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Covalent immobilization of redox protein within the mesopores of transparent conducting electrodes

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ABSTRACT

Redox protein cytochrome c was immobilized at high electrochemically accessible loading on mesoporous films of antimony doped tin oxide (ATO) exhibiting high conductivity, transparency and a large surface area. The grafting was achieved by covalent attachment of the protein to the electrode surface. Alternatively, cytochrome c was effectively adsorbed on the ATO electrode surface due to strong electrostatic interaction between the positively charged cytochrome c and the negatively charged ATO surface. The amount of electrochemically addressable cytochrome c is proportional to the specific surface area, reaching up to 440 pmol/cm^2 and 600 pmol/cm^2 for covalently attached and adsorbed protein, respectively, for the 370 nm thick films. The covalently attached protein exhibits substantially higher stability towards leaching than the adsorbed one. The combination of transparent conducting porous electrode matrix with the electroactive proteins is promising for the development of efficient bio-optoelectronic devices and for in situ spectroelectrochemical studies of biomolecules.

1. Introduction

The use of biomolecules as the principal components of bioelectronic devices offers highly promising possibilities for the application in sensing, diagnostics, catalysis or energy conversion [1–3]. By connecting the evolution-optimized biological units to the artificial man-made circuits, one can imagine benefiting from the advantages of both natural and artificial components and achieving a new type of synergy in their performance. The successful implementation of natural units in artificial circuits demands the availability of electrodes with suitable composition and morphology. Equally important is the way of binding the biological units to the electrodes without any destruction of their biological functionality [4].

Porous electrodes are considered the most attractive platforms for the immobilization of biomolecules. The controlled porosity of electrodes enables to increase biomolecule stability due to their encapsulation within the pores, and to tune their chemical surrounding by a chemical modification of the pore interior. Up to now, a wide range of porous materials with different conductivity of porous network was investigated, such as insulating silica layers [5–7], metal organic frameworks [8], films of semiconducting metal oxides such as TiO_2, SnO_2 and Nb_2O_5 [9], and conducting metal or carbon electrodes [10]. The conductivity of the electrode material is advantageous as it enables a direct electrochemical addressing of the immobilized biological species. A new emerging class of materials which offer optical transparency in addition to a large conducting interface are transparent conducting oxide layers such as indium tin oxide (ITO) and antimony-doped tin oxide (ATO) with porous architecture [11–18]. Very few papers dealing with porous TCO electrodes have already demonstrated their great potential as a platform for the immobilization of biological molecules for photoelectronic and photoelectrochemical applications [13–15,18–20]. In the published communications, the biomolecules were immobilized exclusively by adsorption. While the adsorption procedure is simple, it suffers from rather low leaching stability in the electrolytes. Covalent immobilization of the biomolecules is in many cases more desirable as it substantially enhances the leaching stability. Although the covalent binding of biomolecules was intensively studied for silica materials and for flat ITO electrodes [21], the possibility of such way of grafting in porous TCOs has not been studied yet.

In this communication, we investigate the possibility of covalent immobilization of redox proteins in the 3D-matrix of transparent conducting electrodes. As a porous conducting platform for the protein immobilization we use the recently developed transparent conducting films of mesoporous antimony doped tin oxide (ATO) [11]. We demonstrate that the redox protein cytochrome c can be covalently grafted in the pores of mesoporous ATO layers

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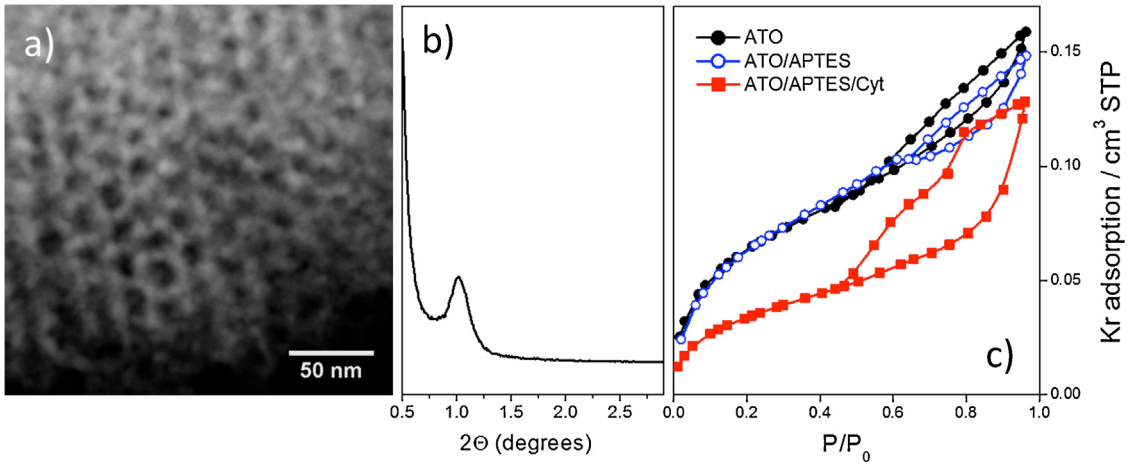

Fig. 1. Morphology of mesoporous ATO films: (a) STEM-HAARD image of the films scrapped off the substrate; (b) small angle XRD pattern of the films on the substrate. (c) Krypton adsoption isotherms on mesoporous ATO film (black solid circles), ATO film functionalized with APTES (blue open circles) and mesoporous film with incorporated cytochrome c (red solid squares). Film thickness is 370 nm. (For interpretation of the references to color in figure legend, the reader is referred to the web version of the article.)

without losing the electrochemical activity and without protein denaturation. Covalent binding greatly enhances leaching stability of cytochrome compared to the adsorbed protein. Electrical conductivity and high surface area of the mesoporous ATO electrode enable direct electron transfer to the immobilized cytochrome c and lead to a greatly enhanced loading proportional to the surface area.

2. Results and discussion

Transparent conducting films of antimony-doped tin oxide (ATO) with a uniform 3D-mesostructure were prepared via the self-assembly of crystalline ATO nanoparticles directed by Pluronic copolymers according to the procedure developed by us previously [11]. For the film preparation, colloidal solutions containing dispersed ATO nanoparticles and Pluronic copolymer were dip-coated onto ITO glass substrates. Films from ca. 100 nm to ca. 400 nm in thickness can be deposited by varying the dip-coating rate. The coated layers were calcined at 500 °C to remove the polymer template and to form porous crystalline conducting ATO electrodes (see Section 4 for further details). The mesostructure of the films prepared in this way exhibits d-spacing of ca. 14 nm as follows from the small-angle XRD patterns (Fig. 1b) and STEM images (Fig. 1a), and pore size of around 10 ± 1 nm. Films with the thickness of ca. 360 nm possess a specific surface area (roughness factor) of about 20 cm^2/cm^2 as measured by the adsorption isotherms of Kr at 77 K (Fig. 1c). The presence of a very narrow hysteresis loop at relative pressure above ca. 0.6 indicates that the films contain only very limited percentage of pores narrower than ca. 10 nm. The obtained data demonstrate that the mesoporous ATO films exhibit a suitable pore width for incorporation of small cytochrome c protein (ca 3 nm in size) within the porous matrix.

For the covalent immobilization of cytochrome c, we used a peptide chemistry approach. Although not orientation-specific, it enables grafting of proteins to the amino-functionalized substrates via formation of amide bonds with glutamic or aspartic acid residuals usually present on the protein surface. The amide bond is formed at mild conditions and is therefore very frequently used for the immobilization of proteins on various substrates.

The covalent attachment of the cytochrome c to the porous ATO was performed in two steps. In the first step, the mesoporous material was amino-functionalized with aminopropyltriethoxysilane (APTES). The Kr adsorption isotherms show that the incorporation of small APTES molecules practically does not affect the porosity of the ATO films, which show a similar surface area as well as the pore size without any pore blocking as the parent ATO film.

In the second step, cytochrome c was grafted to the amino group of APTES via formation of amide bonds with carboxylic groups from the protein. The incorporation of bulky protein molecules changes markedly the character of krypton isotherms. On the protein-modified layers the specific surface area decreases significantly. The widening of the hysteresis loop and shift towards the range of smaller relative pressure show the presence of a substantial amount of pores smaller than ca 10 nm and indicate that the pores have become markedly narrower due to the grafting of protein on the pore walls.

The proof of the cytochrome c integrity in the mesoporous ATO matrix after covalent immobilization is obtained by IR spectroscopy (Fig. 2) and UV–vis spectroscopy (Fig. 3). Most of the vibrations obtained in the IR spectra for the pure cytochrome c [22] are found in the ATO sample with incorporated cytochrome c, however with a lower intensity. The UV–vis spectra (Fig. 3a) of covalently immobilized cytochrome c show a Soret band (409 nm) and weaker Q

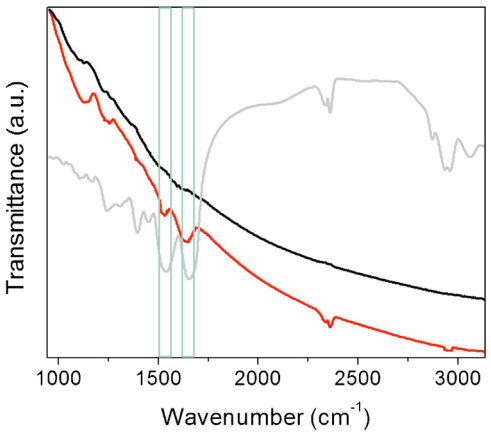

Fig. 2. IR spectra of pristine mesoporous ATO (black line), ATO with incorporated cytochrome c (red line) and pristine cytochrome c (green line). The IR spectra of cytochrome c covalently incorporated in the ATO show the presence of characteristic amide I and II bands at ca. 1650 and 1533 cm^{-1}, respectively, which are also present in the spectrum of pristine cytochrome c and which are related to the secondary structure of proteins [22]. Most of the vibrations obtained for the pure cytochrome c are found in the ATO sample with incorporated cytochrome c, however with the lower intensity. (For interpretation of the references to color in figure legend, the reader is referred to the web version of the article.)