Structural stability study of protein monolayers in air

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The assessment of the folding and of the structural stability of a protein in air, upon immobilization in the solid state, represents a critical point from both a fundamental point of view and for the development of solid state nanobioelectronics. The recent demonstrations by Rinaldi *et al.* [R. Rinaldi *et al.*, Adv. Mater. **14**, 1453 (2002); Appl. Phys. Lett. **82**, 472 (2003); Ann. (N.Y.) Acad. Sci. **1006**, 187 (2003)] of protein-based solid state devices and transistors working in air have raised an intriguing question about the behavior of a biomolecule under nonphysiological conditions. The operation principle of the realized devices is based on the physiological electron transfer function of the metalloprotein azurin. This means that azurin should retain its shape and functionality also in the solid state when utilized in air and at room temperature. In this Brief Report, we prove this claim by analyzing the conformational state of the azurin monolayers developed for such devices by means of intrinsic fluorescence spectroscopy. We show that the immobilization of azurins in the solid state under nonliquid conditions, by means of a specific chemisorption process, does not necessarily lead to protein denaturation. This result is of great importance because it opens up interesting perspectives for the development of solid state hybrid nanodevices for electronic applications requiring nonliquid environments.

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In recent years, the use of biomolecules as electron-conductive materials for bioelectronics has attracted great attention, thanks to the possibility of exploiting their self-assembling capabilities and their specific functionalities at nanoscale. The combination of molecular biology (for engineering proteins with the desired functional and/or self-assembling properties) and nanotechnology (for device fabrication) opens the prospect of a new class of nanoelectronics elements. Progress in this field depends on methods and procedures yielding high-quality molecular monolayers. Chemisorption is an attractive approach since the specific reactivity of biomolecules with other molecules and/or with exposed chemical surface groups may be controlled to realize complex supramolecular architectures.

The creation of protein monolayers onto a specific substrate is a crucial aspect in the construction of a nanodevice, as its functionality strongly depends on the quality of the biomolecular film [1]. The interaction between proteins and solid substrates may lead to alterations of the native structure (and/or aggregation), with consequent loss of functionality. Hence, the development of protein-based devices requires detailed understanding of the immobilization processes and of the conformational changes possibly induced in the biomolecules.

Azurin (Az) from *Pseudomonas aeruginosa* [2–4] is an electron-transfer (ET) metalloprotein. Its redox-active center contains a copper ion having five amino acid ligands arranged in a trigonal bipyramidal geometry. In the last years, azurin has attracted huge attention for nanoelectronics applications, due to the possibility that it can sustain current flow with the possible direct involvement of the redox center [5–11]. Furthermore, our group has recently shown that Az electron transfer activity can be exploited for the realization of solid state biomolecular electronic rectifiers and transistors whose conduction state can be controlled by tuning their redox state through an external voltage source [12–14]. Such devices imply the involvement of the physiological ET function of copper azurin, even though they operate in air, thus demonstrating, though indirectly, that azurin can retain its functionality also in the solid state.

In this Brief Report we provide experimental evidence on this point. We carefully analyze by means of intrinsic fluorescence spectroscopy the conformational state of the azurin monolayers developed for such devices, showing that the immobilized proteins do not undergo denaturation, thus demonstrating the possibility of realizing high-quality protein films by means of a specific chemisorption process. This result is important in view of the development of functional metalloproteins monolayers and discloses interesting perspectives for the fabrication of protein-based hybrid nanodevices operating in nonliquid environments.

The protein monolayers were realized by covalently binding azurin (from a 50 mM NH_4Ac buffer, pH 4.6) to functionalized silicon dioxide, exploiting the formation of intermolecular disulphide bonds between the exposed thiol groups on the substrate and the sulphur atoms of Cys 3 and Cys 26 of azurin [12,13]. After incubation, the substrates were rinsed copiously with deionized water in order to remove physically adsorbed material, and accurately dried by high purity nitrogen flow. The formation of protein monolayers, as well as the coverage uniformity, were assessed by molecular resolution atomic force microscopy (AFM) measurements [12].

Azurin from *P. aeruginosa* contains a single tryptophan residue (Trp48). The fluorescence spectrum of Trp48 is centered at an unusually short wavelength ($\lambda_{max} \approx 308$ nm) ow-

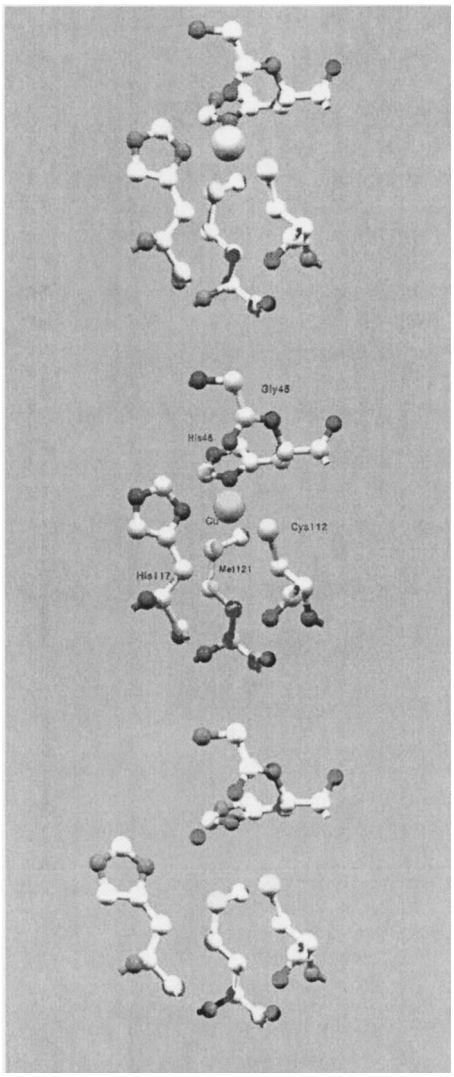

FIG. 1. Structure of azurin active site. From top to bottom: stereo view for zinc, copper, and apo azurin (copper azurin annotated). PDB files: azurin *Pseudomonas aeruginosa*; (i) 1E67 (zinc, pH 5.7; resolution: 2.14 Å); (ii) 4azu (copper, pH 5.5; 1.90 Å); (iii) 1E65 (apo, pH 5.5; 1.85 Å).

ing to the highly hydrophobic microenvironment surrounding it [15–17]. In the native state, azurin, apoazurin (i.e., without Cu^{2+}) and derivatives in which the copper has been replaced by other metals, such as Zn^{2+}, exhibit the same emission and excitation spectrum, clearly showing that no structural change occurs besides the metal site (Fig. 1). This is also confirmed by crystal structures determinations [16,18–20]. In the wild type protein, the copper ion is coordinated by three strong ligands (His46, His117, and Cys112), arranged in a trigonal planar configuration around the metal, and by two weakly interacting groups (Met121 and Gly45), at axial positions [18]. The zinc azurin is characterized by a slight shift (~ 0.3 Å) of the metal ion toward the Gly45 ligand, whereas, in the apo form, a water molecule is found in the empty metal site [19]. The different derivatives display large variations in the fluorescence quantum yield. The exact mechanism underlying the fluorescence quenching by metal ions is still argument of investigation [15,21–25], and a possible role of the Trp48 in the long-range electron transfer processes has been invoked [17].

Intrinsic fluorescence spectroscopy allows accurate monitoring of conformational equilibria induced by the immobilization procedure and of the eventual perturbations in protein folding, possibly resulting in physico-chemical changes of the copper active site. In the present paper, fluorescence spectroscopy experiments were performed both in solution and in solid state films in order to characterize the conformational properties of the azurin molecules. To this purpose, we first investigated the emission spectra of the apo and zinc derivatives (native state, denatured state, and chemisorbed as dehydrated films), and, in a second step, we checked the folded state of the immobilized metalloproteins by analyzing the relative efficiency of the Tyr-Trp fluorescence energy transfer both in solution (when denatured by HCl), and in the solid state. It is noteworthy that, owing to the remarkable experimental complexity, the reported optical characterization represents, to our knowledge, the first direct measurement of intrinsic fluorescence from an azurin monolayer.

The emission spectrum of the chemisorbed apoazurin exhibits a line-shape similar to the free protein in buffer [Fig. 2(a)]. The occurrence of a slight redshift ($\sim 2-3$ nm) is the only minor effect detectable in the immobilized apo proteins. According to the photophysics of aromatic amino acids, such a result reveals that the tryptophan residue in the native and in the immobilized apo protein is embedded in the same hydrophobic environment. The small spectral shift may signal a small internal rearrangement of the protein structure, without effect on the folding pattern. This conclusion is further supported by comparing the photoluminescence spectrum of the apo-protein monolayer with the broadband, redshifted emission of a denatured sample of azurin (6 M guanidine hydrochloride), in which the unfolding process clearly results in a large solvent exposure of the hydrophobic residues which were otherwise shielded in the core of the protein in its native state. Interestingly, also the excitation spectrum of the apo protein was unchanged upon chemisorption (not shown), thus demonstrating the absence of any relevant perturbation in the physico-chemical conditions of the chromophore microenvironment, and indicating that the covalent binding to the functionalized SiO_2 surface does not interfere with the conformational properties of the native protein.

An additional remarkable result was obtained with zinc azurin. In this case, the immobilized protein exhibits a photoluminescence spectrum which is identical to that observed for the native state [Fig. 2(b)]. Since the intrinsic fluorescence in azurin is very sensitive to small perturbations of the protein folding [26], the blue and structured emission of the chemisorbed zinc derivative indicates that the Trp remains buried in the highly hydrophobic and rigid protein core, and that no conformational rearrangements occur upon chemisorption. This result is consistent with the expected structural stability of zinc azurin, which exceeds that of the apo form, due to the presence of the metal ion [26]. However, owing to the higher affinity of the metal-binding site for Cu^{2+} than Zn^{2+} [20,26,27], the wild type copper protein has the highest

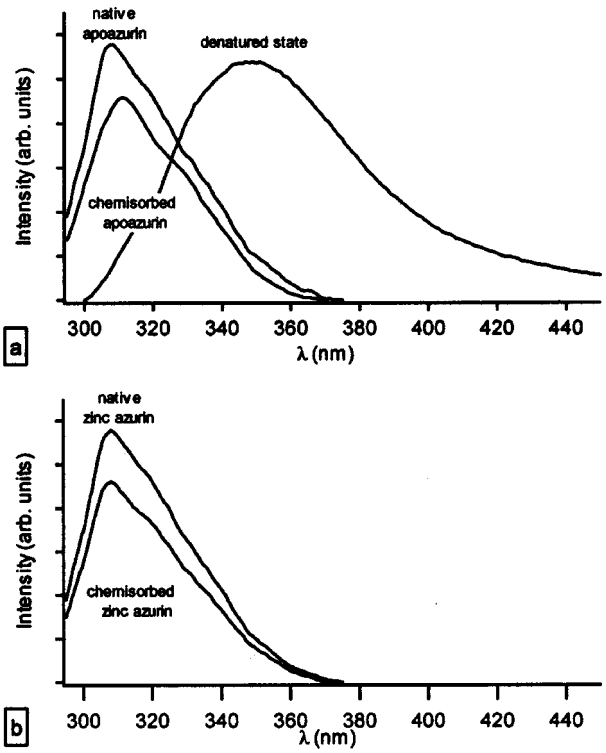

FIG. 2. Azurin fluorescence spectra: (a) free native apoazurin (in 50 mM NH_4 Ac buffer, pH 4.6), denatured apoazurin by 6 M guanidine hydrochloride, and apoazurin chemisorbed in the solid state; (b) free native zinc azurin (in 50 mM NH_4Ac buffer, pH 4.6), and zinc azurin monolayers (the fluorescence intensities are not in scale; the spectra of the chemisorbed proteins are the mean of 10 acquisitions). All the emission spectra were recorded at room temperature (20 °C), atmospheric pressure, 54% of humidity. [As a general comment, we think that, though it is possible to exclude the presence of bulk water onto the substrates (owing to the extensive N_2 drying procedure performed on all the samples), we do believe that the azurin molecules retain their hydratation shells also in the film environment. This is the key reason (obviously together with their high intrinsic stability) for which azurin can retain the native fold pattern also in the solid state.] The excitation wavelength was 280 nm (2 nm bandwidth).

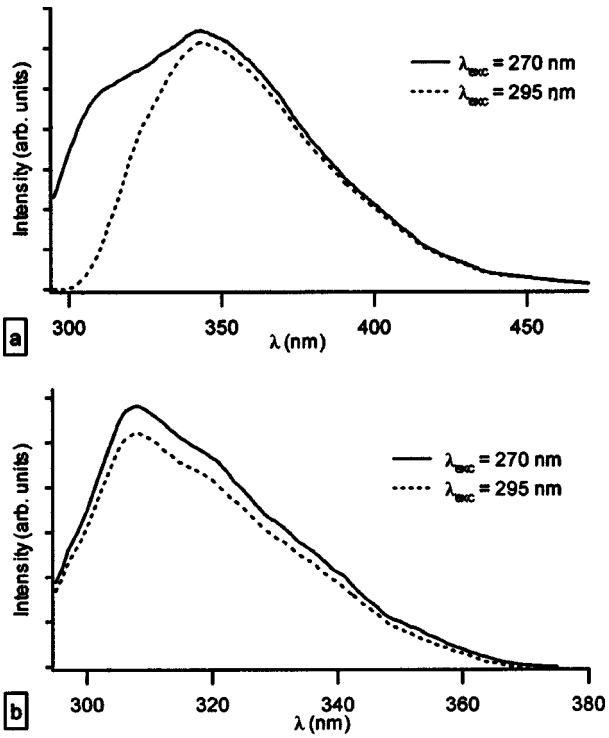

FIG. 3. (a) Free apoazurin photoluminescence at pH 1.5, excited at different wavelengths; (b) solid state apoazurin monolayers (the fluorescence intensities are not in scale; the spectra of the chemisorbed proteins are the mean of 10 acquisitions). All the emission spectra were recorded at room temperature (20 °C), atmospheric pressure 54% of humidity, excitation bandwidth 2 nm.

stability [26]. It is important to note that unlike previous studies performed in aqueous solution, i.e. under physiological conditions, the present result has been obtained with the proteins immobilized in the solid state. In addition, we observed that the almost comparable quantum yield characterizing the apo and the zinc azurins in the native state is retained in the film environment.

The folding properties of the azurin monolayers were further investigated by monitoring the energy transfer processes between the different protein fluorophores. Azurin contains one tryptophan (Trp 48) and two tyrosines (Tyr72 and Tyr108) but, as generally recognized, its photoluminescence spectrum is basically due to Trp emission, due to a very efficient energy transfer process from the tyrosines to the Trp48. This photophysical mechanism explains why native azurin exhibits identical fluorescence spectra (apart from quantum yield) though excited at 270 or 295 nm, as well as identical excitation spectra regardless of the emission wavelength. Energy transfer strongly depends on the distance between donor and acceptor chromophores and it is a very sensitive probe of the protein structure. Hence, while in its native state azurin is characterized by a negligible or very small tyrosine contribution to fluorescence emission, the occurrence of externally induced conformational transitions may result in a significant decrease of the energy transfer efficiency, and in the rise of new spectral features which can be ascribed to tyrosine residues. This is the case, for instance, of azurin emission in acid solutions, where the unfolding action of HCl clearly results in a pronounced change in molecular conformation [15,28]. As shown in Fig. 3(a), the fluorescence spectra of apoazurin at pH 1.5 are characterized by remarkable redshifts of the emission maximum ($\lambda_{max} \approx 343$ nm), reflecting large solvent exposure of the aromatic residues. In particular, unlike the photoluminescence spectra of native apo and zinc azurin in Fig. 2, which are independent on the excitation wavelength, the fluorescence emissions reported in Fig. 3(a) are very different when excited at 270 nm or at 295 nm (where tyrosine absorption is negligible). The pronounced shoulder around 305–310 nm under 270 nm excitation is therefore due to tyrosine fluorescence, and indicates that, owing to the significant conformational changes, the transfer of excitation energy from the tyrosines to the tryptophan residue is now characterized by a very low efficiency. The same experiment was done in the solid state azurin monolayers. In this case, the proper conformation retained by the chemisorbed proteins causes the

photoluminescence spectra to be completely independent of the excitation wavelength, thus confirming that no variations in the folding properties are elicited upon immobilization. As shown in Fig. 3(b), the Tyr-Trp energy transfer process was investigated by exciting the azurin films at different wavelengths and no variations in the shapes of the fluorescence spectra are recorded. Similar results were obtained also for the zinc azurins (not shown). In addition, as opposed to the unfolded azurin by acid pH, also the excitation spectra for different emission wavelengths (300, 310, 330, and 350 nm) were identical in the molecular films, thus reflecting the same behavior of the free native protein [29].

All these conformational features, therefore, indicate the absence of any perturbation in the folding properties of azurins upon immobilization in the solid state. Such results are definitely interesting since they open promising perspectives for the development of biological physics applications to nanoscale technologies (e.g., biomaterials, protein based nanobiosensors, hybrid devices), but they are also relevant from a fundamental point of view, since we have demonstrated, by qualitatively comparing the zinc and the apo systems, an important biophysical issue, i.e., that some azurin properties, highly specific of the native state, are preserved after the transition from physiological conditions to solid state environment.

Work is in progress to evaluate aging effects of the azurin monolayer and to improve the shelf life of the molecular film at a nanoscale level.

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[29] The reported investigation on the fluorescence energy transfer, however, deserves further comment. We know that in recent years the issue of the Tyr to Trp energy transfer for the azurins has been the subject of debate. Nevertheless, it is beyond the scope of this work to characterize such a process or to measure its specific parameters, but to use the possible dependence of the emission spectrum on the excitation wavelength as a probe to monitor the induction of conformational transitions in the protein. Besides the properties of the Tyr residues and the specific controversies about the energy transfer mechanism, it is a fact that the photoluminescence of azurin is independent of the excitation wavelength if the protein is in the folded state, while it can be strongly affected by the excitation wavelength if the protein is denatured. Hence the wavelength independence observed in the fluorescence emission of the azurin monolayers indeed indicates the existence of proteins which retain the nativelike conformation.