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Conductive atomic force microscopy study of plastocyanin molecules adsorbed on gold electrode

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Abstract

The electrical conduction of mutant plastocyanin molecules self-assembled on gold was measured by conductive atomic force microscopy. The copper protein molecules, able to bind to gold through an engineered S–S group, were immobilised on Au(1 1 1) substrates to form a dense monolayer, which was contacted by a conductive atomic force microscopy tip at controlled force. Specifically, the electronic conduction of the gold coated tip–plastocyanin–Au(1 1 1) junction was well characterised between +1 and −1 V for increasing compressional forces. The conduction varies slightly within the force range of 3–9 nN, while it rapidly increases above these force values. The occurrence of a jump in mutant plastocyanin conduction at a critical force value, suggests that the current transport mechanism through the bioelectronic junction can be dominated by protein mechanical characteristics and/or by considerable variations in the protein conduction upon molecular deformation. By operating in imaging mode, we have obtained good topographic images of the protein self-assembled on Au(1 1 1) surface and for the first time simultaneous current images were recorded. Remarkably, a correspondence between the biomolecules observed in the topography images and the current spots was found for negative bias.

Keywords: Metalloprotein; Conduction; Plastocyanin; Conductive atomic force microscopy

1. Introduction

The understanding of electron transport mechanism at the protein–metal interface is one among the fundamental issues in the development of improved biosensors and nanoelectronic devices. Particular attention has been drawn by redox metalloproteins, for which the possibility to be self-assembled on a metallic electrode and to tune their redox activity by an external potential, makes them good candidates for creating hybrid systems, able to convert a biological function (i.e. catalysis,

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recognition, storage, etc.) into a detectable electrical signal [1–3].

There is so far a wide knowledge of the electron transfer properties of metalloproteins due to a combined use of spectroscopic, electrochemical and theoretical approaches which have essentially disclosed an average behaviour of these properties [4–7]. On the other hand, the electron transfer of single molecules adsorbed on a substrate can differentiate from that in bulk solution, disclosing new pathways, dynamical behaviour and phenomena hidden in molecule ensembles, i.e. rectification, single electron tunnelling, molecular switching, etc. The study of biomolecular conduction through single metalloproteins has been mainly addressed by using scanning tunnelling microscopy (STM) [8–10], under electrochemical control [11–15] and by tunnelling spectroscopy (STS) [15,16]. In the latter configuration the STM tip is held stationary over a single molecule and the current flow is measured as function of the bias, after the feedback loop had been disengaged. This technique suffers from the drawback that the current (i.e. the tunnelling resistance) is used to control tip positioning, and this makes difficult to establish a controllable tip–sample contact [17–19].

Conductive atomic force microscopy (CAFM), overcomes these difficulties because an independent feedback, which maintains a constant force, allows to place the tip directly in contact with the sample and, by applying a bias voltage between them, the flowing current can be measured [20]. In addition, the possibility to vary the applied force on the sample allows probing the effect of mechanical deformation on molecular conduction, as already reported for inorganic [21] and organic molecules [22–25]. A further and unique advantage offered by CAFM is that topography and current images can be recorded simultaneously and, in favourable cases, the conductive properties can be coupled directly to the morphology of the sample investigated. Nevertheless, only in few cases restricted to inorganic and small molecules, the correlation between current images and morphology has been reported [25–27].

In the present work, we used CAFM to investigate the conduction properties of the blue copper protein poplar plastocyanin (PCSS) molecule placed within the junction formed by a metal coated AFM probe and a gold substrate. To obtain specific and direct immobilisation on gold, a disulfide group, located in a region opposite to the copper containing active site, was genetically engineered in the wild type protein [14a].

The PCSS molecules were immobilised on Au(1 1 1) substrate and then contacted with a conductive AFM tip to perform spectroscopic and imaging experiments. Due to the experimental conditions (such as tip radius and the size of the molecule) the conduction properties of few molecules, possibly only one, are very likely investigated.

2. Experimental section

Design, expression and purification of plastocyanin mutant was carried out as described previously [14a].

Conductive AFM probes (Mikromasch) consist of standard silicon cantilevers coated with a chromium film of 20 nm and a further gold layer of 20 nm. The measurements were performed by using rectangular cantilevers with a spring constant of 0.05 ± 0.01 N/m. According to their geometry this value was determined by measuring the resonant frequency and quality factor of the cantilever in air [31].

The tip radius was 18 ± 4 nm as measured by acquiring contact AFM images of 5 nm gold colloids assembled on a glass slide functionalised with (3 mercaptopropyl)-trimethoxysilane. All CAFM tips before either measuring or functionalisation, were rinsed with acetone, ethanol and ultra pure water (Millipore 18.2 MΩ cm).

Gold substrates (Arrandee™) with a thickness of 250 ± 50 nm were produced by evaporation on top of an adhesive chromium layer deposited on borosilicate substrates. They were annealed with a butane flame at a temperature of about 1300 °C to obtain re-crystallised terraces of about 400 nm^2.

PCSS molecules were adsorbed on freshly annealed gold substrates by incubation with 20–50 μM of protein solution in sodium phosphate buffer (20 mM, pH 6.0) for 12–36 h at 4 °C. Modulation of protein coverage was obtained by either

varying the incubation time or the protein concentration. After incubation, samples were gently rinsed with ultra pure water blown dry with pure nitrogen. The formation of PCSS monolayer was checked by STM in air.

PCSS molecular conduction was studied by approaching a gold coated AFM probe down to contact the molecule layer assembled on Au(1 1 1) substrate.

I–V measurements as a function of the applied force and topography-current images were carried on by using a CAFM (PicoSPM, Molecular Imaging), which was equipped with a current sensing module with a sensitivity of 1 nA/V. Its operational range is from few pA to 10 nA. Each single *I–V* sweep was registered in 0.06 s. The overall drift in *x–y* plane was evaluated to be less than 1 Å/s, thus not appreciably influencing the tip position during measurements. The drift in *z*-direction was checked by recording the current as function of time without any feedback; a current fluctuation of about 1 pA/s having been observed. All experiments were done under controlled atmosphere; the samples having been sealed into the environmental chamber and flushed with pure dry nitrogen before and during measurements. Under nitrogen, we may be confident that the water layer at the sample interface is reduced to the minimum [32].

3. Results and discussion

A direct immobilisation of PCSS molecules on gold substrate, with the copper active site placed away from the gold substrate, was obtained by chemically anchoring the protein molecules to Au(1 1 1) via the engineered S–S group. The occurrence of a strong bond between the engineered S–S bridge and the gold atoms was supported by STM experiments. Indeed, well resolved and stable images, even after repetitive scans, were demonstrated only with this mutant protein and not with the wild type form, which lacks of an S–S anchoring group [14a,15]. Fig. 1 shows a three-dimensional STM image of a PCSS monolayer obtained by a self-assembling process subsequent to incubation of substrate with the protein solution. Similar

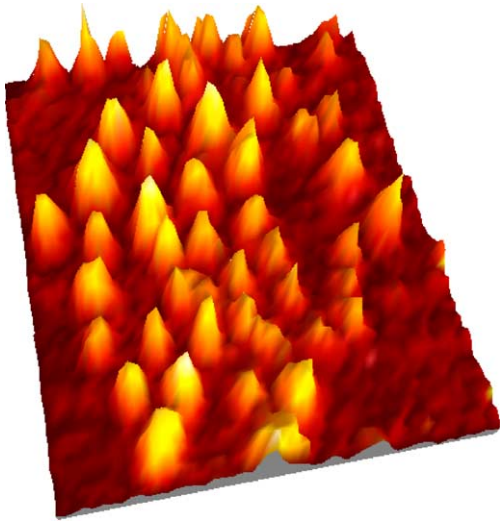

Fig. 1. Three-dimensional view of STM image of PCSS monolayer assembled on Au(1 1 1), obtained in constant-current mode at a tunnelling current of 50 pA, and a bias voltage of 0.2 V. Scan speed is 4 Hz and scan size 90×110 nm.

images were obtained on several sample areas confirming a uniform protein coverage of the substrate. Then, a gold coated AFM tip, able to apply a controlled force, was positioned in contact with the protein monolayer, as schematically shown in Fig. 2. In this figure, PCSS molecules are represented as partially lying down on the gold substrate, according to a previous analysis by tapping mode AFM and molecular dynamic simulations on the same system [15,33]. In order to monitor the current response, a sweep of the tip–substrate voltage in the range of ± 1 V at each applied force was performed. Fig. 3 shows the *I–V* curves, obtained at different forces. Up to applied forces of 9 nN the curves show a sigmoidal behaviour almost overlapping in the ± 0.2 V range; while at higher bias (absolute values) the current changes slightly for different forces. A remarkable rise in current is instead observed when the applied load exceeds 9 nN. These data indicate that the current conduction is non-linearly modulated by the mechanical load, with a negligible effect of the electric field at the higher biases.

I–V curves, especially for low forces, show slightly higher currents for negative biases, thus indicating a small asymmetry. This could be due

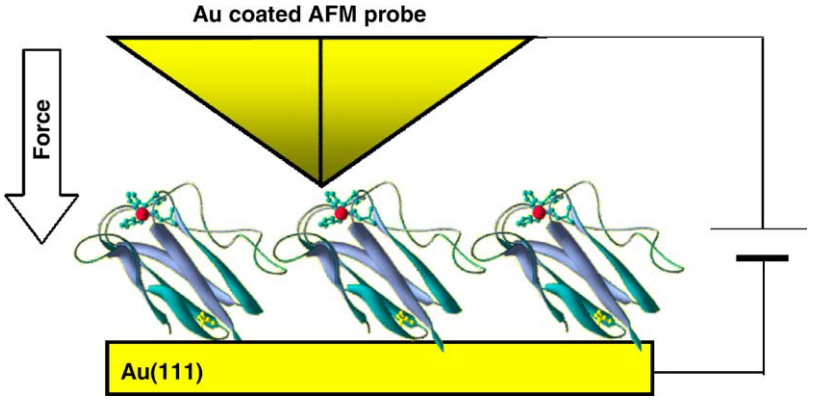

Fig. 2. Scheme illustrating a gold coated AFM probe brought into mechanical contact with PCSS molecules chemically anchored on Au(1 1 1) substrate.

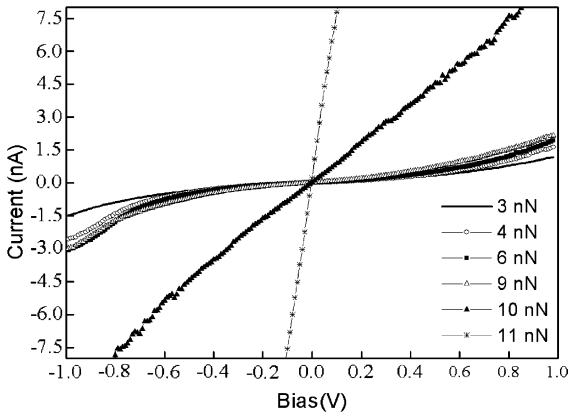

Fig. 3. *I–V* curves, obtained under nitrogen atmosphere, for a gold coated AFM tip in contact with PCSS molecules immobilized on Au(1 1 1) substrate at increasing applied forces.

to either the asymmetric positioning of the redox active centre in the junction as result of specific protein immobilisation [34], or to the different nature of the contacts at the tip and substrate metal–molecule interface [35–37]. These *I–V* characteristics are reproducible when placing the tip on different sample areas and when repeating measurements on several samples with similar protein coverage.

I–V plots, between ±0.2 V, are linear for all applied forces, and the inverse of the slope of the curve can be used to estimate the molecular resistance. When plotting the estimated resistance values as function of applied forces two distinct trends can be revealed (see Fig. 4). For forces ranging over 3–9 nN the junction resistance remains almost constant at a value of $2–3 \times 10^9\ \Omega$. These resistance values are comparable to those obtained in STM configuration for tip–substrate distances high enough to exceed the physical size of the adsorbed copper protein thus enabling a non-perturbative protein imaging [19]. In addition, the CAFM measurements at low forces show *I–V* features qualitatively similar to those obtained in tunnelling spectroscopy studies, performed at distances exceeding physical size of the PCSS [16d]. When applying forces higher than 9 nN the resistance drops rapidly to reach $1.5 \times 10^7\ \Omega$ at 11 nN. This behaviour is reversible when the forces are reduced to the initial values (about 3 nN),

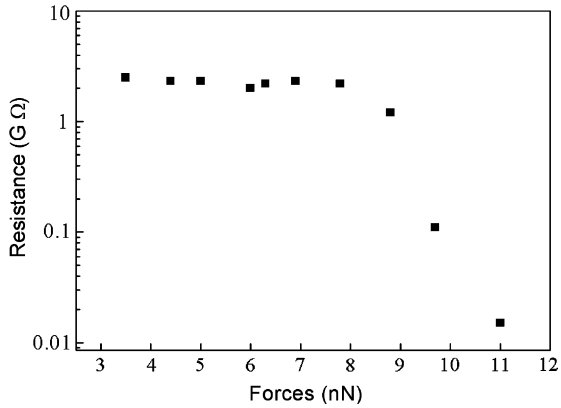

Fig. 4. Semi-logarithmic plot of PCSS junction resistance versus increasing compressional force loads when the gold AFM tip is brought into contact with PCSS monolayer.

thereby indicating that the PCSS molecules are likely elastically compressed in this range of forces and not squeezed out by the tip.

The increase of current conduction (or junction resistance decrease) upon compression, has been already revealed in CAFM measurements on other soft samples such as SAM monolayers [22,37,38], azurin [28–30] and cytochrome [30]. This effect can plausibly depend on several factors: a larger number of molecules contacted when increasing pressure, distance reduction between the AFM tip and the substrate, changes in electronic properties as a result of molecular deformation. In addition, also the type of contacts with the electrodes (PCSS is chemically anchored to the Au(1 1 1) substrate and physically contacted by the gold coated tip) can be responsible of the current dependence on force load. Indeed, in other studies, in which both ends of the molecule are chemically contacted, a current response insensitive to increasing contact force, has been reported [37,39].

We believe that the observed discontinuous increase of conduction with the applied force cannot be ascribed to a progressive involvement of more and more molecules, since in such case the averaged response would lead to a more linear current trend. Based on theoretical and experimental studies the transport mechanism across the molecular junction is generally believed to consist in an electron tunnelling occurring through bonds and/or through space [40]. We would, therefore, expect an exponential current increment on force load, as due to the fact that increasing the force leads to a reduction of the probe–substrate distance. Instead, in our case, two linear conduction trends upon compression were obtained. All these observations suggest that the step in the PCSS resistance is more likely associated with either the mechanical characteristics of the protein, which may be not uniformly and linearly compressible [41], or with a sudden change of protein conductive properties at a critical mechanical pressure. The significant change of PCSS electron conduction properties could be even related to structural perturbations, though they do not seem to severely affect the protein structure. Indeed, when reducing pressure, the electrical characteristics observed for low forces are recovered.

Thanks to the advantage provided by CAFM, combined force and current measurements were performed as function of the tip substrate distance at a fixed bias. In Fig. 5(a) both applied force and current signal as function of tip displacement on a bare gold substrate are shown. Here we observed that when the tip just comes into contact with the metal surface, as shown by the change of the slope of the force curve, the current immediately rises to values that saturate the preamplifier. In contrast, when similar experiments were repeated on PCSS molecules (Fig. 5(b)), as the tip approaches the proteins, the current increases almost linearly until a force of about 8 nN is applied, as inferred by the corresponding force curve. A further increase of the force load induces

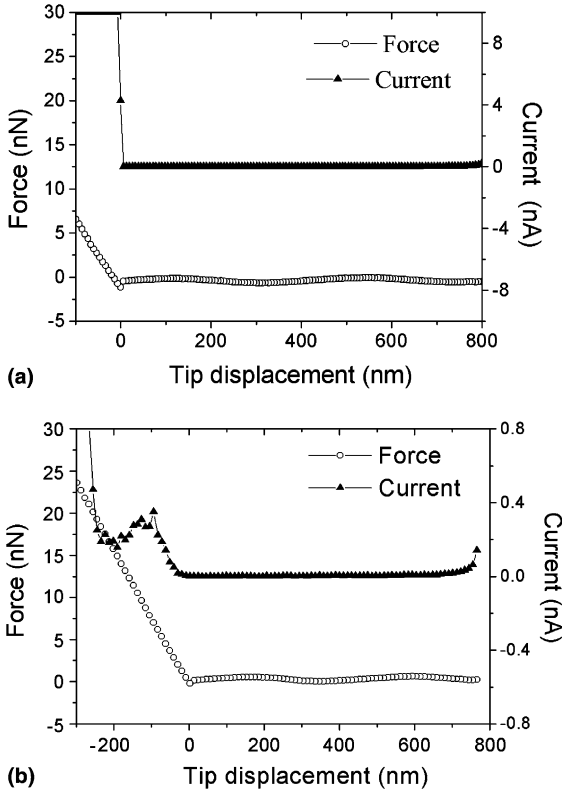

Fig. 5. Current (▲) and force (●) curves as function of the conductive AFM probe extension toward the sample surface at a bias of 1 V for bare Au(1 1 1) (a) and PCSS molecules (b). The experimental error in the current measurement is 2%. The retraction curve is not shown for clarity of the picture.

a slight, but significant, decrease in current up to 20 nN, above which, the current signal quickly reaches high values beyond the preamplifier limit. Such a slight current decrease at forces comprised in the range of 8–20 nN is probably due to protein structural rearrangement; however the overall behaviour indicates that the PCSS protein is directly involved in the electron transport between the AFM tip and the gold substrate and supports the discontinuous characteristic of PCSS conduction on contact force.

In this work, *I–V* measurements on PCSS molecules were performed also by tethering the protein to the gold coated tip, which was approached to the Au(1 1 1) substrate. In this configuration, however, less reproducible *I–V* characteristics were obtained. The reasons for the difference between the two PCSS junction configurations are still under investigations. In contrast, analogous CAFM spectroscopic measurements previously performed on azurin, a copper protein similar to plastocyanin and chemically linked on gold via its native disulfide group, showed reproducible electrical results by either chemisorbing the protein on the underlying gold substrate [30] or on the AFM probe [28,29]. The resistance values estimated for PCSS protein junction were found to be in general agreement with those observed for azurin at low applied forces [28–30]. A major difference between azurin and PCSS, however, observed by us [30] and other groups [28,29] regards the fact that azurin presents a monotonic exponential trend of resistance on applied forces up to 25–30 nN. According to these results the transport mechanism seems to be dominated by a non-resonant electron tunnelling through the protein towards the electrode [28–30]. On the contrary, for PCSS the trend of resistance versus force presents two distinct slopes already at applied forces below 25 nN. Hence, the current transport mechanism in the PCSS biomolecular junction appears to be influenced by the mechanical and/or conductive characteristics of the protein, which acts as dielectric medium able to affect the charge transport across the gap.

A broader and attractive application of CAFM relies on the possibility to acquire topography and current images simultaneously. Besides *I–V* characterisation, we paid a particular attention to CAFM imaging of protein monolayers adsorbed on Au(1 1 1) surface. Such an approach, although up to now poorly exploited, looks very promising to understand the relationship between the single molecule morphology and its conductive properties.

In CAFM a metal coated AFM probe scans the surface in contact mode. In such modality the feedback mechanism measures and keeps constant the cantilever deflection (normal force) while a bias voltage is applied between the tip and the sample during scans. When performing contact mode AFM in air on soft sample, the imaging is complicated by various factors as additional long-range forces due to water adlayer and electrostatic charges, which strongly contribute to increase lateral forces during scan. Therefore, to avoid the damaging of soft protein sample, the images were taken under controlled nitrogen atmosphere and the force load was kept minimal by reducing the set-point value. The images were acquired mainly with Pt coated AFM tips, since gold coated tips gave a quite strong adhesion on the sample. In Fig. 6(a) CAFM image on PCSS monolayer is shown. Fig. 6(a) shows the topography image recorded at positive bias (similar topographic images were obtained between +0.8 and 2.5 V on various samples). In this image it is possible to recognize the globular shape structure of the assembled protein. Their observed vertical size is less than 1 nm, a value considerably reduced respect to that obtained in tapping mode AFM [15] and by crystallography [42]. Such a difference is very likely due to the pressure exercised by the tip during the scan in contact mode. Indeed, as mentioned before, when operating in this mode in ambient conditions, additional capillarity and electrostatic forces may pull the tip downwards, and this could lead to a significant mechanical compression of the soft biological objects with a subsequent reduced estimate of the molecular height. In addition, the monolayer is densely packed and this may complicate the measurement of the vertical size of a single molecule. Regarding current images, we generally do not detect any signal current for positive bias (0.8 up to +2.5 V) (Fig. 6(b)).

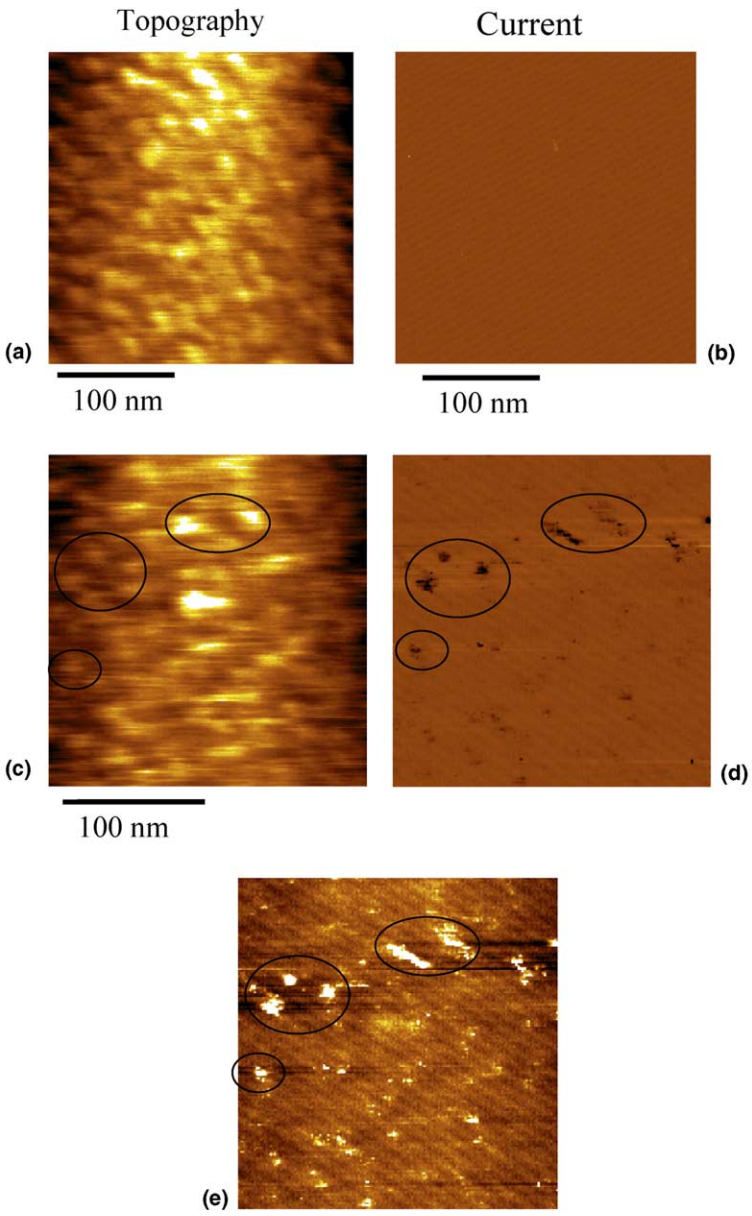

Fig. 6. Topography and current images of PCSS molecules chemisorbed on gold substrate taken under nitrogen atmosphere: (a) topography image at a bias of +0.8 V and force of 0.3 nN; (b) correspondent current image; (c) topography image recorded at a bias of −0.8 V; (d) correspondent current image. Image (e) is obtained by subtracting image (d) from (c). Color scale is 2 nm for the topographic images and for the current images is 0.1 nA. Scan speed 1.1 Hz and scan size 220 × 220 nm.

Subsequent scans of the same area are taken at negative bias (−0.8 up to 2.5 V). The topography image is slightly perturbed but still single molecules can be discerned (Fig. 6(c)). This effect on topography may be due to electrostatic forces, which are added to the mechanical load when a bias is applied between the tip and the substrate. However, we noticed that this effect seems to be

minimal as demonstrated by recording force–distance curves at positive and negative biases (data not shown). In the corresponding current images spots appear with intensities generally comprised between 10 and 100 pA (Fig. 6(d)). Conventionally, in current images negative biases gave a negative contrast, in other words the darker are the spots the more conductive is the object. A comparison between the topography and the current image shows that a good correlation between the current spots and the topography can be observed for some molecules. These molecules and the spots where this correlation seems to be more clear are evidenced with black circles (see Fig. 6(c) and (d)). We noticed, however, that other spots present in the current image can be hardly related to molecules of the topographic image. Even if this aspect deserves further investigations, we should remark that some variability in the current conduction among the PCSS molecules could occur even though they may show similar topological features.

The correlation between the molecules in the topography images and the current spots is clearly depicted in Fig. 6(e), obtained by subtracting current from the corresponding topography image. The molecules appearing in topography which match the holes of current images generate spots with a high brightness, thus indicating an exact correspondence between the molecule and the current spots. When the bias was reversed to positive values, the topography image was still rather clear but no current could be detected.

Similar images were obtained for different protein samples and various sample areas, although numerous scans of the same sample areas seem to affect the topography, probably due to the friction exerted by the tip during scans. Moreover, when initially scanning a sample area at negative bias, current spots corresponding to the molecules of topography were immediately detected, even if it was noticed that the topographic images quickly deteriorate upon scanning.

According to these CAFM images, the assembled PCSS molecules seem to allow current flowing only for negative bias. However, this was not observed in the I–V spectra (see Fig. 3), where a current flow was detected both for positive and negative biases. Such behaviour is still puzzling, if we consider that the main difference between the spectroscopic and the imaging mode is that I–V measurements were performed just by placing the tip in contact with the protein sample, without scanning the surface. It could be assumed that the PCSS molecules may undergo a variation of their conductive properties (charging effect) when the metal coated tip scans the protein surface at positive bias, blocking the current flow at this bias polarity. Actually, a similar behaviour was found for semiconductor nanocrystals inserted into a thin polymer film imaged by CAFM [26]. In this work, current was detected only over nanocrystals and an excellent and stable relationship between current and topography images was observed at negative bias, while at positive bias the current image vanished after only one scan. This phenomenon has been ascribed to a charging effect of nanocrystals upon scanning at certain bias, which may block the charge transport through the nanoparticles. Analogously, such effect may also be involved in the electron transport variations of the PCSS proteins, though no current image has been revealed at positive bias, perhaps because the current passing through the protein in these imaging conditions is below our detection limit (about 5 pA).

A further explanation for the discrepancy observed between spectroscopy and imaging mode could be also associated with the fact that forces, greater than those used for imaging, were applied in the spectroscopic mode.

Finally, comparing the CAFM images of the PCSS proteins immobilised on Au(1 1 1) with those generally obtained in STM, we can observe that no considerable variations in STM image contrast was found, for positive and negative biases. We suppose, that the protein mediates the tunnelling current similarly both in STM and CAFM, but the additional effect of the friction resulting from scanning of the tip may strongly influence the electron transport through the adsorbed protein for certain biases. However, the difference between STM and CAFM images deserves further investigations, because it may lead to a better understanding on the mechanism responsible of the contrast formation in STM imaging of biomolecules.

4. Conclusion

Among a few others, the present work reports on a CAFM study on molecular conduction of redox metalloproteins within a metallic nanojunction. In this study, a stable biomolecular junction was formed by bringing a gold coated AFM tip down to contact PCSS molecules chemically anchored on a Au(1 1 1) substrate. The characterisation of PCSS bioelectronic junction, within ± 1 V for increasing compressional forces, has shown that the conduction slightly changes under the force range of 3–9 nN, while above this value it suddenly increases. For applied forces of 3–9 nN, the resistance has been estimated to be of $2\text{–}3 \times 10^9\ \Omega$, and falls to lower values upon increasing compressional forces. This step in the PCSS conduction at a critical mechanical pressure and the comparison with the studies carried out on azurin suggest that the electron transport across a bioelectronic junction is likely a tunnelling process, which can be differently influenced by the protein mechanical characteristics and/or by abrupt changes of protein conductive properties upon compression.

Finally, operating in imaging mode, well resolved and reproducible topographic images of PCSS molecules were obtained by CAFM both for positive and negative bias. No signal current was detected when CAFM images were acquired at positive bias, whereas at negative biases current spots were revealed, showing also a correlation with the topography images. At present, a possible explanation for such behaviour may be found in a charging effect of the protein molecule as result of scans under certain biases. These results, however, point out for the first time that a spatially resolved current image directly related to the protein topography can be obtained. This represents an important step towards the elucidation of electron transport properties of single protein molecules.

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