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Self-assembling of proteins and enzymes at nanoscale for biodevice applications

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Abstract: Different nanotechnological strategies have been selected to implement biomolecular devices following a bottom-up or top-down approach depending on the biomolecule and on its functionality. Biomolecules have particular functionality and self-assembling capabilities that can be exploited for the implementation of both bioelectronic devices and multipurpose engineered biosurfaces. Surface preparation with supramolecular methods and microcontact printing have been developed and optimised to realise suitable functionalised surfaces. These surfaces can be used to link metalloproteins and enzymes for the implementation of nanobioelectronic devices and planar biosensors or to bind cells in order to promote their growth along predefined tracks and grooves. Some possible applications of these biosurfaces are shown and discussed. Results are presented for the realisation of a biomolecular nanodevice working in air based on the metalloprotein azurin immobilised in the solid state, the formation and characterisation of functional glutamate Dehydrogenase monolayers for nanobiosensing applications, the results of soft lithography processes on azurin for biosensor implementation, and the development of physiological self-assembled patterns of laminin-1 for cell culture applications and hybrid devices.

1 Introduction

In recent years, much attention has been paid to developing and investigating the use of biomolecules as electron-conductive materials for bioelectronics or for the construction of amperometric devices. Proteins and enzymes are extremely interesting for nanobiotechnology and nanoelectronics due to their capability of self-assembling, which opens the attractive possibility of nanoscale surface patterning, and because their great potential in controlling highly specific reactions can be optimised towards different technological targets. The physiological function of natural electron-transfer proteins is a key phenomenon since it can be exploited in many reactions of biotechnological interest. For all these reasons, the formation and the study of protein film assemblies on solid substrates are fundamental for a wide range of bioelectronic applications, such as protein chips, nanobiosensors, new hybrid electronic devices, etc.

Protein immobilisation is, thus, an important aspect in the construction of nanodevices, as their functionality strongly depends on the quality of the biomolecular film. However, the interaction between proteins and solid substrates may lead to alterations of the native structure, with consequent loss of functionality. Self-chemisorption is an attractive approach since the specific reactivity of biomolecules with other molecules and/or with the exposed chemical surface groups may be controlled to realise complex supramolecular architectures.

The development of protein-based devices requires, therefore, detailed understanding of the immobilisation processes and eventual conformational changes of the biomolecule. The investigation on the intrinsic stability of proteins provides a better understanding of the mechanisms affecting shelf life and operational stability upon immobilisation on to a solid surface. Structure-sensitive experiments capable of monitoring protein structure at interfaces are thus crucial for the technological progress of the field.

In this paper, we present and discuss some possible applications of these biosurfaces in the field of nanobioelectronics and nanobiosensors. We also show detailed characterisations of the protein monolayers developed for such applications, demonstrating, by means of a variety of techniques, that the biomolecules retain the native-like conformation upon immobilisation in the solid state. In particular, we present the results of the implementation of a solid-state protein transistor working in air, based on the physiological electron transfer function of the metalloprotein azurin. We then analyse the possibility of realising high-quality glutamate dehydrogenase monolayers on silicon for nanobiosensing applications, and report the results of soft lithography processes on azurin for biosensor implementation. Finally, we show the interesting perspectives related to physiological self-assembled patterns of laminin-1 for cell culture applications and hybrid devices.

2 Bioelectronic applications

2.1 Protein monolayers

Recently, different molecular electronic devices have been fabricated [1–4]. These hybrid devices exploit individual molecules to perform electronic functions now performed by semiconductor devices. In this perspective, the use of electron-transfer proteins appears to be particularly attractive due to their natural charge transfer activity in biological systems. We developed a protein immobilisation method on

both conductive and insulating surfaces aiming at achieving reproducible monolayers and avoiding the perturbation of the active site. The blue copper protein *Pseudomonas aeruginosa* azurin (14.6 kDa) is one of the best characterised redox metalloproteins [5]. It mediates electron transfer in the denitrifying bacteria. In this work, we exploit the surface disulphide bond of the wild-type azurins to chemisorb them on to functionalised SiO₂ substrates.

The immobilisation procedure is a particularly demanding aspect; it should assure an appropriate and permanent localisation of biomolecules on a support and it must preserve its necessary function [6]. The technique for protein immobilisation is based upon the self-assembly of 3-mercaptopropyltrimethoxysilane (3-MPTS) on SiO₂ surfaces. A very dilute solution (2%) of 3-MPTS has been used to obtain ordered monolayers and to prevent multilayer formation. Sulphidic groups are absorbed from high-purity ethanol solution and at the solid-liquid interface a strong chemisorption of the head group (Si) gives rise to highly ordered architectures.

Scanning probe microscopy allows the study of structural and functional properties of adsorbed proteins with molecular resolution. Tapping mode atomic force microscopy (TMAFM) is suitable to image biomolecules because the tip is periodically in contact with the surface and the lateral force applied to the soft materials is considerably reduced. Figure 1 displays a TMAFM image of an azurin monolayer self-assembled on functionalised SiO₂ with a scan size of 0.88 $\mu\text{m} \times 0.88 \mu\text{m}$. The recognisable granular structures are adsorbed proteins and the surface is totally covered by proteins. This is evidence for azurin adsorption since it reveals features uniformly distributed across the surface, which were not present on silanised substrates. The presence of these topographic features was reflected in the roughness. The protein adsorption, in fact, is responsible for an RMS roughness increasing from 0.3 nm to 3.17 nm. The width of these structures is overestimated due to the geometrical effect of the AFM tip.

The assessment of the folding properties of the azurin monolayers has been performed by intrinsic fluorescence spectroscopy. This technique provides very accurate analyses of the integrity of the protein globular fold, as the fluorescence parameters of the aromatic residues are highly sensitive to the microenvironment of fluorophores in protein structures [7]. In the following, we demonstrate that the immobilised protein does not undergo denaturation.

Azurin from *Pseudomonas aeruginosa* has only one tryptophan residue (Trp48) which is responsible for protein fluorescence [8, 9]. Due to the hydrophobic microenvironment surrounding Trp48 [8, 10], ultraviolet excitation (280–300 nm) of azurin results in a structured photoluminescence with a maximum around 308 nm [9]. Despite the fact that the holo- and apo-azurins exhibit identical fluorescence spectra, the emission of the holo-protein is strongly quenched by the presence of the copper. Though the exact mechanism underlying the quenching process is still a matter of investigation [11–13], it is thought that Trp48 plays an important role in the long-range electron transfer processes [8]. Therefore, we utilised the apo-form of azurin to optically investigate the protein monolayers on SiO₂.

As reported in Fig. 2, the emission spectrum of the chemisorbed apo-azurin exhibits a comparable lineshape with respect to the free protein in buffer. A slight red shift (~ 2 –3 nm) is the only minor effect detectable in the immobilised apo-protein, indicating that the covalent binding to the functionalised SiO₂ surface does not interfere

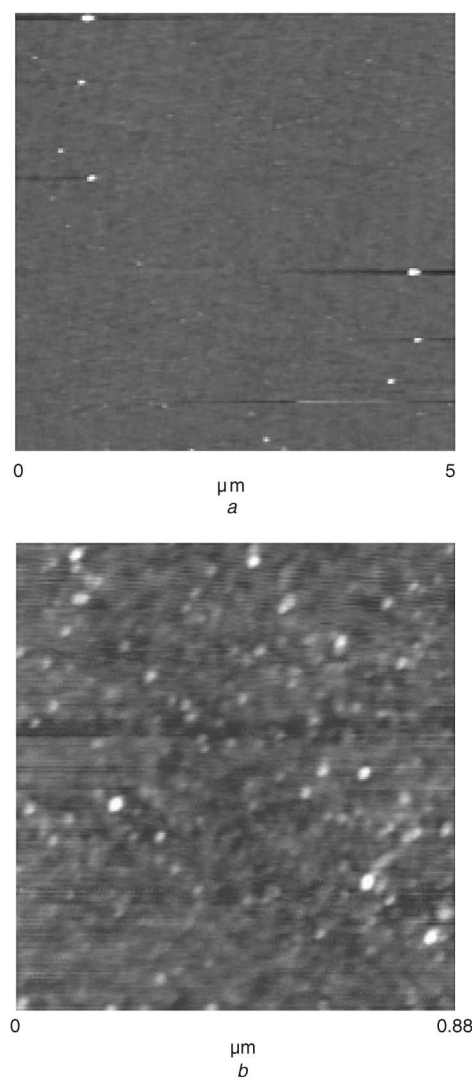


Fig. 1 Tapping mode atomic force microscopy (TMAFM) topography images

a Uncovered silanised substrate
b Azurin immobilised on self-assembled silane film

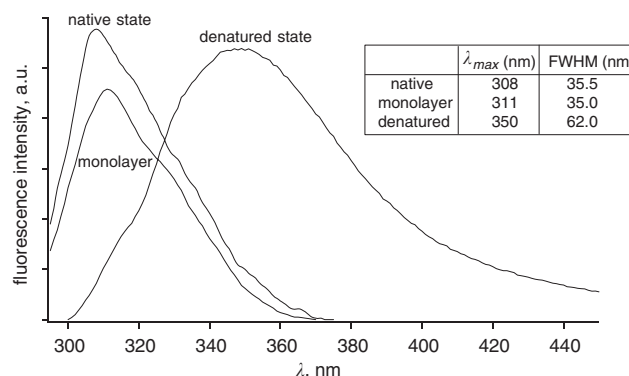


Fig. 2 Fluorescence emission of chemisorbed apo-azurin and comparison with the spectra of apo-azurin in solution, in the native and fully denatured states (the fluorescence intensities are not in scale)

Inset: optical parameters of the three spectra

with the fold pattern of the native protein. This evidence is additionally supported by comparing the photoluminescence spectrum of the apo-protein monolayer with the broadband, red-shifted emission of a denatured sample of azurin, in which the unfolding process clearly results in a

large solvent exposure of hydrophobic residues that were shielded in the core of the protein in its native state (see also the inset of Fig. 2). According to the photophysics of aromatic amino acids, the experimental evidence reveals that tryptophan residues in native and immobilised apo-proteins are found in similar locations. The small spectral shift may suggest the possibility of a very weak internal rearrangement due to the interaction of the ligand binding, without affecting the proper fold pattern. 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 physicochemical conditions of the chromophore micro-environment.

Preparation of the molecular devices begins with the fabrication of two Cr/Au arrow-shaped electrodes to interconnect proteins, by means of electron-beam lithography and the lift-off process. Between the different alternatives, planar nanojunctions permit transport experiments at the surface of a solid, allowing the investigation of conduction in molecular monolayers immobilised on functionalised substrates by self-assembly. Moreover, a field effect device can be easily fabricated by simply introducing gate electrodes on the back of the substrates. Nanojunctions were fabricated on the oxide surface of thermally oxidised silicon wafers (the thickness of the gate oxide is 100 nm). A silver back electrode, deposited on the degenerated doped silicon substrate, was chosen as the gate electrode. Typical source-drain distances were in the range 50–100 nm. The success of the whole technological process was checked by inspecting the fabricated nanodevices by plan-view scanning electron microscopy (SEM). All nanojunctions were then tested by current-voltage measurements and open-circuit resistances as high as 100–200 G Ω were obtained when the technological process succeeded. Typical process yield, i.e. the percentage of good nanojunctions useful for transport experiments and device implementation, is around 90%.

In the protein transistor [14], the azurin disulphide bridge S-S was broken to form covalent bonds on SiO₂ substrates, as described above. The electronic coupling between the disulphide bridge and the Cu redox site by means of an electron tunnelling pathway enabled us to fabricate protein FETs. Figure 3 shows a 3-D plot of the drain-source current I_{ds} as a function of the drain-source voltage V_{ds} and

the gate voltage V_G . The current in the protein FET is low and then rises up to hundreds of pA. The transfer characteristic (see the projection in Fig. 3) exhibits a pronounced resonance with a Gaussian-like shape centred at $V_G = 1.25$ V. This unusual behaviour, if compared to standard FETs, originates from the unique properties of the redox site. Azurin exists in two stable configurations, Cu(I) and Cu(II), and its capability to mediate the electron transfer depends on the equilibrium between these two oxidation states by the reversible redox reaction $\text{Cu}^{2+} + e^- \rightleftharpoons \text{Cu}^+$ which continuously reconverts the copper oxidised state Cu(II) into the reduced state Cu(I). Electron hopping from one reduced (Cu(I)) molecule to an adjacent oxidised (Cu(II)) molecule mediates the transport of electrons through systems containing redox sites [15]. Therefore, two adjacent Az molecules in the Cu(I) and Cu(II) redox states are required to have conduction between two planar electrodes. This explains the existence of a resonance, since V_G modifies the oxidation state of the redox site and the current as a function of V_G is maximum when the populations of protein in the Cu(II) and Cu(I) state become equals, otherwise the current is lower. From an electronic viewpoint, the device switches from an n-MOSFET behaviour before resonance to a p-MOSFET after resonance. This is a key result because it allows us to exploit the advantages of a complementary logic, fabricating both p-type and n-type devices on the same chip.

2.2 Glutamate dehydrogenase monolayers for electrochemical planar biosensors

In this Section we show a novel experimental approach to form high-quality glutamate dehydrogenase (GDH) monolayers on silicon for nanobioelectronics applications. The reported characterisations on immobilised GDH revealed that no significant conformational changes occurred in the enzyme upon immobilisation, indicating that such a method may be extremely attractive for the development of alternative and stable enzyme-based nanosensors. The implementation of a glutamate biosensor with a spatial resolution up to few nanometers would permit continuous monitoring of glutamate release from individual cells, subcellular structures or into the extracellular space of the central nervous system, resulting in an effective detection technique for the mapping of its concentration.

The enzyme glutamate dehydrogenase (GDH) reversibly catalyses the oxidative deamination of glutamate to α -ketoglutarate and ammonia, using NAD⁺ or NADH as coenzymes [16]. Glutamate favours the transfer of amino groups among different amino acids, assuming the role of key intermediary in amino acid synthesis and degradation. Moreover, as glutamate acts as an excitatory neurotransmitter within the central nervous system, disruptions of its metabolism are related to other clinical disorders, including Alzheimer's disease and schizophrenia.

In these mental diseases, the brain shows changes in the levels of glutamate and in the function and expression of its transporters and receptors. Since the levels of glutamate are largely determined by the rate of its metabolism, the changes of its concentrations may be associated with dysfunctions of appropriate enzymes. However, monitoring of glutamate release at the synaptic levels is very difficult owing to its extremely low concentration and to the small size of the synapses. As a consequence, glutamate monitoring requires highly sensitive detectors in the submicron range. Techniques such as HPLC analysis [17] cannot provide both spatial and temporal resolution. Therefore, the characterisation of the spatiotemporal nature of its release would be of particular interest.

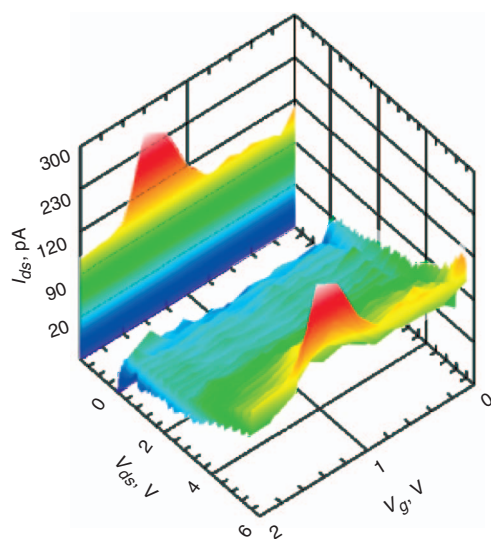


Fig. 3 In the protein transistor, current shows modulation against gate voltage, and an onset with respect to drain-source voltage

In the perspective of realising a solid-state nanobiosensor, utilising high-quality monolayers of GDH as the sensitive medium, we have carefully analysed the enzyme properties and functionality both in the native state and when deposited on functionalised Si/SiO₂ substrates. One important issue regarding the applicability of the biosensor is the activity of immobilised GDH on the sensor surface, which can be affected by several experimental parameters. The enzyme monolayers were characterised by atomic force microscopy (AFM), enzymatic assay and fluorescence spectroscopy.

Surface functionalisation with 3-aminopropyltriethoxysilane was performed by means of a three-step procedure [18]. Enzyme immobilisation was achieved through Schiff base formation incubating the activated chips in a solution of glutamate dehydrogenase (2 mg ml⁻¹ in 50 mM sodium phosphate buffer, pH 7.0) at 19°C. After 12 h the sample was rinsed with 4.0 M urea in 50 mM sodium phosphate (pH 7.0) in order to get rid of physically adsorbed molecules. After these treatments all samples were stored at 4°C.

Morphological information gives a physical picture of how the GDH molecules are assembled on a biosensor surface. The mean roughness value (RMS) of images can also be analysed and related to the properties of surfaces. Figure 4a shows the AFM image of the silicon surface after the silanisation of silicon substrate. The surface is quite homogeneous (with a mean roughness of 0.4 nm) and occasional peaks of 1 nm average height and 40 nm average diameter due to the formation of silane aggregates. Figure 4b shows the silicon surface after the glutaraldehyde treatment. The surface morphological changes and the increase of the mean roughness value up to 0.7 nm indicates the occurrence of the adsorption of glutaraldehyde molecules. Figure 4c shows the AFM image after the glutamate dehydrogenase immobilisation on the surface derivatised by the three-step procedure. The surface morphology after protein immobilisation is quite different from that without GDH, the mean roughness value being increased up to 1.6 nm. Globular structures of ~5 nm in height and ~70 nm in width are clearly observed. From X-ray analyses, the 5 nm dimension corresponds to the size of a single glutamate dehydrogenase molecule. Thus, these structures may be assigned to monomolecular clusters of protein.

The activity of immobilised GDH was directly measured by spectrophotometry, following the decrease in absorbance of NADH (at 340 nm) using the reverse reaction catalysed by GDH [16]. The dependence of absorbance on reaction time revealed a good degree of activity, without any observable saturation even after 2 h operation. The reaction rate for the immobilised enzyme, $dA/dt = (-1.67 \pm 0.03) \times 10^{-3}$, is appreciably larger than that characteristic of the spontaneous oxidation of NADH, $dA/dT = (-0.40 \pm 0.02) \times 10^{-3}$ (Fig. 5). This result confirms that our immobilisation method can be successfully exploited for the realisation of a GDH-based nanobiosensor.

In a second step, we utilised the potentiality of fluorescence spectroscopy for probing the conformational and structural states that GDH molecules undergo during their transfer from solution to immobilisation on solid films. GDH contains all three aromatic aminoacids [19], but its fluorescence is dominated by the contribution of the tryptophan residues [20, 21]. GDH is readily amenable to intrinsic fluorescence studies, since it contains three Trp per subunit. This circumstance, however, does not facilitate the interpretation of photoluminescence data. The fluorescence

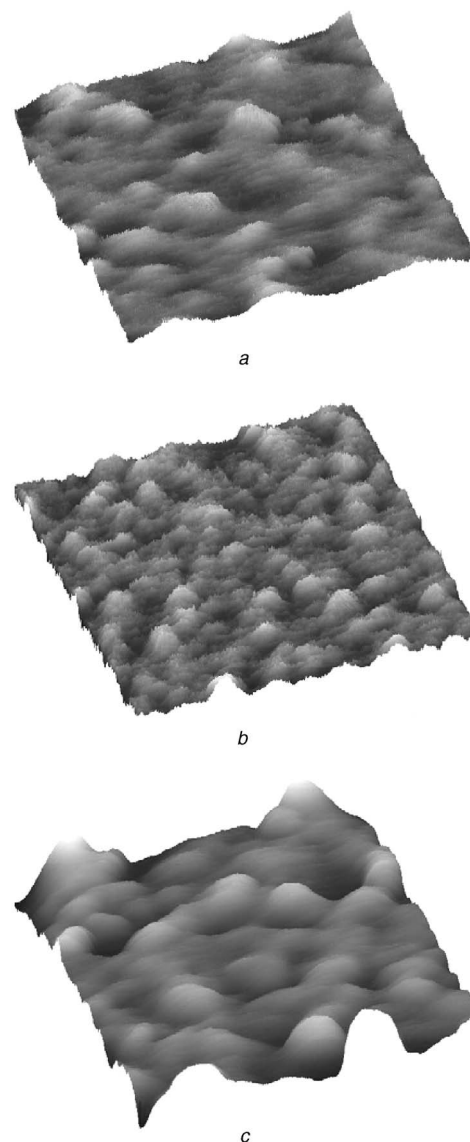


Fig. 4 Three-dimensional AFM images of the silicon surface of the three-step GDH immobilisation procedure

a First step 500 × 500 nm, Δz = 10 nm, scanning frequency 0.7951 Hz
b Second step 500 × 500 nm, Δz = 10 nm, scanning frequency 0.7951 Hz
c Third step 500 × 500 nm, Δz = 0.20 nm, scanning frequency 1.007 Hz

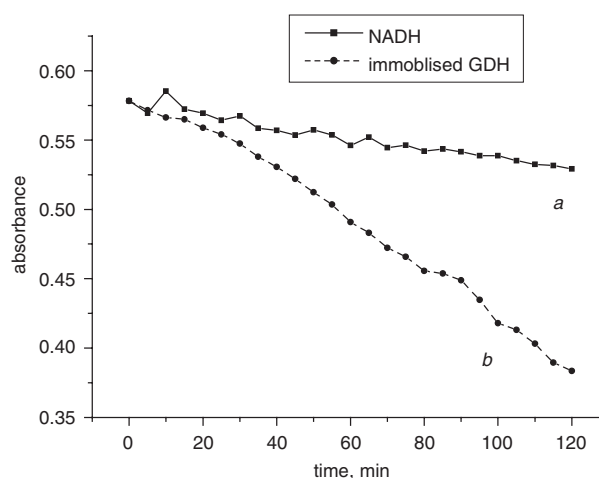


Fig. 5 Comparison of reaction rates obtained by the decrease of the absorbance of NADH at 340 nm

a NADH oxidation
b Immobilised GDH

emission spectrum of native GDH in a phosphate buffer at pH 7.2, upon excitation at 292 nm, exhibits a broad band with a maximum emission (λ_{max}) at 340.5 nm (Fig. 6). Such a λ_{max} value shows that the aromatic residues responsible for the fluorescence emission of GDH are located in a rather polar environment. The microenvironment of every residue is characterised by a particular set of physicochemical conditions that influence the fluorescence of the chromophore. As a consequence, the protein fluorescence results from the convolution of fluorescent contributions from individual tryptophan residues, which may vary over a rather wide range [22]. Figure 6 illustrates the deconvolution procedure; the broad band emission spectra of GDH is deconvolved by means of three Gaussians, representing the contributions of the individual emitting species. According to the photophysical characteristics of Trp, the three different classes are identified as the most inner, inner and external bands. This kind of analysis may lead to a deeper comprehension of the conformational changes than the basic study of the integrated behavior alone [23].

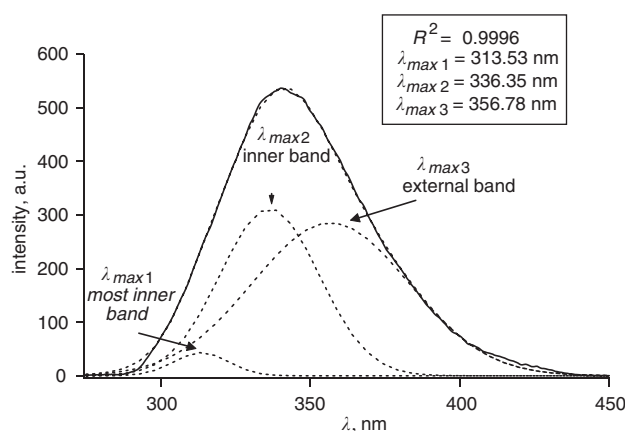


Fig. 6 Native GDH fluorescence and deconvolution analysis

The binding of small molecules to proteins may not only alter the structure of proteins, but may also modulate both its functions and its properties. With respect to GDH fluorescence in the buffer, the spectrum of the enzyme immobilised in the film is not shifted (Fig. 7a), accounting for the fact that immobilisation binding does not interfere with the fold pattern of the native enzyme. According to this investigation, it seems that tryptophan residues in native and immobilised proteins are found in identical locations. In addition, the deconvolution analysis (Fig. 7b) confirms this experimental evidence; the three individual emitting bands maintain identical characteristics, with respect to their wavelength of maximum emission (λ_{max}). This indicates that the single contributions are equally influenced by the microenvironment. Furthermore, as reported in Table 1, the individual contributions to the integrated enzyme fluorescence are also very similar.

In conclusion, intrinsic fluorescence spectroscopy reveals that no variations in the folding properties are present between the GDH native state and the film environment.

3 Soft lithography processes on biomolecules

3.1 Microcontact printing of azurin

The adsorption of biomolecules on solid substrates in controlled patterns of microscopic dimensions is a fundamental requirement for fabricating miniaturised biosensors. Here, we apply an easy soft lithography method, namely

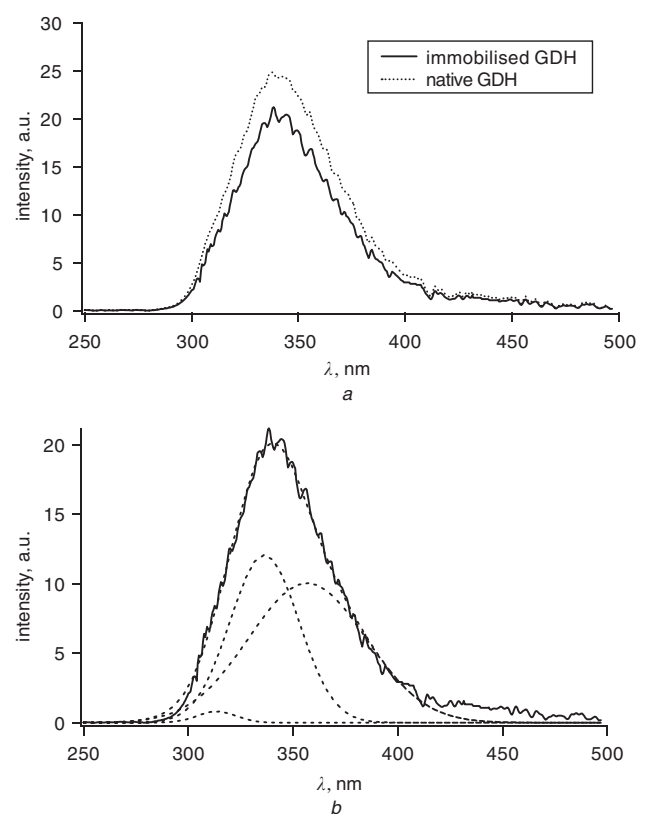


Fig. 7 Photoluminescence of GDH immobilised on Si/SiO₂ substrates

a Comparison with the native GDH spectrum (the fluorescence intensities are not in scale)

b Deconvolution analysis

Table 1: Individual contributions to fluorescence emission

Enzyme	Individual contributions to GDH fluorescence (%)		
	$\lambda_{max 1}$	$\lambda_{max 2}$	$\lambda_{max 3}$
Native	3.04	39.57	57.38
Immobilised	1.53	40.72	57.75

microcontact printing, for the surface patterning of azurin proteins on Au(111) substrates, and describe its characterisation by scanning probe microscopy. Soft lithography gives the ability to pattern biomolecules on to surfaces without the use of high-energy exposure beams and of the denaturing reagents necessary for photolithographic-based techniques. On gold substrates, the chemical adsorption of proteins occurs directly via the disulphide bond, which links covalently to Au, and no functionalisation procedures are required.

Master patterns for microcontact printing [24, 25] made by parallel stripes of periodicity between 20 and 30 μm were fabricated by photolithography on Si/SiO₂ (SiO₂ thickness $\cong$ 100 nm) substrates. After the deposition of a standard photoresist (Clariant AZ5214E:AZ EBR Solvent 1:1), UV exposure (8'30" at 45 W) and developing, we etched the thermal dioxide by using NH₄F/HF/H₂O (6.6 g/1.6 ml/10 ml). The SiO₂ mask obtained was then employed to etch silicon by 5.9 M solutions of KOH at 80°C. Elastomeric replicas of hard masters are realised by *in situ* polymerisation of poly(dimethylsiloxane) (PDMS) (Sylgard 184, A:B 1:9, Dow Corning) at 140°C for 15 min. A few drops of the protein solutions (10^{-8} $\times$ 10^{-5} M) are then cast on the

patterned side of the replica. After solvent evaporation, the protein film is deposited on the elastomeric element, which can be placed on to the substrate under its own weight for a few minutes. The protein layer is adsorbed on to the target substrate by physisorption and/or chemical linking (Fig. 8). After peeling off the replicas, the patterned substrates are vigorously rinsed several times in order to remove the unattached protein molecules.

Printing metalloproteins can be an advantageous approach for the easy realisation of biosensors and bioelectronic circuits. An Az pattern, showing protein stripes about $9\ \mu\text{m}$ large, separated by $16\ \mu\text{m}$ exposed gold regions, is shown in Fig. 9. To have a deeper insight into the morphology of printed layers, we investigated the inner

region of the protein stripes. The lateral size of the visible features is strongly affected by the convolution effect between the sample and the AFM tip, whereas the measured height is around $4\ \text{nm}$, which is consistent with the size of single proteins. An alternative soft lithography approach for biomolecules, based on the microfluidic filling of elastomeric capillaries [26–28], can also be employed in order to achieve high-resolution patterns on to chemically linking surfaces. Further experiments are currently underway in our laboratory to apply micromoulding in capillaries to metalloproteins for the realisation of interconnected protein circuits.

3.2 Patterned laminin-1 physiological network

In recent years, much progress has been made in the knowledge of the interactions between a cell and its surroundings [29]. It is well known that cell physiological phenotype is strongly affected by the extracellular environment, with specific cell–cell and cell–matrix interactions, both topographical and functional. Among the extracellular matrix (ECM) proteins, laminin-1 (LN-1) plays an important role in the stimulation of the growth and differentiation of various cell types, supports cell adhesion, induces cells polarity and organises the basement membrane. Moreover, it strongly modulates the morphogenesis of the nervous system and promotes neurite outgrowth [30]. These functions are enabled by its cruciform shape, which is well suited for bridging between distant sites on cells and in the ECM, and are strongly dependent from the laminin polymerisation process in mesh-like matrices [31].

We have obtained self-assembled LN-1 networks on glass, realised by one-step microcontact printing (μCP) lithography [32], without any preliminary functionalisation of the substrates. We investigated the realised patterns by indirect immunofluorescence (Fig. 10) and atomic force microscopy (AFM) (Fig. 11), in order to assess their uniformity, the well retained antigenicity of the proteins, and their supramolecular structural organisation. The AFM images clearly show the characteristic protein self-assembling in polygonal networks with well defined sub- $100\ \text{nm}$ quaternary structures of laminin. However, our recent studies [32] demonstrate that this result is strongly

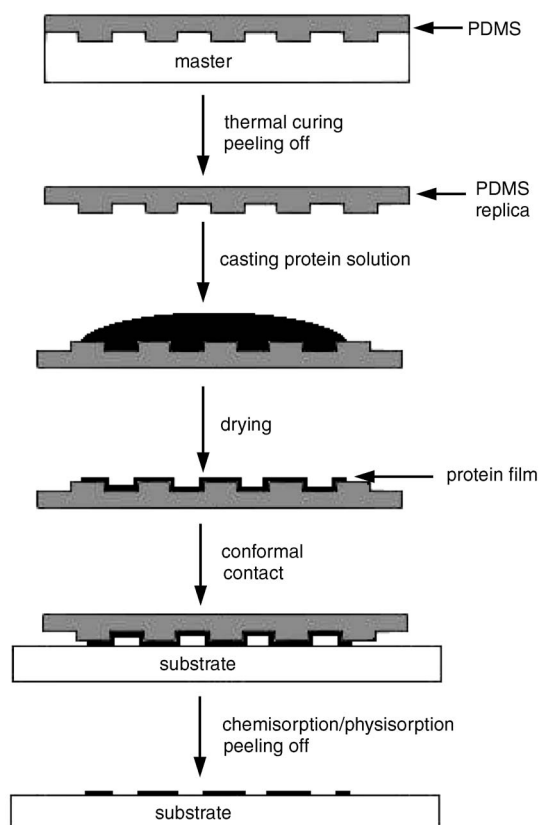


Fig. 8 Scheme of the microcontact printing (features not in scale)

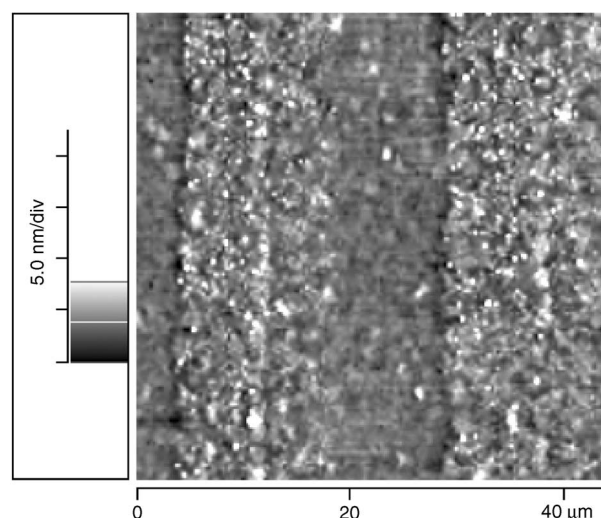


Fig. 9 $43.8 \times 43.8\ \mu\text{m}^2$ topography image of printed Az layer
Scan rate was $1\ \text{Hz}$

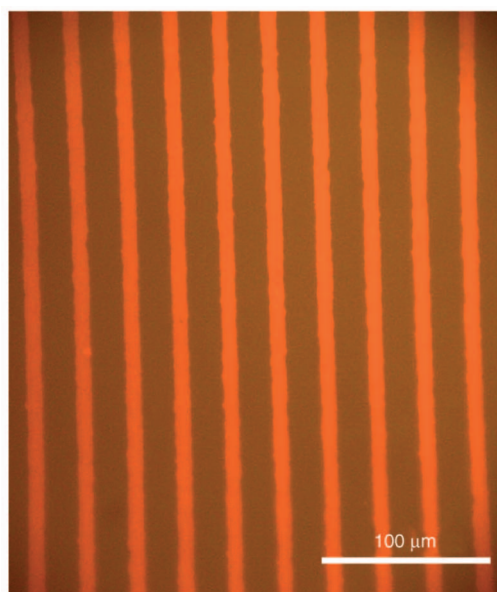


Fig. 10 Indirect immunofluorescence image of printed laminin stripes on glass

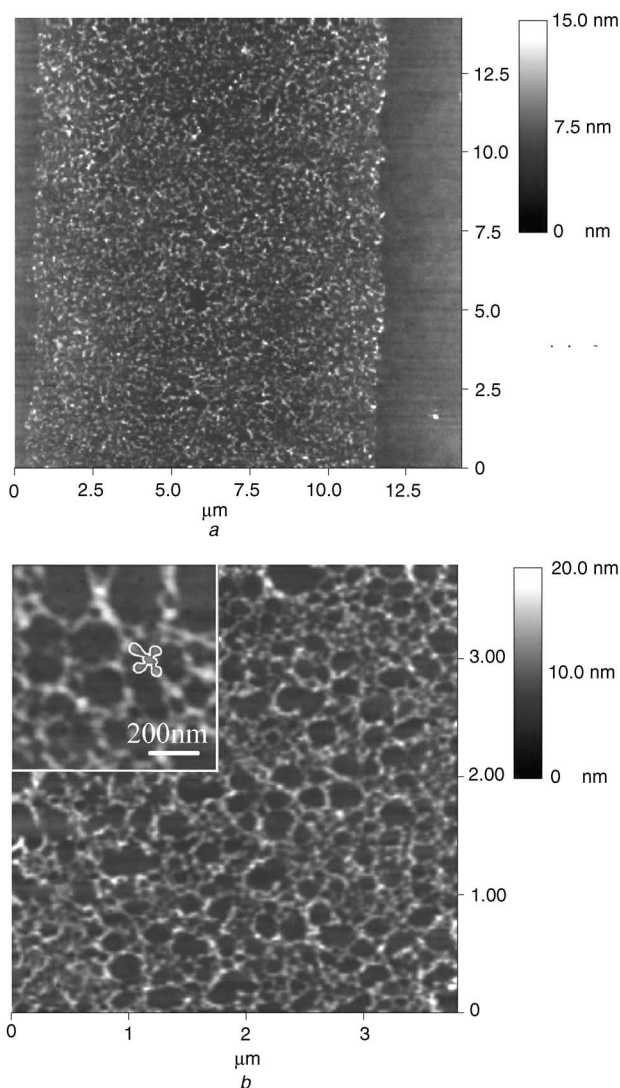


Fig. 11 AFM images of a printed LN-1 stripe and self-assembled protein network

a Printed LN-1 stripe

b Self-assembled protein network within the printed region

Inset: higher resolution view, in which the single proteins are clearly appreciable. The edge of a single LN-1 molecule is marked by the white line

dependent on the LN-1 initial concentration. This finding powerfully suggests one-step soft lithography as a powerful technique to realise both micropatterned and physiologically self-assembled layers with biocompatible properties for cell culture applications in both basic biology studies and biosensors, drug discovery and hybrid devices.

4 Conclusions

In this paper, we have shown several applications of biosurfaces at the nanoscale in the field of molecular electronics and biosensing. The following topics have been presented and discussed: the realisation of functional azurin monolayers for the implementation of a solid-state protein transistor working in air; a novel experimental approach to produce high-quality Glutamate dehydrogenase monolayers for nanobiosensing applications; and the results of soft lithography processes on biomolecules, namely, microcontact printing of azurin for biosensor implementation, and physiological self-assembled patterns of laminin-1 for cell culture applications and hybrid devices.

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