Chapter 2

METALLOPROTEIN-BASED ELECTRONIC NANODEVICES

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Abstract: A key challenge of the current research in nanoelectronics is the realization of biomolecular devices. The use of electron-transfer proteins, such as the blue copper protein, azurin (Az), is particularly attractive because of its natural redox properties and self-assembly capability. This chapter discusses results of fabrication, characterization, and modeling of devices based on this redox protein. The prototype of biomolecular devices operate in the solid state and in air. The charge transfer process in protein devices can be engineered by using proteins with different redox centers (metal atoms) and by controlling their orientation in the solid state through different immobilization methods. A biomolecular electron rectifier has been demonstrated by interconnecting two gold nanoelectrodes with an Az monolayer immobilized on SiO₂. The device exhibits a clear rectifying behavior with discrete current steps in the positive wing of the current—voltage (I – V) curve, which is ascribed to resonant tunneling through the redox active center. On the basis of these results, an Az-based three-terminal device has been designed. The three-terminal device exhibits an ambipolar behavior as a function of the gate bias, thus opening the way to the implementation of a new generation of logic architectures. This peculiar characteristic allows the implementation of a fully integrated nanoscopic logic gate.

Key words: biomolecular devices, nanotechnology, three terminal electronic devices, proteins

1. INTRODUCTION

In the last decade there have been dramatic advances toward the realization of molecular scale devices and integrated computers at the molecular scale.¹ First pioneering experiments were performed demonstrating that individual molecules can serve as nanorectifiers² and switches,^{3,4} 1000 times smaller than those on conventional microchips. The assembly of tiny computer logic circuits built from such molecular scale devices has been demonstrated.⁵

Researchers are working to join biology and nanotechnology, fusing useful biomolecules to chemically synthesized nanoclusters in arrangements, which do everything from emitting light to storing tiny bits of magnetic data. The result is a merger that attempts to blend biology's ability to assemble complex structures with nanoscientists' capacity to build useful devices. One of the biggest drivers behind nanotechnology's enthusiasm for biological systems revolves around the organism's impressive ability to manufacture complex molecules such as DNA and proteins with atomic precision.

One of the fundamental goals of bioelectronics is the realization of nanoscale devices in which a few or a single biomolecule can be used to transfer and process an electronic signal. The biomolecules have particular functionality that can be exploited for the implementation of electronic devices. In order to design and realize such devices, several steps are required: (i) choice and characterization of the suitable biomolecular system, (ii) immobilization of the molecule onto an electronic substrate, (iii) interconnection to contacts, (iv) device fabrication processing, (v) read-out, recognition of molecular information, and (vi) processing of the information. The nanobiotechnology community has started a big effort along these lines to realize biomolecular devices for information technology.⁶ Among biomolecules, proteins have a fundamental role in biological processes. The combination of molecular biology (for engineering proteins with the desired, either functional and/or yield or self-assembling, properties/capabilities) and nanotechnology (for device fabrication), thus becomes the tool to realize a new class of nanoelectronic elements.⁷ Different nanotechnological strategies have been selected to implement the biomolecular devices, following a bottom-up or a top-down approach depending on the biomolecule and its functionality.

Thanks to their functional characteristics, metalloproteins appear to be good candidates for biomolecular nanoelectronics. Among them, blue copper proteins⁸ and, in particular Az, appear to be the best candidates of choice due to some specific structural properties and intrinsic functionality in biological environments. They can bind gold via a disulfide site present on the protein surface, and their natural electron transfer activity can be exploited for

the realization of molecular switches, whose conduction state can be controlled by tuning their redox state through an external voltage source (gate). In order to realize a real biomolecular device in the solid state operating in air, a comprehensive study of the redox, electronic, and electrical properties of the metalloproteins linked to an inorganic substrate under nonphysiological environments has been conducted.⁹ Besides all those devices based on proteins and enzymes, which belong to the family of biosensors, having typical size of hundreds of microns or more, a novel example of protein-based active electronic device has been demonstrated by the group of Rinaldi and coworkers.^{9,10} This chapter describes the implementation techniques and the basic principle of operations of such class of novel protein-based nanodevices.

2. DEVICE FABRICATION

2.1 Protein Monolayers

In the field of biomolecular electronics, the function of proteins adsorbed at solid interfaces is of fundamental importance. Moreover, since electronic devices fabrication aims at producing a device working in air, another critical treatment is the drying procedure that can influence the protein activity. Therefore, the optimization of the chemisorption process of a protein at a surface has to be carefully developed, and the protein functionality has to be checked after each step.

For the implementation of protein monolayers, surface functionalization by means of silanes, which enables the adsorption process, has been developed.

Azurin⁸ from *Pseudomonas aeruginosa* is a small (molecular mass 14.6 kDa, Fig. 1) and soluble metalloprotein involved in the respiratory phosphorylation of its hosting bacterium. The protein folds into an eight-stranded Greek key-barrel motif with only a helix present (Fig. 1).

Given to this secondary structure and also to the presence of the disulfide bond, it can be considered as a very stable protein. Structural and electronic studies have shown that the Az capability to function as a one-electron carrier in the biological environment is due to the equilibrium between the two stable oxidation states of the Cu ion, Cu⁺ (reduced) and Cu²⁺ (oxidized), and the structural stability of the active site. The redox active center of Az contains a copper ion liganded to 5 amino acid (aa) atoms in a peculiar ligand-field symmetry, which endows the protein with unusual spectroscopic and electrochemical properties, such as an intense electron absorption band at 628 nm, a small hyperfine splitting in the electron paramagnetic spectrum, and an

Figure 1. Schematic structure of Azurin representing eight β -barrel strands (yellow) and just one α -helix (pink).

unusually large equilibrium potential (+116 mV vs SCE) in comparison to the Cu (II/I) aqua couple (−89 mV vs SCE).

Commercial natural Az from *Pseudomonas aeruginosa* (Sigma) was used without further purification after having checked that the ratio OD_{628}/OD_{280} (OD = optical density measured at nm) was in accordance with the literature values (0.53–0.58).

Two different immobilization procedures were developed for Az on Si/SiO₂, exploiting different structural features of the protein and resulting in: (1) random orientation (three-step procedure) and/or (2) oriented immobilization (two-step procedure) in the protein monolayers. Following the three-step procedure (1), the immobilization of the proteins is achieved

through the formation of imido bonds between the exposed amino groups on the Az surface and the carbonyl heads exposed on the functionalized SiO₂ substrate. This procedure is based on a three-step chemical reaction performed first, by incubating the Si/SiO₂ substrates with gold electrodes in 3-aminopropyltriethoxysilane (3-APTS) [diluted to 6.6% (V/V) in CHCl₃ immediately prior to use] for 2 min and then by rinsing in CHCl₃, in order to remove 3-APTS molecules not linked to the surface. The second step consisted in exposing the sample already reacted with silanes that was exposed to glutaric dialdehyde (GD) (diluted in H₂O to a final concentration of 4×10^{-4} M) for 10 min and successively followed by a thorough washing in ultrapure H₂O. Finally, the precoated substrates were exposed to Az solution for 10 min and rinsed in NH₄Ac to get rid of physisorbed molecules. For this step a working solution of 10^{-4} M Az in 50 mM NH₄Ac (Sigma) buffer, pH 4.6, was prepared. The buffer was degassed with N₂ flow prior to use. Milli-Q grade water (resistivity 18.2 MS/m) was used throughout all the experiments. In the three-step supramolecular reaction, the immobilization involves the chemical bonding of the exposed amino groups. Since the Az molecule contains 13 surface-exposed amino groups (found in the Lys residues and in the N-terminus), random orientation of the proteins is obtained in the solid state by this immobilization method.

In contrast, a highly ordered phase is achieved following procedure (2), which exploits a different two-step immobilization protocol acting on the unique disulfide bond found in the protein between Cys3 and Cys26. Such a procedure not only provides a special orientation of the protein molecules, but also facilitates the chemisorption process by avoiding the GD exposure. In this case, the SiO₂ substrates were cleaved and washed with organic solvents and then incubated with H₂SO₄ and bidistilled deionized water for 1 h. After washing with abundant water, the substrates were incubated for 2 min with a 2% ethanol solution of mercaptopropyltrimethoxysilane (3-MPTS) and then washed in absolute ethanol. After this step, the sample was dried under nitrogen (N₂) stream. A drop of the protein solution (0.6 g/ml) was deposited onto the silane layer, and the substrate is incubated for 5 min at room temperature and continuously shaken in order to avoid stagnant conditions. Then it was rinsed for 5 min with the buffer and finally with deionized water. The samples were dried under N₂ stream for noncontact atomic force microscopy (NC-AFM) imaging. A typical AFM image of Az monolayer is reported in Fig. 2.

Protein immobilization took place via the reaction of the free thiol groups of 3-MPTS with the surface disulfide bridge of Az, giving rise to substrate/overlayer disulfide bonds. By using the two-step method for the immobilization, the orientation of the molecule is expected to be well defined, as confirmed by electrochemical (cyclic voltammetry) and scanning force microscopy

Figure 2. Non contact atomic force microscopy image of a self assembled azurin monolayer, realized by means of the two step immobilization procedure.

(SFM) measurements.¹¹ Samples covered just by the first molecular layers (namely 3-MPTS, 3-APTS, and 3-APTS+GD) were also produced for reference.

The last procedure is specific for all those proteins with S atom that can covalently bind the substrate. In the specific case of Az, it exploits the presence of the disulfide bond Cys-3–Cys-26, present on the protein external surface.

Synthetic wild-type Az and Az with zinc as a metal ion and without the metal (APO form) have been obtained using the recombinant DNA technique. The gene coding for the protein of interest has been introduced in a plasmid employed to transform *E. coli* cells. The two-step immobilization procedure was used to produce monolayers with synthetic proteins having the same orientation with respect to the substrate surface.

Intrinsic fluorescence experiments performed on the immobilized proteins showed no protein denaturation after the chemisorption and draying procedures.

Other blue copper metalloproteins, for example the Plastocyanin, can be used to implement the electronic devices described in the next section. In this case, however, mutants (e.g., PCSS and PCSH) have to be produced to achieve a stable and oriented immobilization of plastocyanin onto gold substrate. In the first mutant (PCSS), a disulphide bridge can be inserted within the protein. The residues Ile-21 and Glu-25 can be mutated to Cys by structurally conservative mutagenesis. In the second mutant (PCSH), usually a

residue tail (threonine, cysteine, and glycine) at the C-terminal position is inserted. The single exposed thiol group should provide a further possibility for plastocyanin immobilization.

2.2 Implementation of the Two- and Three-Terminal Protein-Based Nanodevices

Nanoelectrodes were fabricated by electron beam lithography and lift-off on a Si/SiO₂ substrate. The geometry of the device comprises two planar Au/Cr contacts separated by a gap in the range 50–100 nm. An Az monolayer was then immobilized by means of surface functionalization and chemisorption in the gap between the electrodes of the planar circuit. Both commercial natural Az and synthetic purified Az were used to implement the devices, following the methods described in Section 2.1. In the case of three-terminal devices the contact separation defined the gate width and a silver electrode was added on the back of the Si substrate as the gate to control the source–drain conduction. A schematic diagram of the device geometry and implementation is reported in Fig. 3. All the fabricated devices were tested at room temperature and ambient pressure. Prior to protein deposition, a negative control on the empty devices was performed to check the effective insulation between the source (S), drain (D), and gate (G) terminals along the different current pathways. All these tests revealed drain–source (I_{DS}) and source–gate (I_{SG}) currents lower than 20 pA and typical open circuit resistance larger than 100 G.

3. NANO-ELECTRONICS WITH PROTEIN

3.1 Two-Terminal Protein Devices

The achievement of oriented immobilization is extremely crucial for electronic applications in which the charge transport benefits the long-range order of the transporting material. Orientation can in principle affect conduction in two ways: (i) it allows increased protein coverage, thus favoring electron transfer among neighboring molecules and (ii) it enhances, for a given coverage, the intermolecular electron transfer (due to the fact that the positions of the Cu sites are approximately coplanar, thus offering more favorable pathways for conduction).

In addition, the molecular electrostatic potential (MEP) of the protein in solution is known to be important in protein interaction properties at

Figure 3. A schematic diagram of the device geometry and implementation procedure.

medium- and long-range distances in solution and plays a fundamental role in the recognition processes of biomolecules. It is therefore expected that electrostatics will influence both the deposition kinetics of the proteins in solution and their self-assembly. The charge distribution on the Az surface gives origin to a strong intrinsic dipole (150 Debye), suggesting that two-terminal circuits, interconnecting solid state films of immobilized Az molecules, should have an intrinsic polarity which depends on the value and the orientation of the molecular dipoles. Both oriented and nonoriented Az films should exhibit a macroscopic polarization. However, the total dipole is enhanced by depositing oriented self-assembled films with parallel dipoles. If the dipole distribution is preserved in the device after drying, it is expected to induce a macroscopic electric field favoring conduction.

The comparison between the I - V curves measured in the oriented and randomly oriented Az layers is reported in Fig. 4. The continuous and dotted lines represent the downward and upward sweeps, respectively. Three important effects can be deduced from this comparison. Both curves are asymmetric, with a strong rectifying behavior. The value of the current measured under

Figure 4. Current voltage curves of two terminal devices interconnecting proteins films with the same orientation and with random orientation. The gap between the gold electrodes was 70 nm.

forward bias between the nanoelectrodes suggests that the electron transfer mechanisms in the protein, between the Cu site and the edges, are quite effective in determining the conduction processes. The difference between the positive and negative wings of the current curve of both samples may be attributed to the presence of the dipole in the Az molecules, which sets the polarization of the planar devices.⁹

The current flowing through the device with the oriented layer is about ten times larger than that flowing through the device with the nonoriented layer. In fact, the regular orientation of the Az molecules, determined by the unique sticking site on the protein (the Cys3–Cys26 bridge) exploited for the layer formation, drives the self-assembly on the substrate, resulting in a distribution of parallel dipoles. This induces a macroscopic electric field favoring conduction. In the sample with random orientation of the proteins, although the electrostatic long-range interaction may favor some dipole alignment on the surface, a complete parallel orientation of the molecular dipoles cannot be achieved as a consequence of the many possible sticking sites on the protein surface used in the immobilization procedure.

In the oriented protein layer under forward bias, the current is step-like with a smooth exponential rise in the region between 1.9 and 2.3 V and a steep rise around 4.9 V (Fig.4). The step centered around 2.1 V corresponds to the energy required by the protein molecule to reduce the Cu atom by means of the electronic transition involving the S(Cys)Cu charge transfer. The step around 4.9 V corresponds to the energy required to perform resonant tunneling via the redox levels of Az. Such a process occurs through a coherent two-step tunneling in which the electrons go from the negative to the positive electrode via the molecular redox level. This is consistent with the electrochemical STM measurements and *in situ* cyclic voltammetry curves, showing a maximum at -4.96 eV (measured with respect to the vacuum level). This step is also observable in the I - V curve of the sample with randomly oriented proteins, though less pronounced and with a small hysteresis.

In order to further elucidate the role of the metal ion and the effects of purity of the protein sample on the device performances, three different types of high purity of engineered Azs were used for implementing oriented layer devices as shown in Fig. 5.¹⁰

- (i) A highly purified synthetic Az, referred to as “recombinant Az,” in sample (A) of Fig.5. These proteins are identical to the natural proteins (Fig. 13a), though with a higher degree of purity

Figure 5. Current voltage curves of (A) recombinant Az (black), (B) Zn-Az (red), and (C) APO-Az (blue). The gap between the gold electrodes was 70nm.

- (ii) A modified synthetic Az with the Cu atom replaced by a Zn atom (Zn-Az), in sample (B) of Fig. 5
- (iii) A modified Az without metal atom (called APO-Az), in sample (C) of Fig. 5.

Sample (A) shows the same rectification and step-like character observed in the devices fabricated with oriented commercial (nonpurified) Az layers, reported in Fig. 4. However, the current flowing through device (A) with recombinant proteins is two orders of magnitude larger. This suggests that highly purified proteins are best-suited for molecular electronic applications. Reasonably, a high degree of purification favors a closer packing of the proteins onto the device substrate and avoids passivation phenomena of the substrate. Sample (B) shows a less-pronounced rectifying behavior, without the redox-induced steps characteristic of the Cu atom, and a current intensity about one order of magnitude lower than sample A. This is ascribed to the electronic properties of Zn that are different from those of Cu. Zn has in fact only one stable redox state, Zn^{2+} , which is redox inert, thus preventing Zn-Az molecules from being efficient redox species-mediated electron carriers. The importance of a metal atom in the protein structure is demonstrated by a device (C) that was built using APO-proteins obtained from recombinant Az. In the absence of metal redox center in the protein structure, the biodevice does not show any measurable conduction. This indicates that the metal in the protein is responsible for the electron transfer as reflected in the transport characteristics of Az biomolecular diodes.

3.2 Three-Terminal Protein Devices

The natural electron transfer activity of the Az can be exploited for the realization of molecular switches, whose conduction state can be controlled by tuning their redox state through an external voltage source (gate).

Fig. 6a shows the I - V characteristics of a protein device for $V_{\text{DS}} > 0$ as a function of V_{G} in the range between 0 and 4 V (first active region). As a general feature, the I_{DS} remains low (< 20 pA) up to a V_{DS} bias voltage of about 2.3 V and then starts to increase reaching 100 pA intensity at 6 V. A clear modulation effect is visible as a function of V_{G} . The dependence of the I_{DS} current as a function of the V_{G} intensity is reported in Fig. 7a for a fixed value of V_{DS} ($V_{\text{DS}} = 4.5$ V). The current increases up to a maximum value of 200 pA at $V_{\text{G}} = 1.1$ V, then it decreases to the 100 pA level at $V_{\text{G}} = 2$ V, and finally falls down to the open circuit value at the higher applied gate voltages.

Similar set of measurements were performed under positive and negative V_{DS} bias with positive (Figs. 6a and 6c, respectively) and negative (Figs. 6b

Figure 6. I - V characteristics of the protein transistor in the four different active regions : a) $V_{DS} > 0, V_G > 0$; b) $V_{DS} > 0, V_G < 0$; c) $V_{DS} < 0, V_G > 0$; d) $V_{DS} < 0, V_G < 0$.

and 6d, respectively) gate bias. Four different active regions resulted: $V_{DS} > 0, V_G > 0$ (16a); $V_{DS} > 0, V_G < 0$ (16b); $V_{DS} < 0, V_G > 0$ (6c); $V_{DS} < 0, V_G < 0$ (6d). In Fig. 6b, by decreasing the gate potential the current increases up to 160 pA at $V_{DS} = 6$ V. The corresponding dependence of the I_{DS} current as a function of V_G is reported in Fig. 7b for a V_{DS} value of 5 V. In this case, we did not observe a peaked dependence, like in the case of Fig. 7a, but a smooth increase and a saturation at 11.5 pA in the range between -2.5 and -4 V. In the last two situations, i.e., $V_{DS} < 0, V_G > 0$ (Fig. 7c) and $V_{DS} < 0, V_G < 0$ (Fig. 7d), we observed a decrease and an increase (in absolute values) of the I_{DS} negative currents in the two cases, respectively, with increasing the gate voltage. As a general trend, the current increases linearly for V_{DS} voltages higher than 3 V and then saturates in the range between 4.5 and 6 V. In this voltage range the operation of the protein transistor resembles that of an inorganic MOS-FET in the saturation region, with constant I_{DS} current value for each curve. In the case of the Az monolayer deposited in the channel of our FET, both superexchange charge transfer inside the single Az units and sequential hopping between adjacent proteins are involved in the

Figure 7. I_{DS} current intensities as a function of V_G at fixed V_{DS} for the four active regions reported in figure 6: a) $V_{DS} = 4.5$ V; b) $V_{DS} = -5$ V; c) $V_{DS} = 5$ V; d) $V_{DS} = -5$ V.

conduction process. The existence of a strong internal dipole due to the surface charge distribution on the protein can affect the planar conduction in the gate channel depending on whether the dipoles of the proteins in the layer are aligned or not along the source-drain axis. In the case of the characteristics of the device fabricated with commercial Az, a similar effect is observed for positive gate voltages values (Figs. 6a and c). In the case of the I - V measurements in Fig. 6c, current intensities are higher than the corresponding values in Fig. 6a, at least by a factor 10. For negative gate voltages, the measured current intensities in the 6b and d diagrams are similar.

The change of the gate voltage acts in different ways depending on the cross correlation of the signs of the bias and the internal dipole. As a general trend, the I_{DS} current decreases with the increase in the absolute value of the applied V_G . However, a different trend is usually observed in the case of $V_{DS} > 0$, $V_G > 0$ (Fig. 6a) in which a peaked dependence is observed in most of the realized devices. The maximum of the current is always found around $V_G = 1$ V. This is a peculiar behavior of the protein device that is not found in any inorganic counterpart and could be of interest for the realization of electronic devices with novel functionalities. In the case of three-terminal devices realized with synthetic and purified Az, the current increases by a factor 10 to 100 and the V_G resonance becomes sharper. The physical origin of this trend can be found in the effect of the gate voltage on the molecular levels of the protein, and in turn on the conduction pathways inside the molecule. The protein is chemisorbed onto the SiO_2 surface by the formation of a thiolate onto the silanized surface. In this way the protein sits on the surface with the axis connecting the thiolate with the Cu ion almost perpendicular to the surface. The vertical field that propagates along this direction (gate field) modifies the electronic properties of the molecule and the oxidation state of the Cu ion, since it is strongly coupled to the thiolate by the electron transfer pathway inside the protein.¹² This resonance effect has been already observed in electrochemical STM experiments¹³ and standard STM experiments performed on Az molecules chemisorbed on Au(111) substrates at room temperature and ambient pressure. It has been demonstrated that the STM detection of the protein at the surface strongly depends on the applied bias between the tip and the gold substrate.

4. CONCLUSION

We have demonstrated the first implementation of protein-based electronic nanoscale devices working in air and at room temperature by using Az metalloproteins. Both rectifying (diode-like) and amplifying (transistor-

like) devices have been implemented. We have demonstrated the first protein transistor working in air and at room temperature by using Az metalloproteins. Though this field is rather young and very open, the results obtained so far are promising and deserve further studies to determine the actual potentiality of these biodevices and elucidate a number of important issues such as the reproducibility and ageing of the nanodevices.

ACKNOWLEDGEMENTS

We are thankful for the invaluable support and exciting collaboration by various colleagues. We would like to thank Elisa Molinari, Rosa Di Felice, Francesca De Rienzo, Paolo Facci at S³ – INFN research center in Modena (Italy), Gerard Canters and Martin Verbeet at Leiden University (NL).

Financial support by NNL-INFN and by EC through SAMBA project is gratefully acknowledged.

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