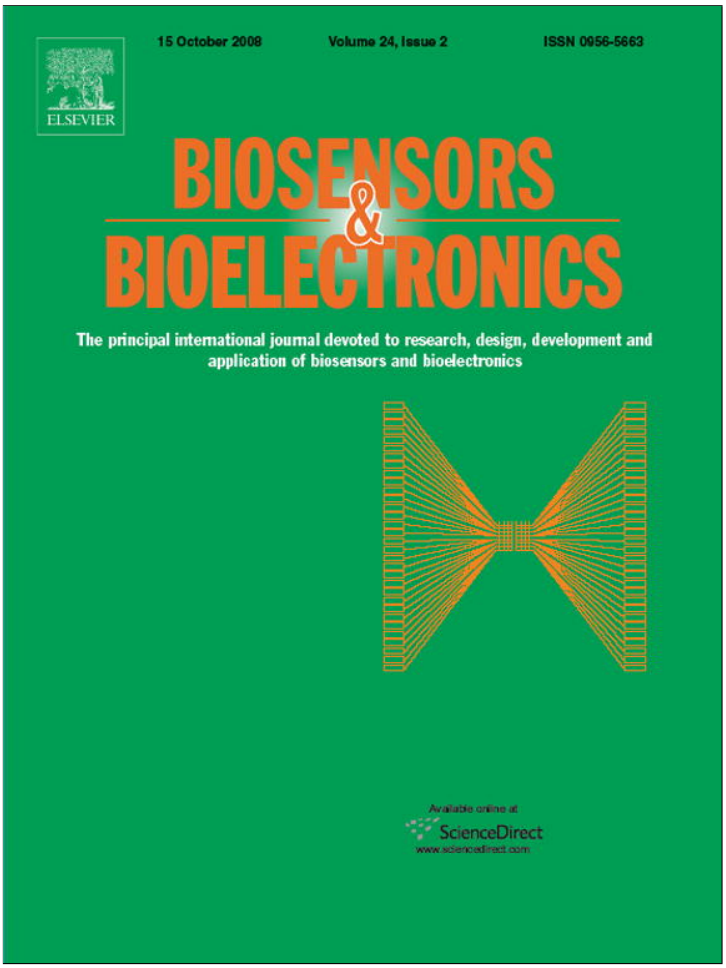

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Optical and electronic coupling of the redox copper Azurin on ITO-coated quartz substrate

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ABSTRACT

We have investigated the hybrid system constituted by the redox copper protein Azurin integrated with the semiconductor indium tin oxide (ITO) coated on quartz substrate. The system appears to be a good candidate for bio-sensing and bio-optoelectronics applications, especially due to the coupling between the optical and electron transfer features of Azurin with the conductive properties and optical transparency of ITO. The optical, morphological and electrical properties of the system have been investigated by combining optical absorption and transmission, steady-state fluorescence, resonance Raman spectroscopy and scanning probe microscopies. We found that Azurin molecules are firmly anchored on ITO and retain their structural and optical features underlying the physiological electron transfer activity. Scanning tunnelling spectroscopy evidenced a good electric coupling between the protein molecules and the substrate and a concomitant modulation of the ITO semiconductor properties upon deposition of Azurin. Some interplay between the conduction and valence bands of ITO and the electronic levels of Azurin is therefore suggested. These results are of a significant relevance in the perspective of developing bio-nanodevices able to process both optical and electrical signals, in conjugation also with the biorecognition capability of the protein molecules.

1. Introduction

Integration of biomolecules with inorganic conductive substrates has opened new perspectives in nanotechnological applications. Indeed, natural biological functions, such as electron transfer, electrical response to optical excitation, biorecognition, catalysis, etc., can be suitably combined with the processing power of microelectronics (Willner and Katz, 2005; Bonanni et al., 2007).

Gold electrodes are commonly used to build up hybrid systems, allowing to achieve a stable and efficient electrical communication with the biomolecules through covalent links due to the high affinity of native, or engineered, disulfide or thiol groups for gold (Davis et al., 2005; Bonanni et al., 2003; Zhang et al., 2002; Andolfi et al., 2006). However, processing of optical signals within these substrates, is somewhat prevented by the significant absorption in the visible spectral region from the surface-plasmons of gold. On the other hand, the simultaneous detection of optical and electrical signals of biomolecules integrated with a substrate could be of utmost relevance in the perspective of developing bio-optoelectronic nanodevices. In this respect, materials able to combine efficient optical and conductive properties are required. Recently, platinum has been proposed as a possible alternative to gold (Conoci et al., 2006), even if its capability to form a stable binding with biomolecules with a preservation of their structure and functions is still unexplored. Another extremely promising substrate, alternative to gold is represented by indium tin oxide (ITO) (Mitsubayashi et al., 2003; Campbell et al., 2007; Lin et al., 2007). Indeed, ITO, a n-type semiconductor material, is characterized by optical transparency to near UV, visible and near-infrared light conjugated to a good electrical conductivity (Alam and Cameron, 2000; Gassenbauer and Klein, 2006). Additionally, it possesses stable electrochemical and physical properties with a good substrate adherence and chemical inertness (Chen and Zu, 2007; You, 2007). Protein molecules can be firmly anchored on ITO substrates by covalent attachment through their carboxyl groups (Ng et al., 2002; Bermudez et al., 2006; Lin et al., 2007; Matsuda et al., 2003; Martin et al., 2002); even though the effective capability of ITO to preserve the structure and functionality of biomolecules, integrated with inorganic substrates, is to be explored with special care.

In this respect, to test the optical and electrical response of biomolecule-ITO-based systems and to assess their suitability for applications in bio-nanodevices, we have chosen to study the blue copper protein Azurin (AZ) assembled on ITO-coated quartz. AZ is a very well-characterized redox protein from *Pseudomonas aeruginosa*, with peculiar electron transfer and optical features (Nar et al., 1991; Solomon et al., 1992; Adam, 1991) which make it especially

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valuable for bio-optoelectronics applications (e.g. photo-induced charge transfer, optoelectrical switching, light harvesting elements, etc.). Actually, AZ possesses an inherent, efficient electron transfer capability, occurring at level of single electron in a very fast, directional way (Gray and Winkler, 1996). These properties, coupled to a high stability and intrinsic robustness, have been recently exploited in nanoscale bioelectronics (Bonanni et al., 2007; Zhang et al., 2002; Zhao et al., 2004; Rinaldi et al., 2002). Interestingly, optical excitation of AZ in the visible region, involving a ligand-to-metal-charge transfer (LMCT) transition within the active site, well mimics the thermal electron transfer process (Book et al., 1998; Baker, 1988). The active site is constituted by a copper ion ligated to two sulphurs (Cys112 and Met121), two nitrogens (His46 and His117) and an oxygen (Gly45), organized in a distorted trigonal bipyramidal geometry; the LMCT transition occurring between Cu and S (Cys112) (Solomon et al., 1992; Gray and Winkler, 1996; Baker, 1988). Furthermore, its isolated tryptophan residue (Trp48), embedded in a highly hydrophobic environment and involved in the physiological electron transfer path, represents a valuable intrinsic fluoro-phosphorescent probe to get information on both the electron transfer process and protein conformational changes (Gabellieri and Strambini, 2001; Gilardi et al., 1994).

By combining optical techniques (optical absorption and transmission, steady-state fluorescence, resonance Raman spectroscopy) with scanning probe microscopies (atomic force microscopy (AFM), scanning tunnelling microscopy/spectroscopy (STM/STS)), we have tried to enlighten the potentialities of the AZ-ITO hybrid system for applications in bio-sensing and bio-optoelectronics.

Our results indicate that AZ molecules can be firmly anchored on the ITO substrate by fully retaining their native structural and spectroscopic features underlying the native electron transfer activity. An investigation of the conductive properties of the AZ-ITO hybrid system put into evidence a good electric contact between the protein molecules and the substrate with a modulation of the ITO semiconductor properties upon deposition of AZ. These evidences, in connection with the interplay between the optical and electron transfer properties of AZ and the conductive and transparency properties of ITO, suggest that the AZ-ITO system is a promising candidate for simultaneous processing of optical and electrical signals in bio-nanodevices. The biorecognition ability of AZ toward biological partners of relevance in nano-biomedicine (Apiyo and Wittung-Stafshede, 2005; Bonanni et al., 2005; Taranta et al., 2008), adds a further ground to the perspective of integrating AZ in biosensing lab-on-chip nanodevices.

2. Materials and methods

Indium tin oxide (ITO)-coated quartz substrate (No. CQ-50IN, Delta Technologies) consists of fused quartz slides (thickness 1 mm) with a transmittance of 85%, coated by ITO (nominal coating thickness 150–200 nm) with a sheet resistance of R_s, 10–20 Ω.

Azurin (AZ) from *P. aeruginosa* (14.6 kD) was purchased from Sigma and dissolved in MilliQ water. AZ-ITO hybrid system (AZ-ITO) was prepared by drying a drop of the AZ solution at a concentration of 50 μM onto the ITO substrate and then rinsed by MilliQ water (Millipore). AZ molecules are expected to be covalently anchored to indium or tin atoms through protein carbonyl groups, in agreement with the Ng et al. (2002).

Steady-state fluorescence emission spectra were measured by using a Spex FluoroMax (Jobin-Yvon) spectrofluorometer at room temperature. The excitation wavelength was 290 nm with a slit width of 5 nm for the excitation and emission monochromator.

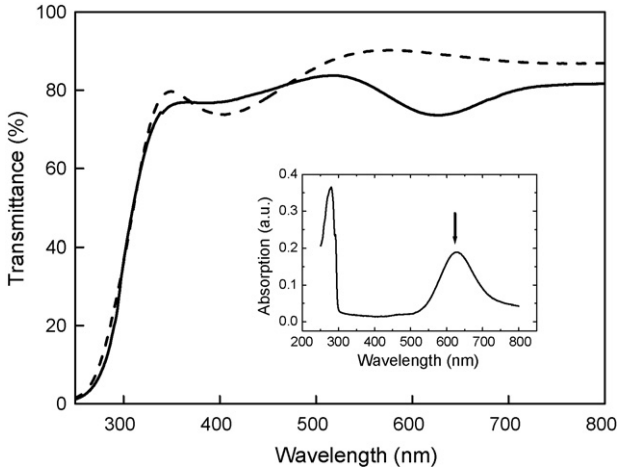

Fig. 1. Transmittance of the AZ-ITO system (continuous line) and of bare ITO (dashed line) as a function of incident wavelength. *Inset*: optical absorption of AZ in aqueous solution at a concentration of 50 μM; the arrow indicates the LMCT absorption band.

Raman spectra were recorded by Labram confocal set-up (Jobin-Yvon) equipped with a charge coupled device (CCD) Peltier-cooled detector, and a single-grating spectrograph with an 1800 g/mm grating allowing a resolution of 5 cm^{-1}. The microscope objective was a 100× with a numerical aperture N.A. = 0.9 producing a laser spot size of about 1 μm in diameter. The source was a HeNe ion laser (MellesGriot) providing a 632.8 nm radiation, with power kept below 5 mW.

Tapping mode atomic force microscopy (TM-AFM) images were acquired, in MilliQ water, by a Nanoscope IIIa/multimode scanning probe microscope (Veeco) equipped with a 12 μm scanner. The typical scan rate was 1 Hz. T-shaped silicon nitride probes (Veeco), 85 μm long, with nominal radius of curvature between 10 and 20 nm and spring constant of 0.5 N/m were used. Resonance peaks in the frequency response of the cantilever were chosen for the TM oscillation in the range 8–30 kHz. Free oscillation of the cantilever was set to have a root-mean-square amplitude approximately corresponding to 1.2 V; after engaging, the set point was adjusted to minimize the tip–sample interaction forces. Scanning tunnelling microscopy (STM) and scanning tunnelling spectroscopy (STS) measurements were performed in constant current mode with a Picoscan system STM (molecular imaging) equipped with a 10 μm scanner (final preamplifier sensitivity of 1 nA/V). The measurements were done in air at room temperature. STM tips for measurements were mechanically cut from 0.25 mm Pt/Ir wire (Goodfellow).

The morphology of both TM-AFM and STM images was performed by WSxM software (Horcas et al., 2007). The root mean square roughness was defined by $R_{rms} = 1/N\sqrt{\sum_{mn=1}^{N\times N}(h_{mn} - \bar{h})^2}$ where N × N is the number of pixels of the image (256), h_{mn} is the height of the pixel mn, and $\bar{h}$ is the related average height.

3. Results and discussion

3.1. Optical transmission

With the aim to fully exploit the optical transparency properties of ITO, we have used ITO-coated quartz substrates, instead of using ITO-coated glasses, as commonly done. The optical transmission of both bare ITO and ITO with adsorbed AZ molecules (AZ-ITO) is shown in Fig. 1. Transmittance values higher than 74% are observed in the whole spectral visible window for both the samples. The

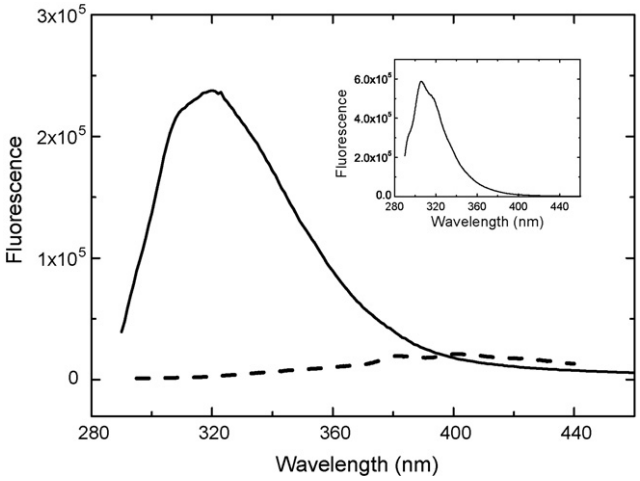

Fig. 2. Steady-state fluorescence emission spectra of the AZ-ITO system (continuous line) and of bare ITO (dashed line). *Inset*: fluorescence emission of AZ in aqueous solution at a concentration of 50 μM. All the spectra have been obtained by an excitation wavelength of 290 nm.

presence of AZ molecules on ITO is clearly signalled by the appearance of a negative peak at about 625 nm that corresponds to the LMCT absorption band of AZ (see the inset of Fig. 1). Therefore, the AZ molecules adsorbed on ITO retain the absorption band which underlies their electron transfer functionality.

3.2. Steady-state fluorescence

Further information on the optical properties of the AZ-ITO system can be derived from steady state fluorescence spectroscopy. Excitation at 290 nm of AZ-ITO results into a strong fluorescent band at 315 nm which can be attributed to AZ, since bare ITO shows a negligible fluorescent signal (see dashed line in Fig. 2). Indeed, AZ in solution shows a fluorescence peak at 310 nm, upon excitation at the same wavelength (see the inset of Fig. 2). This is due the single tryptophan residue (Trp48) which is buried in a highly hydrophobic environment and is extremely sensitive to slight conformational changes in the overall protein structure (Kroes et al., 1998). This result indicates that the native structure of AZ adsorbed on ITO, is substantially preserved; such a robustness having been already observed in AZ chemisorbed on functionalized silicon dioxide (Pompa et al., 2004). On the other hand, the slight broadening observed in the fluorescence band of AZ adsorbed on ITO with respect to that of AZ in solution, might be ascribed to a small increase in the protein conformational heterogeneity; a similar behaviour having been registered by phosphorescence and molecular dynamics simulation for AZ adsorbed on solid matrices (Gabellieri and Strambini, 2001; Bizzarri, 2006). On the other hand, the broadening of the fluorescence band might be due to a modulation of the fluorescence lifetime; such an aspect should deserve further investigations. In fact, the occurrence of an electron transfer from the excited state of organic molecules towards the ITO conduction band may result into an increase of the nonradiative rate with a substantial reduction of the fluorescence lifetime (Holman et al., 2004).

3.3. Raman spectroscopy

Additional information on the optical behaviour of the AZ-ITO system can be obtained by Raman spectroscopy. Actually, a Raman spectroscopy experiment, upon an excitation in resonance with the LMCT absorption band at 625 nm (see the inset of Fig. 1), provides rewarding information on the vibrational modes of the AZ active site. These modes are coupled to protein atomic motions and yield a fine-tuning of the ET process (Cimei et al., 2002; Baker, 1988). Fig. 3 shows the resonance Raman spectrum of bare ITO and of the AZ-ITO system. While the former one (dashed curve) shows only a very weak signal, the latter (continuous curve) reveals an intense pattern around 400 cm^{-1}, and weaker signals at about 280 and 750 cm^{-1}. These vibrational features are closely reminiscent of those shown by AZ in solution (see the inset of Fig. 3), whose main peaks have been attributed to the Cu–S (Cys) stretching modes, with a smaller contribution from the Cu–N (His) modes (Webb and Loppnow, 1999). In this respect, we mention that the resonance Raman spectrum of copper proteins can be well reproduced by calculating the power spectrum of the temporal evolution of the Cu–S (Cys84) bond distance, as extracted from molecular dynamics simulations (Bizzarri and Cannistraro, 2001).

The full preservation of the vibrational features in the resonance Raman spectrum of AZ indicates that the protein copper active site maintains its structure and symmetry properties upon adsorption onto ITO. The possibility that the AZ molecules, immobilized onto ITO substrate, may undergo an electron transfer process, upon an optical excitation of the LMCT band, could deserve some relevance also for applications requiring a charge separation process, e.g. in light harvesting structures. It should be remarked that the resonance Raman spectrum of AZ, directly linked to gold substrates through its native disulphide group, does not reveal any spectral feature (see the dotted line in Fig. 3). In this respect, ITO offers an additional, relevant advantage with respect to gold in building hybrid system exploiting the functional capabilities of biomolecules. Furthermore, it is interesting to note that the vibrational features of AZ on ITO have been found to be stable for long time (more than 1 month, at room temperature); a similar behaviour having been obtained for both transmittance and fluorescence.

3.4. Morphological investigation by TM-AFM and STM

Before testing the conductive features of the AZ-ITO system, we have investigated its morphological properties at nanoscale by both force and current imaging by using AFM and STM, respectively. While ITO-coated glass substrates have been well characterized by both AFM and STM (You, 2007; Ng et al., 2002; Matino et al., 2005),

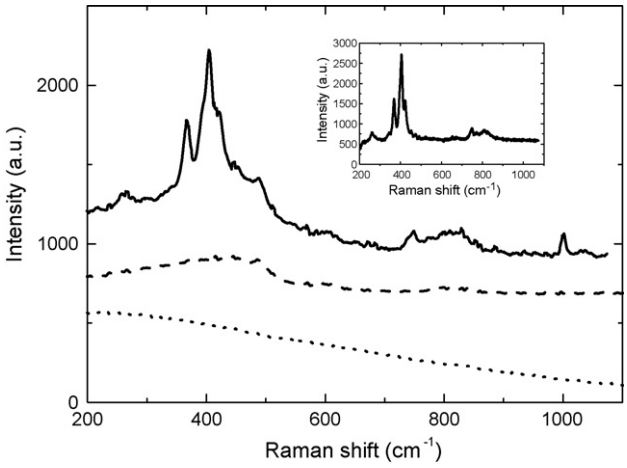

Fig. 3. Resonant Raman spectra of the AZ-ITO system (continuous line), of bare ITO (dashed line) and of AZ on gold substrate (dotted line). *Inset*: Resonant Raman spectrum of AZ in aqueous solution at a concentration of 50 mM. All the spectra have been obtained by an exciting wavelength of 632.8 nm.

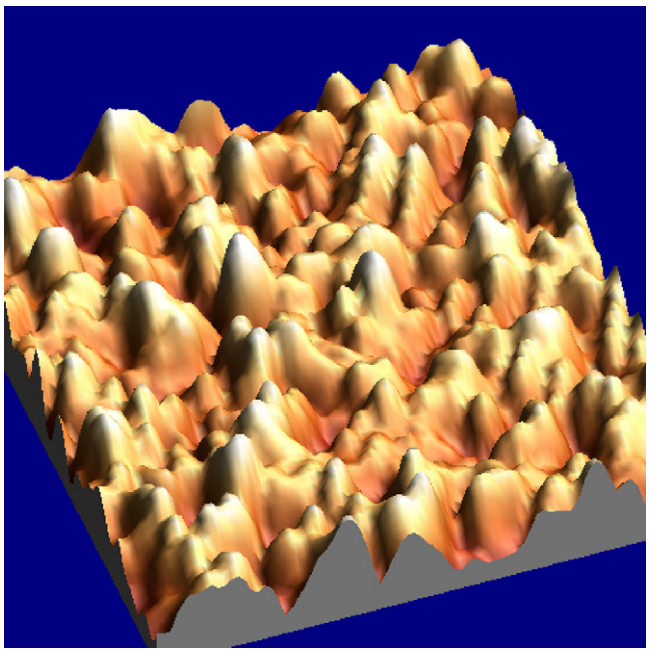

Fig. 4. TM-AFM image (500 × 500)μm of bare ITO in MilliQ water at room temperature.

no data are available for ITO-coated quartz. A typical TM-AFM image of ITO is shown in Fig. 4, and displays a grainy structure with a grain diameter ranging from 15 to 60 nm; the corresponding root mean square roughness, R_{rms}, being (4.9 ± 0.5)nm. Such a value is higher than that usually measured for ITO-coated glass substrates whose R_{rms} is in the 2.5–3.9 nm range, depending on the deposition procedure and on the mechanical and chemical treatments of the surface (You, 2007). Notably, TM-AFM images of the AZ-ITO system do not reveal, at visual inspection, significant changes with respect to bare ITO (image not shown). A morphological analysis put into evidence comparable grain size range, 15–60 nm, and roughness (5.5 ± 0.5)nm, consistently with the low dimension of AZ in comparison to the grain size. However, the absence of significant differences before and after AZ deposition, supports the hypothesis that AZ biomolecules uniformly cover the ITO surface, as already suggested (Ng et al., 2002).

STM imaging of bare ITO reveals again a rough surface, with rather sharp structures (see Fig. 5A). The measured roughness (1.9 ± 0.3)nm, is higher than that detected by STM for ITO-coated glass substrates, which is around 1.1 nm (Ng et al., 2002). At variance with what observed by TM-AFM, the STM images of the AZ-ITO system exhibit significant changes with respect to bare ITO: hills appear drastically smoothed, suggesting the occurrence of some changes in the conductivity of ITO as induced by the adsorption of AZ molecules (see Fig. 5B). However, the roughness (2.3 ± 0.3)nm, is not changed within the error. It should be remarked that both the TM-AFM and STM images of the AZ-ITO system are found to be stable under repeated scans, suggesting that AZ molecules are firmly anchored to the substrate, in agreement with the hypothesis that their immobilization preferably occurs via covalent attachment through protein carbonyl groups to the substrate indium or tin atoms (Ng et al., 2002).

3.5. Conductive properties by STS

The conductive properties of the AZ-ITO system have been investigated by STS. Once the tip has been positioned on the substrate at a height set by the engage tunnelling current and bias, the feedback was disabled and the current registered as function of the applied bias. The characteristic *I–V* curve of bare ITO obtained by averaging over 10 different sites, is shown in Fig. 6 (see the dashed curve). The typical asymmetric trend of semiconductors is clearly evident. Actually, for negative voltages, the current is very low and positive, while at positive polarity, the current is negative and rather high. The variability of the *I–V* curves of bare ITO from site to site (see the dashed region in Fig. 6) is in agreement with literature data for ITO-coated glass substrates (Liau et al., 2001). The Fermi level of ITO has been found to be zero, and it indicates the absence of band bending, similarly to what observed for other ITO films (Kasiviswanathan et al., 1997). The conduction band minimum, corresponding to the voltage asymptotically traced by the current, is around −2.2 V; while the valence band maximum, as extracted from the derivative of the *I–V* curve, is 1.2 V (see the inset in Fig. 6). It turns out then an energy gap of about 3.4 V; a value being consistent with that found for other ITO samples (Kasiviswanathan et al., 1997).

The *I–V* curve of the AZ-ITO system, obtained by averaging over *I–V* curves from 10 different sites, are shown in Fig. 6 (continuous curve). It reveals the same asymmetric trend observed for bare ITO. Positive and very small values are registered for negative bias, while

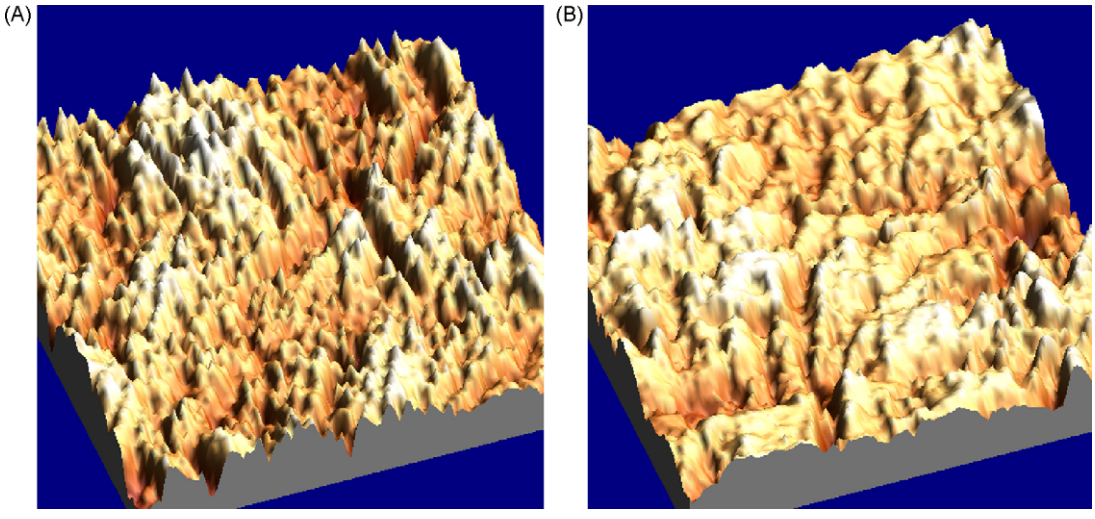

Fig. 5. (A) STM image (500 × 500)μm of bare ITO. (B) STM image (500 × 500)μm of the AZ-ITO system. Both the STM images have been recorded in air with a tunneling current of 50 pA and a bias of 0.5 V.

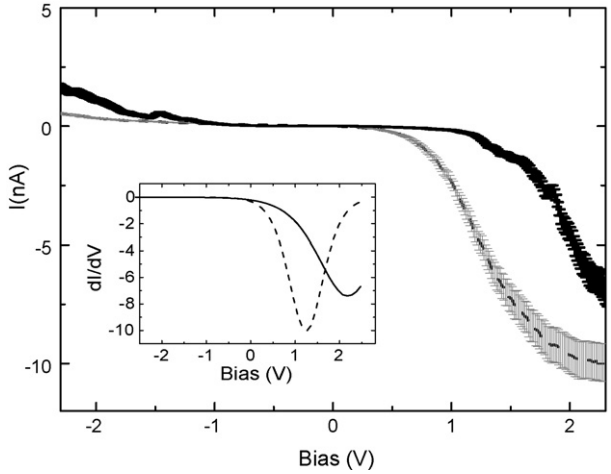

Fig. 6. *I–V* curves of the AZ-ITO system (black, continuous line) and of bare ITO (grey, dashed line) at an engaged tunnelling current of 50 pA and at engaged bias of 0.5 V. Each curve has been averaged over 10 different sites; for each site an average over 25 curves has been performed. Bars indicate the variability from site to site. Inset: derivative of the *I–V* curves of the AZ-ITO system (continuous line) and of bare ITO (dashed line).

higher current values are found for positive bias. A decrease in the absolute currents at fixed bias in both the negative and positive region for the AZ-ITO system with respect to bare ITO is registered; these changes being well beyond the variability from site to site (see error bars in Fig. 6). Similarly to what observed for the spectroscopic properties, the *I–V* curves of the AZ-ITO system remain stable over time.

The conduction band minimum of the AZ-ITO system is around −2.1 V, very close to the value detected for ITO. At variance, the valence band maximum (see inset in Fig. 5), is located at 2.0 V, shifted to higher values with respect to that of the ITO substrate. Consequently, an energy gap of 4.1 V can be calculated. The observed changes in the conduction band, and then in energy gap, point out a significant modulation of the conductive properties of ITO when AZ proteins are deposed on it. This interesting result suggests the occurrence of some interplay between the ITO bands and the electronic levels of AZ. The conduction and the valence bands of ITO might interfere with the HOMO and LUMO level of the protein, likely through a charge transfer mechanism. In this respect, we mention that a recent, theoretical study has shown that isolated aminoacids deposed on the surface of a n-type semiconductor could induce a carrier injection depending on the charge and polar character of the aminoacids (Oda and Nakayama, 2005). We can therefore reasonably hypothesize that a charge transfer between AZ and ITO could result into a modulation of the conduction properties of ITO. Therefore, ITO could act as a transducer of biomolecule-generated events provided that they are able to inject charges into the ITO layer; these charges might arise both from optical excitation of AZ and biorecognition processes. These capabilities, even if they should be investigated more deeply, deserve a remarkable interest for designing artificial hybrid systems.

As we have already mentioned, many attempts to conjugate biomolecules with conductive substrates have been done by directly anchoring proteins to gold substrates, through intrinsic specific groups, such as thiol groups (Davis et al., 2005; Andolfi et al., 2006; Zhang et al., 2002). Generally, this has allowed to achieve a stable and efficient electrical communication between biomolecules and substrates, resulting into high current values in the *I–V* curves. The evidence that the AZ-ITO system is characterized, at positive bias, by current values in the same range, or even higher than those observed for gold substrates, indicates that a good electric contact is established between AZ molecules and ITO.

4. Conclusions

Using biomolecules as electronic components in hybrid systems may prove a new way in the science and technology of nanometre-scale devices. The ability to design nanodevices integrating biomolecules and conductive substrates, with suitable and *ad hoc*, optical electronic and conductive properties, is a crucial step to exploit these systems in bio-sensing and biorecognition applications. In this respect, the hybrid system constituted by the redox copper protein AZ integrated with ITO-coated quartz substrate is an extremely promising candidate. It couples optical transparency with good conductive properties. We found that AZ molecules upon deposition on ITO fully retain their optical features, which underline their physiological electron transfer activity. Furthermore, AZ molecules anchored onto ITO exhibit the capability to modulate the semiconductor properties of ITO. This aspect can be of utmost relevance if we take into account the peculiar interplay occurring in AZ molecules between the optical excitation of the LMCT band and the electron transfer process in AZ. In summary, these results shed light on a new approach to build innovative nanodevices based on the simultaneous processing of optical and electrical signals in hybrid systems for bio-sensing and bio-optoelectronics.

Acknowledgments

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