced cyt-c determined from the relative absorbance at 419 nm after stepping the potential (top, red line) from 0.3 to −0.8 V and then back to 0.3 V *vs.* Ag/AgCl. The reduction (■) and oxidation (●) kinetics were fitted to an exponential equation (black lines) leading to $k_{red} = 0.8\ s^{-1}$ and $k_{ox} = 0.015\ s^{-1}$; (○) corresponds to the spontaneous reoxidation process. Right: difference spectra of Fe^{II}-cyt-c minus Fe^{III}-cyt-c calculated (A) in the TiO_2 network during oxidative titration (recorded after 5, 10, 60 and 300 s, respectively) and (B) in solution ($[cyt\text{-}c]_{sol} = 9\ \mu M$ in a quartz cell of 1-cm path length). In (A), the small shift in the difference spectra at high wavelengths was due to reduced TiO_2 (*i.e.*, $TiO_2(e^-)$).[18]

and the two isosbestic points at 411 and 498 nm (indicative of equilibrium between two redox species) were the same than those of the protein in solution. This is a good indication that the structural integrity and redox activity of both redox states of cyt-c remain preserved in the TiO_2 film even after redox switching.[17] The reoxidation rate was however much slower than the reduction (Fig. 3), a behaviour that was similar to those previously observed for the reoxidation of iron protoporphyrin-IX incorporated in a thick film of interconnected TiO_2 nanoparticles.[19] Assuming a first-order kinetics law, the nonlinear fitting of kinetics curves in Fig. 3 leads to reductive and oxidative rate constants of $k_{red} = 0.8\ s^{-1}$ and $k_{ox} = 0.015\ s^{-1}$, respectively. The fast and almost complete electrochemical reduction of cyt-c ($t_{1/2} < 1$ s) suggests a direct electron transfer from the semiconductive TiO_2 mesostructure to the protein, although an electron transport by intermolecular electron hopping (self-exchange) cannot be totally excluded.[19]

It is worth noting that, even after carefully bubbling the cell with argon for a long period, an unexpected spontaneous reoxidation of cyt-c was reproducibly observed once the applied cathodic potential was switched off (Fig. 3). A same spontaneous oxidation was noticed during the incorporation of ferrocytochrome c (10 μM, Hepes 10 mM, pH 7.4) in a TiO_2-film as the resulting UV-spectrum of the incorporated protein was characteristic of Fe^{III}-cyt-c, a process that was also observed to occur in the dark. This appears to be indicative of a thermodynamically favoured reaction $TiO_2\square + Fe^{II}$-cyt c → $TiO_2 + Fe^{III}$-cyt c due to the presence of electron traps at non-stoichiometric lattice sites at the TiO_2 surface.[20]

In summary, we have demonstrated that highly ordered mesoporous thin films of nanocrystalline anatase TiO_2 allows for fast and non-denaturing incorporation of a small redox protein such as cyt-c. Moreover, we have shown by spectroelectrochemistry that the semiconducting properties of TiO_2 allow for reversible and rapid reduction (over a time scale of a few seconds) of the adsorbed cyt-c without need of an electron transfer mediator, suggesting a direct electrical communication between the redox protein and the TiO_2 matrix. These attractive properties should thus open new opportunities for the development of bioanalytical devices that combine optical and electrochemical detections. The range of proteins that may be adsorbed is currently limited by the pore size, but work is in progress to increase the porosity and to achieve immobilization of larger proteins such as redox enzymes.

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