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Organic-inorganic Interfaces for a New Generation of Hybrid Biosensors

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1. Introduction

Biosensors have by far moved from laboratories benches to the point of use, and, in some cases, their represent technical standards and commercial successes in applications of social interest, such as medical diagnostic or environmental monitoring. Based on biological molecules, but also on their bio-inspired synthetic counterparts, biosensors employ different transducers (optical, potentiometric, volt-amperometric, colorimetric, and so on) converting the molecular interaction information into a measurable electric signal. As the result of a real multi-disciplinary field of science and technology, biosensors can take advantage from each improvement and progress coming from other disciplines: new features and better performances have been reached in the last year due to simplified fabrication methodologies, deep integration of optical or electrical transducers, and, last but not least, microfluidic circuits. More recently, nanostructured components dramatically increased biosensors reliability especially in public health and environmental monitoring. Nevertheless, there is still a pressing demand of innovations which could lead to smaller, faster, and cheaper biosensors systems with ability to provide not only accurate information but also feedback actions to the real world. The fabrication of a new generation of hybrid biodevices, where biological, or bio-inspired, molecules are fully integrated with a micro or a nano technological platform, strongly depends on the bio-compatibilization treatments of the devices surfaces. The design and the realization of bio/non-bio interfaces with specific properties, such as chemical stability, wettability, and biomolecules immobilization ability, are key features in the miniaturization and optimization processes of biosensors. In particular, protein immobilization is a hot topic in biotechnology since commercial solutions, as in the case of DNA microarrays, are not still available. Proteins are, due to their composition, a class of very heterogeneous macromolecules with variable properties. For these reasons, it is extremely difficult to find a common surface suitable for different proteins with a broad range in molecular weight and physical–chemical properties such as charge and hydrophobicity. A further aspect is the orientation of the bound proteins, that could be of crucial relevance for quantitative analysis, interaction studies, and enzymatic reactions. Many different surfaces, and chemical treatments of these surfaces, have been

investigated in the last years, but an universal solution for all the applications aforementioned could not be identified.
Following this very actual theme, our main focus in this chapter is to discuss different applications in biosensing of a special class of amphiphilic proteins: the hydrophobins. These proteins self-assemble in a nanometric biofilm at the interfaces between water and air, or on the surfaces covered by water solution. New functionalities can be added to the biosensors surfaces without using any chemical or physical treatment, just covering them by a self-assembled protein biofilm.
The main topics covered in the following paragraphs are: origin and properties of the hydrophobins; deposition methods of the hydrophobins biofilm on different surfaces and the characterizations techniques we use to determine the physical properties of these bio-interfaces; the features exhibited by the hydrophobins covered surfaces, and finally, the biosensors systems based on hydrophobins biofilms.
We outline in this chapter how the peculiarities of these proteins can be of interest in the technological field, beyond their large utilization in biotechnology, nowadays at industrial level. Moreover, the experience matured on this subject can be the paradigm of a new kind of approach in design and realization of the next generation of biosensors.

2. Hydrophobins: surface active proteins

Proteins are actually polymers whose basic monomer units are amino acids, the so called *residues*. In nature, the building blocks of the protein structure are 20 different amino acids that, on the base of their physical-chemical properties, can be classified as hydrophobic or hydrophilic. The sequence of hydrophobic and hydrophilic residues in the primary structure will give rise to an hydropatic pattern on the protein. As consequence, in water, they behave like amphiphilic molecules, giving rise to structures that maximize the number of interactions between hydrophilic groups and water and, at the same time, minimize those between hydrophobic groups and water.
Hydrophobins (HFBs) are a large family of small proteins (about 100 amino acids) that appear to be ubiquitous in the Fungi kingdom. The name *hydrophobin* was originally introduced because of the high content of hydrophobic amino acids (Wessels J.G.H, *et al*., 1991). They fulfil a broad spectrum of functions in fungal growth and development. They are ubiquitously present as a water-insoluble form on the surfaces of various fungal structures, such as aerial hyphae, spores, and fruiting bodies, etc., and mediate attachment to hydrophobic surfaces. HFBs are very efficient in lowering the surface tension of water allowing the hyphae to escape from the aqueous medium and grow into the air. As the fungal hyphae grow through the air-water interface into the air, the hydrophobins at the interface are believed to coat the emerging hyphae as they penetrate through the interface, as shown in Figure 1. *In vitro* hydrophobins are able to self-assemble at hydrophilic/hydrophobic interfaces into an amphipathic membrane, resulting in the change of nature of surfaces.
Hydrophobins have been split in two groups, class I and class II, based on the differences in their hydropathy patterns, spacing of aminoacids between the eight conserved cysteine residues and properties of the aggregates they form (Linder *et al*. 2005). Class I hydrophobins generate very insoluble assemblies, which can only be dissolved in strong acids (i.e. 100% trifluoroacetic acid) and form rodlet structures outside the fungal cell wall. Assemblies of Class II can be more easily dissolved in ethanol or sodium dodecyl sulphate

and form assemblies that lack a distinct rodlet morphology. Despite these morphological differences, no obvious distinction between the functions of class I and class II hydrophobins within the fungal life cycle has yet emerged.

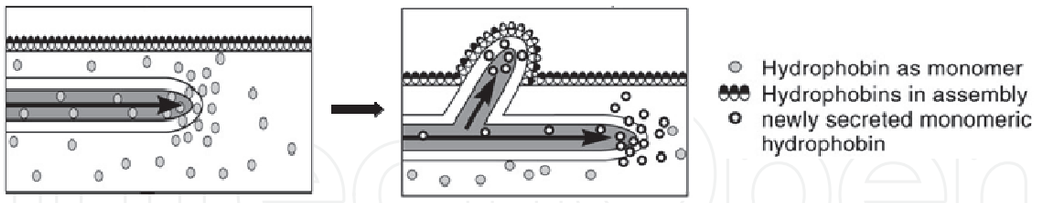


Fig. 1. Schematic of HFB role in fungal hyphae growth.

2.1 Hydrophobins structures

In order to provide a complete molecular description of hydrophobins, two complementary points of view have to be considered: the structure of non-assembled hydrophobins and the features of the assembled form. The structure of a protein is characterized in four ways: the primary structure is the order of the different amino acids in a protein chain, whereas the secondary structure consists of the geometry of chain segments; the main types of secondary structure are two, called the α-helix and the β-sheets. The tertiary structure describes how the full three dimensional arrangement of the chains and all its side groups, revealing how a protein folds in on itself, and finally the quaternary structure of a protein describes how different protein chains hook up with each other.

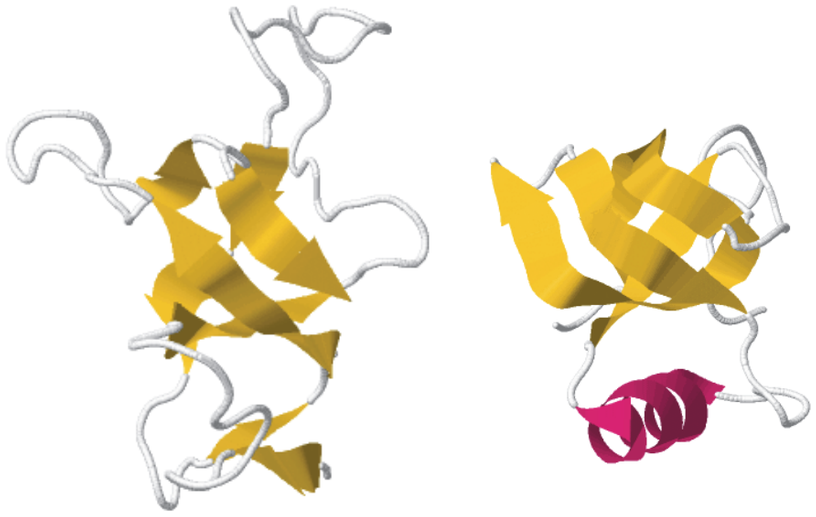
HFBs of both types I and II, although share quite a low sequence similarity, feature a clear signature, namely eight Cys residues in a characteristic pattern. In this pattern, the third and fourth as well as the sixth and seventh Cys residues are always adjacent in the sequence. In the protein folded state, this special pattern gives rise to four disulfide bonds spanning over the entire structure of the protein.

Class I HFBs consist of a four-stranded β-barrel core, an additional two-stranded β-sheet and two sizeable disordered regions, as it can be seen in Figure 2. Notably, the charged residues are localized at one side of the surface of the protein. This strongly suggests that the water-soluble form is amphipathic (Zampieri *et al*., 2010). This structure is consistent with its ability to form an amphipathic polymer.

Class II HFBs consist of a core with a β-barrel structure (Fig. 2), nevertheless do not contain the two disordered loops. Furthermore, the additional two-stranded β-sheet in class I hydrophobins is replaced with an α-helix, in the same region (Kwan, A.H.Y *et al*., 2006; Hakanpää, J, *et al*., 2006). One side of the monomer surface contains only aliphatic side chains. This creates a hydrophobic patch which constitutes 12% of the total surface area (situated on the top of the structure showed in figure 2). The protein surface is otherwise mainly hydrophilic, and thus the surface is segregated into a hydrophobic and a hydrophilic part. This amphiphilic structure governs the properties of class II hydrophobins, such as surface activity and surface adsorption.

2.2 The assembly process

The characteristic property of HFBs is adsorption at hydrophobic-hydrophilic interfaces, at which they form amphiphilic films (Wessels J.G.H, *et al*., 2007; Wösten H.A.B, *et al*., 2007). The interface can occur between solid and liquid, liquid and liquid or liquid and vapour. In early studies, hydrophobins were found to self-assemble into aggregates and form various



Class I **Class II**

Fig. 2. HFBs structures.

types of self-assembled structures. Rodlets were first observed on the outer surface of spores from *Penicillium* (Sassen *et al*., 1967; Hess *et al*.,1968) and *Aspergillus* (Hess *et al*., 1969; Ghiorse and Edwards, 1973).

Class I HFBs at low concentration are in monomeric form, while at higher concentrations they are mainly in a dimeric form (Wang, X *et al*., 2002; Wang, X *et al*., 2004). Self-assembly proceeds through the formation of an intermediate form, the α- helical state (De Vocht, M.L *et al*., 2005, Wang, X *et al*., 2005). Upon transfer to the β-sheet state, the content of β-sheet structures increases. This is accompanied by the formation of a mechanically stable protein film. However, during this transition the proteins forms nanometric wide fibrils, which are known as rodlets. SE measurements have shown that the film is about 3 nm thick (Wang, X *et al*., 2005). This and the fact that the diameter of the β-barrel of the protein is approximately 2.5 nm suggest that the rodlets could be formed by a molecular monolayer (Kwan, A.H.Y *et al*., 2006). The charged patch on the protein surface would face the hydrophilic side of the interface, while the hydrophobic diametrically opposite site would face the hydrophobic side of the interface. This arrangement is consistent with the way other surface active molecules orient themselves at hydrophilic-hydrophobic interfaces (Kwan, A.H.Y *et al*., 2006).

Like class I, class II HFBs exist as monomers at low concentration (Szilvay, G.R et al., 2006). When the concentration is increased, they form dimers and, at higher concentrations, tetramers (Torkkeli, M et al., 2002). The monomers have a higher affinity for surfaces than for formation of oligomers (Linder *et al*. 2005; Szilvay, G.R et al., 2006). These oligomers would dissociate at a hydrophilic-hydrophobic interface, which would result in the formation of a film which consists of a monolayer of the class II HFB: a scheme of these differences is reported in Figure 3. In contrast to class I, self-assembly of class II HFB at the water-air interface is not accompanied by a change in secondary structure (Askolin, S. et al., 2006), furthermore this layer is not rodlet-like as in the case of class I HFBs.

Moreover, as described above, the end state of class I HFBs is very stable and cannot be dissociated by pressure, detergent or 60% ethanol. In contrast, the end form of class II HFBs readily dissolves under these conditions.

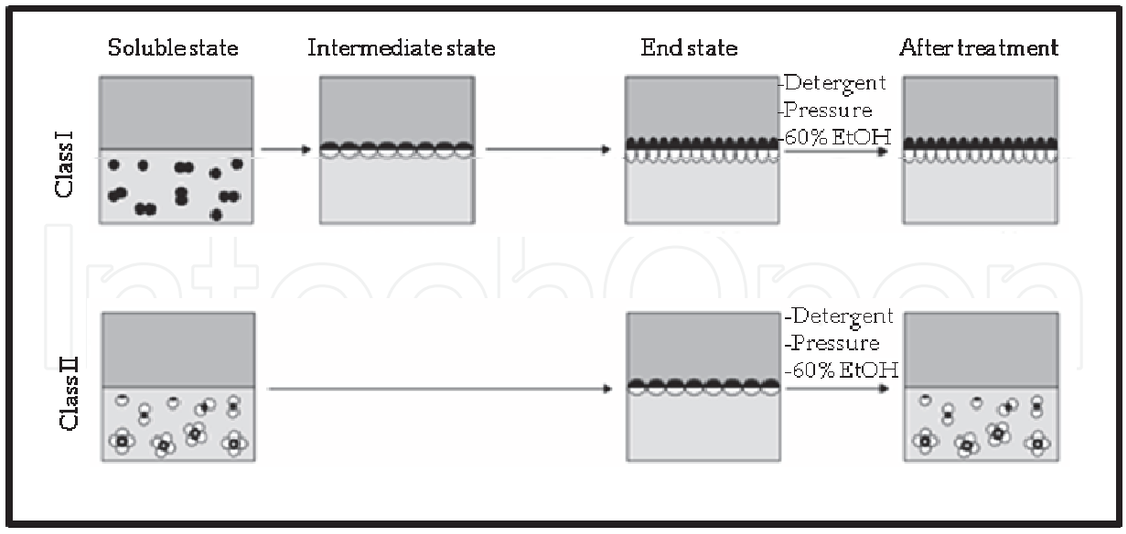


Fig. 3. Main differences between class I and class II HFBs.

3. Hydrophobins self-assembling on solid surface: methods and characterizations

A simple technique used to induce the self-assembling of the HFB biofilm on a solid substrate is the drop-deposition method, where the drop is a micro-litre volume of a liquid solution containing the proteins in their monomeric state. Even if this kind of film casting is not a perfectly controlled process, i.e. the protein concentration increases in an uncontrollable way during solvent evaporation, it is possible, by using proper starting conditions of some parameters, such as temperature, surface cleaning, and so on, to obtain reproducible results in term of film thickness and surface wettability. By using this technique, different kind of surfaces have been conditioned: in the next paragraph we report the main experiences in worldwide laboratories on this subject. Here, we present the standard processes to obtain self-assembled HFB biofilms and the main characterization methods we use. Biofilm drop casting is normally used in our laboratory to give new functionalities to silicon surface: silicon, and silicon related materials, is the most used solid support in the microelectronic industry. For this reason, silicon is widely used in all the application of electro-optic and photonic devices. At this aim, highly doped p^+ silicon wafer, <100> oriented, 0.003 Ω cm resistivity, 400 μm tick, was cut into 2 x 2 cm^2 pieces. The silicon substrates were cleaned using the standard RCA process and dried in a stream of nitrogen gas. RCA is based on a combination of two cleaning steps, one using solutions of ammonium hydroxide/hydrogen peroxide/deionized water, and the other using hydrochloric acid/hydrogen peroxide/deionized water, both at the temperature of 80 °C. The samples were prepared by coating the silicon chips with 200 μl of HFB solution (0.2 mg/ml of protein dissolved in an ethanol-deionized water (60/40 v/v) mixture) for 1 h, drying for 10 min on a hot plate at 80°C, and then washing with the ethanol-water mixture. The incubation process was repeated two times. Then, the samples were treated for 10 min at 100°C in 2% Sodium Dodecyl Sulfate (SDS), so as to remove the protein not assembled into the biofilm, and again washed in deionized water.

The Langmuir technique is the most accurate way to get mono-molecular films of amphiphilic molecules. A known quantity of the material is spread on the free surface of a suitable liquid (the subphase) contained in a trough. The quantity of the material has to be predetermined on

the base of the molecular and trough areas, in order to know the surface molecular concentration. In doing this, one should take care to use a sufficiently low surface concentration in order to have an interfacial film of non-interacting molecules (the so-called gas phase). Moreover, as one desires to have all molecules at the interface, it is very important to adjust the subphase pH in order to match the isoelectric point of the used protein.
Once the interfacial film is formed, the presence of movable barriers on the Langmuir trough allows to compress the film in a controlled manner, varying in this way the surface molecular density and consequently the surface tension. The latter can be measured with several techniques, but the most used is the Wilhelmy plate tensiometer, consisting of a thin plate made from glass, platinum or even paper, attached to a scale or balance via a thin metal wire.

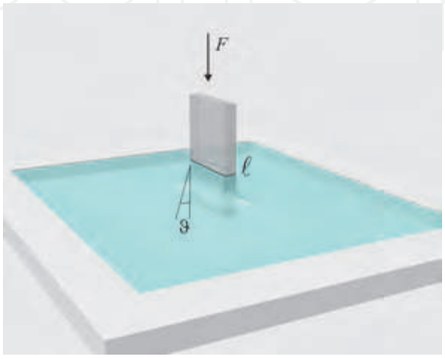


Fig. 4. Scheme and reference angle for Wilhelmy equation.

The force on the plate due to wetting is measured via a microbalance and used to calculate the surface tension (σ) using the Wilhelmy equation (see Figure 4):

$$\sigma = \frac{F}{\ell \cos \vartheta}$$

where λ is the wetted perimeter of the Wilhelmy plate and θ is the contact angle between the liquid phase and the plate. In practice the contact angle is rarely measured, instead either literature values are used, or complete wetting (θ = 0) is assumed.
The most important feature of the interfacial film is the pressure Vs. area isotherm, obtained by recording the surface pressure as a function of the trough area. If the number of molecules present at the interface is known, as in the case of water insoluble amphiphiles, the surface pressure can be plotted as a function of water surface available to each molecule. This curve can reveal several details about the interfacial film properties, in particular phase transitions and collapses of the molecular film. In such cases the steric factor plays a relevant role in the film stability and the entire sequence of phases transitions can be sperimentally observed (see Fig. 5).



a) b) c)

Fig. 5. Cartoon sequence of the possible molecular arrangement for an amphiphile monolayer as function of the molecular density: a) gas phase; b) liquid-expanded phase; c) condensed phase.

When proteins are used for making a Langmuir film things can be quite different, as a considerable number of other factors has to be took into account: the state of the protein (folded or unfolded) and her shape (in general almost globular), the presence of multiple hydrophobic patches. In figure 6 a typical isotherm for Vmh-2 from *Pleorotus Ostreatus* is shown. It is evident that many features are missing with respect to the case of a fatty acid. In particular only one critical point is present. If one keeps in mind the quasi-globular shape of this protein can easily realize that the gas - expanded liquid – solid transitions are in some way continuous, without any abrupt molecular rearrangement as in the case of an elongated molecule.

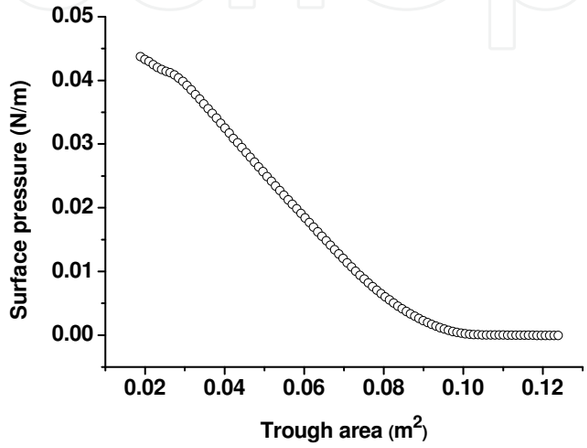


Fig. 6. Typical isotherm measured for the hydrophobin Vmh-2 during film formation.

If the used amphiphilic protein is even only partially soluble in water, although the isotherm features still hold, one has to face with the problem to determine the number of molecules present at the interface and hence the surface molecular density. This occurrence can be made evident by isobaric measurements, in which the ratio between the trough surface S and the initial trough surface S_0 is plotted as a function of time at a given constant surface pressure value. The plot shown in Fig. 7 refers to Vmh2 HFB from the fungus *Pleurotus Ostreatus*. The decreasing of the trough area in time is due to a surface molecular depletion that could be ascribed both to a bare solubilisation of the film or to some more complex process involving the creation of soluble assemblies. From the same plot one can also argue that an increasing in the surface pressure has the effect of stabilizing the film, reducing the molecular depletion ratio, as the decreasing in the curve slope with the increasing of the surface pressure demonstrate.

One possible method for at least estimate the surface molecular concentration is the fitting of the experimental isotherm with a suitable 2-D equation of state, leaving the surface density as fitting parameter. In the simplest case holds a Vollmer-like equation of the kind.

$$\Pi = \frac{mkT}{A - \omega} - \Pi_{coh} \tag{1}$$

where Π is the surface pressure, k is the Boltzmann constant, T is the temperature, ω is the limiting area of a molecule in the gaseous state, A is the area per molecule, Π_{coh} is the

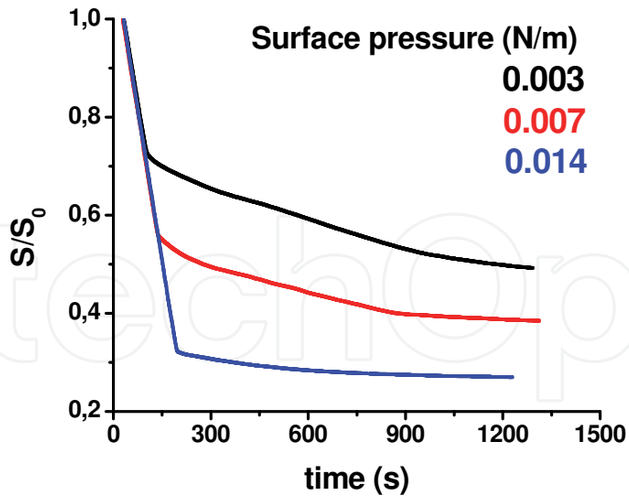


Fig. 7. Isobaric measurement at different surface pressures for the Vmh-2 hydrophobin.

cohesion pressure accounting for the intermolecular interactions, and m is a parameter accounting for the number of kinetically independent units (fragments or ions). The parameter A is actually the inverse of the surface molecular density and can hence be expressed as

$$A = \frac{S}{n}, \tag{2}$$

where S is the trough area and n the number of molecules present at the interface.
Once the Langmuir film has been characterized, it can be transferred onto a solid substrate for applications or subsequent analyses. The most used methods are the vertical lift/dipping of the substrate through the interfacial film (Langmuir-Blodgett technique) and the horizontal plate lift from the interface (Langmuir-Shaeffer technique). When hydrophilic subphases are used, the lift method allows to transfer the monolayer with his hydrophilic side facing the substrate (also hydrophilic), leaving the hydrophobic side exposed to the air.



a) b) c)

Fig. 8. Schematic of Langmuir-Blodgett technique: with the barriers opened, in the gas phase, the substrate is dipped in the subphase (a); then the film is compressed at the desired surface pressure (b); finally the substrate is lifted through the interfacial film, dragging a portion of it. In the meanwhile the closed-loop control closes the barriers in order to keep constant the surface pressure (c).

Alternatively, the film can be transferred with his hydrophobic side facing an hydrophobic substrate by dipping the latter through the interfacial film. The monolayer side exposed to the air in this case will be hydrophilic. In the Langmuir-Blodgett deposition technique, the trough control system allows to perform the film transfer at constant surface pressure, closing the barriers in order to compensate the surface molecular depletion due to the film transfer itself.

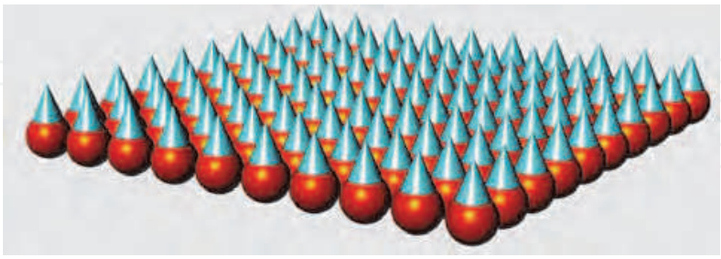


Fig. 9. Cartoon of ordered protein monolayer for Langmuir- Schaefer

The Langmuir-Shaeffer technique allows to remove a whole patch of the interfacial film at once. Under the same initial conditions as above, this method allows the film sticking from the hydrophobic side onto an hydrophobic substrate, leaving thus the hydrophilic side of the monolayer exposed to the air. In this case, the closed-loop active surface pressure control isn't strictly required, providing that the monolayer is stable at the interface.

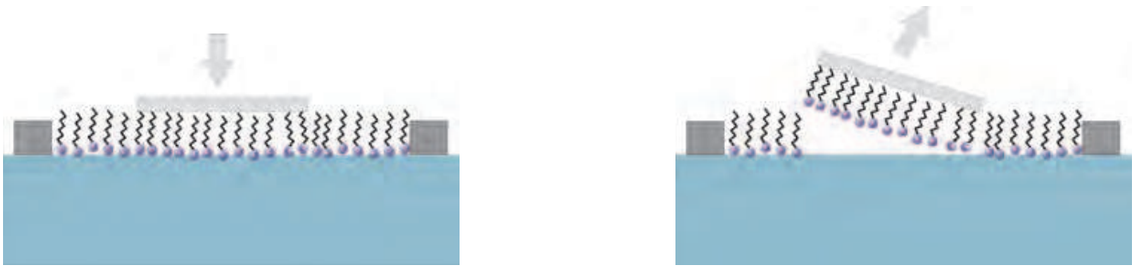


Fig. 10. Schematic of Langmuir-Shaeffer technique.

Sometimes it can be useful to lift the molecular film as self-standing, in order to eliminate the interactions between molecules and an underlying substrate. This task is often accomplished using metallic grids, of the kind used in TEM specimen preparation, featuring a suitably fine mesh.
The HFBs film self-assembled on the silicon surface or by LB methods can be characterized by means of several methods; the most common characterization techniques are the spectroscopic ellipsometry, the atomic force microscopy, and the water contact angle.

3.1 Spectroscopic ellipsometry

Spectroscopic ellipsometry (SE) allows to determine the optical properties (i. e., the refractive index n and extinction coefficient k) and the thickness of the HFB biofilm assembled on a solid surface. The method is based on the measurement of the change in the polarization state of the light over the spectral range after the reflection from the sample surface. Ellipsometry measures the complex reflectance ratio (ρ) defined by:

$$\rho = \frac{R_p}{R_s} = \tan\psi e^{i\Delta} \tag{3}$$

where R_p and R_s are the complex reflection coefficients of the light polarized parallel and perpendicular to the plane of incidence. Thus, ψ and Δ are, respectively, the amplitude ratio and the phase shift between *s* and *p* components of polarized light.
We have used a Jobin Yvon UVISEL-NIR phase modulated spectroscopic ellipsometer, at an angle of incidence of 65° over the range 320-1600 nm with a resolution of 5 nm. The properties of the biofilm have been extracted from the SE measurements using the analysis software Delta Psi (Horiba Jobin Yvon).
The optical properties, *n* and *k*, as functions of the wavelength have been determined by fitting the experimental results using the Tauc-Lorentz model, firstly proposed in 1996 by Jellison and Modine as a new parameterization of the optical functions of amorphous materials. The imaginary part of the dielectric function is based on the Lorentz oscillator model and the Tauc joint density of states:

$$\varepsilon_2 = \begin{cases} \frac{1}{E}\frac{AE_0C(E-E_g)^2}{(E^2-E_0^2)^2+C^2E^2} & E > E_g \\ 0 & E \le E_g \end{cases} \quad (4)$$

The real part of the dielectric function is given by Kramers-Kronig integration:

$$\varepsilon_1 = \varepsilon_\infty + \frac{2}{\pi} P \int_{E_g}^{\infty} \frac{\xi \varepsilon_2(\xi)}{\xi^2 - E^2} d\xi \quad (5)$$

These equations include five fitting parameters: the peak transition energy E_0, the broadening term *C*, the optical energy gap E_g, the transition matrix element related *A*, and the integration constant ε_∞.
In Figure 11, *n* and *k*, as functions of the wavelength, are reported for the Vmh2 biofilm self-assembled on silicon together with the values of the fitting parameters and the χ^2.

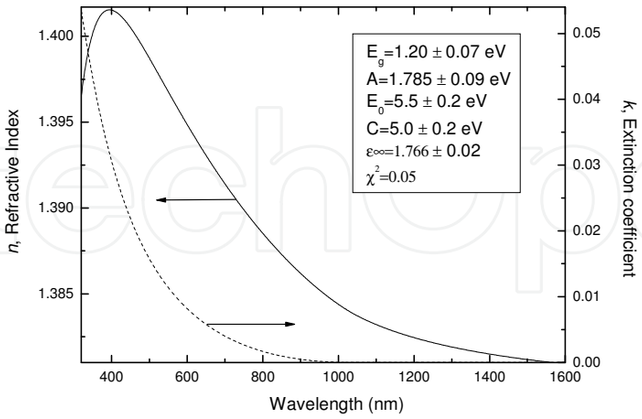


Fig. 11. Optical properties, *n* and *k*, of the Vmh2 biofilm self-assembled on silicon surface as functions of the wavelength.

Starting from these results, we can use the ellipsometric technique to estimate the thickness of the biofilm depending on the concentration or on the post deposition washing procedure, for example. We notice that before SDS washing the thickness of Vmh2 biofilm could be of

tenths of microns, also depending on protein concentration in the starting solution. We have verified that a step-by-step deposition allows the assembling of biofilms of increasing thicknesses: after three consecutive depositions, for a total time of three hours, we have obtained biofilms assembled on crystalline silicon up to 40 nm thick, that is, thicker than those reported in literature. After hot SDS washing the biofilm is very much thinner: a value of 3.91 ± 0.06 nm has been calculated modelling the HFB sample by a simple homogeneous layer. We believe that this is the thickness of a monolayer of HFBs when self-assembled on hydrophobic silicon: this value is consistent with a typical molecular size and comparable to atomic force microscopy measurements. According to the above described model, the washing step of the chip is strong enough to remove the proteins aggregates deposited on the HFB monolayer that directly interacts with the hydrophobic silicon surface. This behaviour points out the stronger interactions between the silicon surface and the HFB monolayer with respect to those between the HFB aggregates and the HFB monolayer. The experimental spectra Ψ and Δ, together with the calculated ones, are shown in Figure 12. The persistence of Vmh2 biofilm on the silicon surface depends strongly on its chemical nature: we have thus verified that the same deposition procedure on silicon dioxide, which is a hydrophilic surface, does not give the same results in terms of biofilm chemical stability. After washing the biofilm in hot SDS solution only sparse islands of protein biofilm can be found on the silicon dioxide chip. This different behaviour can be ascribed to the greater number of hydrophobic residues constituting the protein with the respect to those hydrophilic.

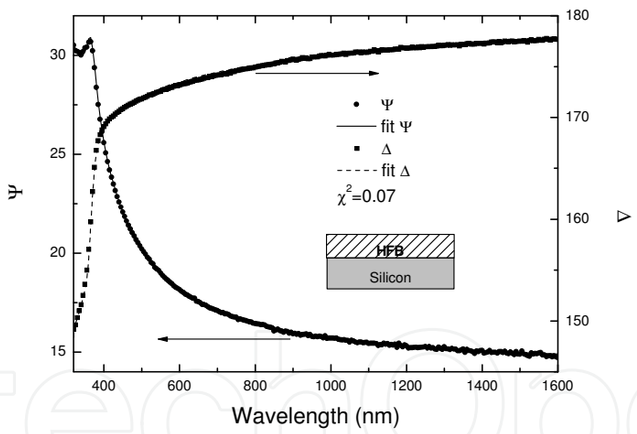


Fig. 12. Measured and calculated spectra (Ψ, Δ) of the HFB biofilm self-assembled on a silicon surface.

3.2 Atomic force microscopy

In the early 1980s the first Scanning Probe Microscope (SPM) has been introduced. It was actually a Scanning Tunneling Microscope, exploiting the tunnelling current between the surface sample and a very sharp conductive tip sweeping back and forth a few tens nanometers over the sample surface. The electron tunnelling is a quantistic effect connected to the probability for an electron to cross a gap between two conductive solids. Its development was driven by the need of the semiconductors industry to characterize the semiconductor inhomogeneities at the nanoscale, this issue becoming of increasing importance with the

progresses in the miniaturization of the electronic devices. A few years later, a similar instrument was released, the Atomic Force Microscope (AFM) exploiting the van der Waals-like interaction forces between the sample surface and the tip, instead of the tunnelling current and allowing the imaging of non-conductive samples as well, down to the atomic scale.

In their essential parts, an AFM is made of a closed loop controlled scanning head and an acquisition/digital signal processing board slotted in a PC running a devoted software. Samples are under the form of small chips or thin films with *x-y* size ranging from the sub-millimiter to a centimeter. The *z* size is usually in the order of a nm. The sample is placed at the free end of a piezoelectric scanner; depending on the piezo characteristics, the scanned area can range from a few tens nm^2 to ~10^4 sq. microns with a resolution down to a few tenth nm. The AFM probes can feature pyramidal or conical shape, with a height in the range of a few microns and a tip radius down to a couple nanometers; this makes a really sharp tip, carring only a few atoms at the end! The tip is placed at the end of a flexible cantilever; both the tip and the cantilever are usually made of silicon, although other materials are now available, as diamond or carbon nanotubes.

When the tip is brought close to the sample surface, the van der Waals-like forces between the sample surface and the tip cause the cantilever bending; if one knows the cantilever spring constant, the bending measurements allows to deduce the interaction force. The detection technique exploits the reflection of a laser beam from the upper side of the cantilever; the bending is measured by the laser spot displacement over a two or four quadrants photodetector.

An AFM can operate essentially in two different modes according to the sample surface-tip distance range. When this distance is small, in the order of few Angstrom, one operates in the repulsive region of the interaction potential and the operating mode is named *contact mode*. On the contrary, when this distance is "large", in the order of 1 – 10 nm , the attractive region of the interaction potential is involved and the corresponding mode is named *no-contact mode*. The difference between the contact and the no-contact mode is remarkable, because of the difference in the range of the forces involved in the interaction between the AFM tip and the sample surface; in the contact mode case, the repulsive forces are in the order of tens of nN, al least one order of magnitude stronger than the case of the attractive forces involved in the no-contact mode. Because of the extreme force weakness, the detection technique used in no-contact AFM is very sensitive: driven by a piezo element, the cantilever oscillates with a typical amplitude of few nm at a frequency slightly above the resonance, while "flies over" the surface of the sample. The presence of Van der Waals and other long-range attractive forces shifts the cantilever resonant frequency which, in turn, causes the oscillation amplitude to decrease.

The NC-mode is useful in imaging soft samples, as is the case of biologic molecules, given the weakness of the involved forces that prevents or minimizes the sample damage.

An evolution of the AFM no-contact operating mode is the *tapping* or *intermittent mode*.

In tapping mode the cantilever oscillates at its resonant frequency or slightly below. The amplitude of oscillation typically ranges from 20 nm to 100 nm. Differently from the case of no-contact mode, in which the tip never comes in contact with the sample surface, in the tapping mode the tip lightly "taps" on the sample surface during scanning, contacting the surface at the bottom of its oscillation.

Beside the bare imaging of soft samples, the AFM dynamical modes (NC and tapping) can supply information about other features of the sample at the nano scale, as its wetting and visco-elastic properties. In order to achieve such information, a fundamental measurement is

the phase lag between the signal driving the cantilever piezo element and the cantilever response. Then the experimental curves are fitted with a model describing the cantilever dynamics. The recording of the phase lag at constant amplitude for each point of the acquisition *x-y* grid will produce a phase image that closely relates to a dissipation map.
In our experience, a NanoScope V Multimode AFM (Digital Instruments/Veeco) was used for the imaging of the HFB biofilm. The sample was imaged in air in tapping mode. The scan frequency was tipically 1 Hz per line and images were flattened to a second order polynomial.
The AFM images of the HFB silicon coated sample are reported in Figure 13; the formation of a homogeneous biofilm can be observed in the phase image (right picture in Figure 13). The AFM characterization also reveals the presence of rodlets-like structures on top of the biofilm (Rodlet average height 4.11 ± 0.08 nm; Rodlet average width 23.9 ± 0.6 nm; Rodlet average length 64 ± 3 nm; Mean roughness 3.32 nm).

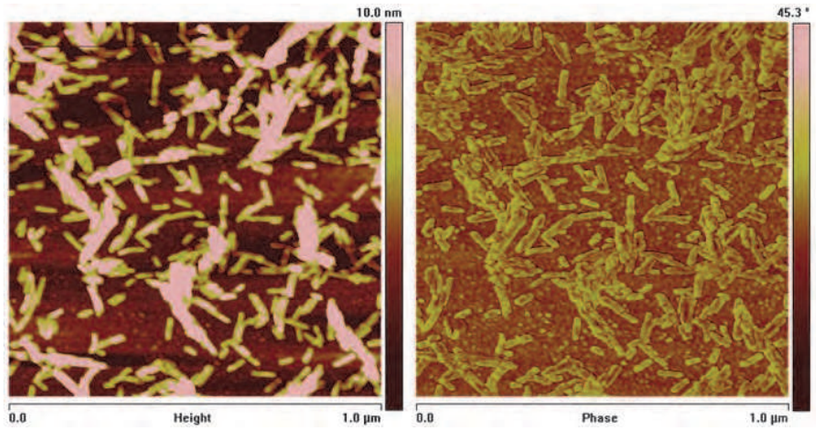


Fig. 13. Height and phase atomic force microscopy images of silicon surface coated with HFB biofilm.

3.3 Water contact angle measurements

The most common method for the determination of the surface wettability is the water contact angle (WCA) measurement. The technique simple, quick, and cheap is based on the analysis of the contact angle formed between a surface and a water droplet placed on it.
At the aim to determine the wettability of the HFB biofilm, we have used the sessile drop method for WCA measurements on a OCA 30 – DataPhysics coupled with a drop shape analysis software. Five measurements were analyzed for each sample.
The silicon surface, after the removal of the native oxide layer in hydrofluoridric acid, is characterized by a WCA of (90.0±0.3)° (Figure 14 (A)). The presence of the HFBP biofilm lowers the WCA down to (44±1)° (Figure 14 (B)): this interface is more hydrophilic due to the assembly of the protein into a film with apolar groups disposed towards the hydrophobic silicon and the polar groups on the other side.

4. Functional surfaces based on hydrophobins biofilms

The capability of hydrophobins to adhere to various surfaces was one of the first observations among hydrophobins functions. An early finding on the behaviour of the class I hydrophobin SC3 from *Schizophyllum commune* was that when it binds to for example

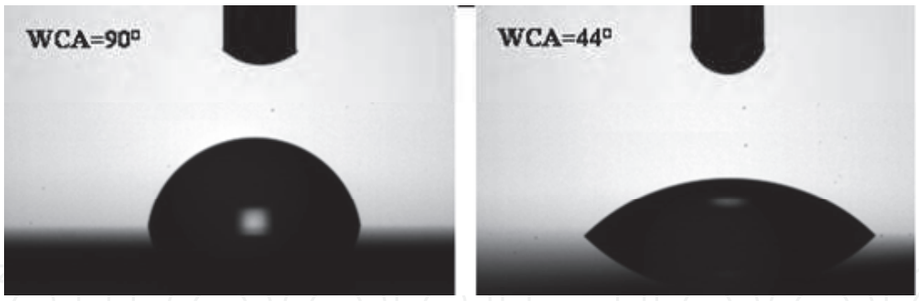


Fig. 14. Water contact angle measurements of bare silicon (A) and silicon coated with HFB (B).

Teflon, it can form a very insoluble layer (de Vocht et al. 2002). All hydrophobins adhere to surfaces, but there is a difference in the binding characteristics (Askolin et al. 2006). While class I members adhere very strongly, this is not seen for class II members which dissociate more easily.

Glass has been one of the widely used substrates for protein chips. Qin et al (2007a) have compared two methods for protein immobilization on glass slides, one is the traditional method of silanization (Mezzasoma et al. 2002); the other is the coating with the class II hydrophobin HFBI from *Trichoderma reesei*. The modified glass surfaces were characterized with X-ray photoelectron spectroscopy (XPS), water contact angle measurement (WCA) and immunoassay. The results have shown that HFBI coating can achieve the same result of protein immobilization on glass slides as silanization did, or even better. Moreover the HFBI coating does not require the complex procedure and strict surface cleanness as the silanization needs. HFBI-coated surfaces can be used immediately or preserved for some days prior to use. Therefore hydrophobin self-assemblies seems to be a simple and generic way for protein immobilization on glass slides.

Mica and polydimethylsiloxane (PDMS) are other substrates used for patterning of biomolecules. Freshly cleaved mica is a promising substrate for patterning applications, because it is the most readily available surface with atomic-scale flatness. Biomolecules, such as proteins, could not be immobilized on mica surfaces effectively without surface modification. PDMS is a kind of soft polymer with attractive physical and chemical properties: elasticity, optical transparency, flexible surface chemistry, low permeability to water, low toxicity, and low electrical conductivity. Thus, it has been widely used in microfluidic devices and microcontact printing technology. A simple method to modify both mica and PDMS surfaces by HFBI for protein immobilization has been recently developed (Qin et al. 2007b). XPS and WCA measurements illustrated that the wettability surface can be changed from superhydrophobic (PDMS) or superhydrophilic (mica) to moderately hydrophilic. The same surfaces, mica, glass, and PDMS, have been modified using the class I hydrophobin HGFI from *Grifola frondosa* (Hou et al. 2009). The surface wettability was efficiently changed by HGFI modification, as XPS and WCA measurements indicated. Furthermore data showed that self-assembled HGFI has better stability than type II hydrophobin HFBI: HGFI self-assembly was stable against rinsing by several solutions, i.e. 2% hot SDS solution and 60 vol.% ethanol.

Polystyrene and its variations are extensively used as solid supports to produce, for example, polystyrene-microtitre plates and tubes in immunoassays. Wang et al. (2010a) have

used the Class I hydrophobin HGFI to increase the hydrophilicity of polystyrene for facilitating immobilization of biomolecules. The adsorption process of HGFI on the polystyrene surfaces was studied by quartz crystal microbalance at different pH values, by XPS, WCA measurements and AFM analyses. By self-assembling, hydrophobin easily formed an intact charged film on the hydrophobic polystyrene that enhanced the hydrophilicity of the polystyrene for a long time (Wang et al. 2010a).

Silicon is the most used solid support in all micro- and nanotechnologies developed for the integrated circuits industry. For this reason, silicon is also used in many commercial technological platforms for biomedical and biosensing applications. The anisotropic wet micromachining of silicon, based on a water solution of potassium hydroxide (KOH), is a standard fabrication process that is extensively exploited in the realization of very complex microsystems such as cantilevers or membranes. A nanostructured self-assembled biofilm of Vmh2, was deposited on crystalline silicon and since this procedure formed chemically and mechanically stable layers of self-assembled proteins, the biomolecular membrane has been tested as masking material in the KOH wet etch of the crystalline silicon. The process has been monitored by SE and atomic force microscopy measurements. Because of the high persistence of the protein biofilm, the hydrophobin-coated silicon surface is perfectly protected during the standard KOH micromachining process (De Stefano et al. 2007). In Figure 15 the optical photos of the two silicon samples after this treatment, are shown: on the A image is clearly visible the etched surface whereas in the B image the HFB covered surface is perfectly homogeneous.

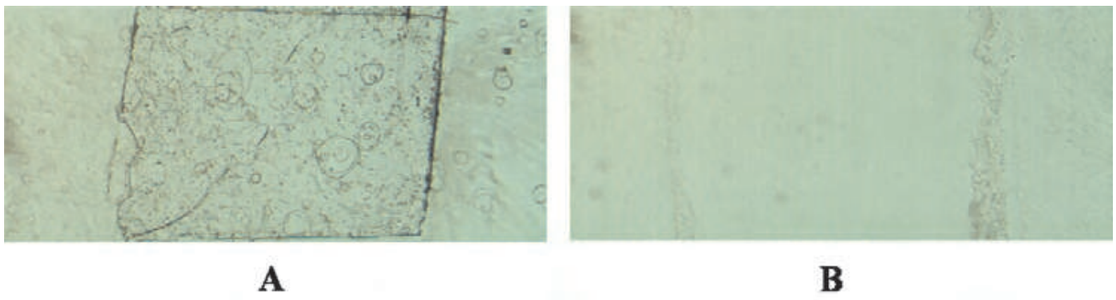


Fig. 15. Optical images of the silicon surface after the KOH etching: (A) bare silicon; (B) silicon coated with HFB.

This qualitative result is quantitative confirmed by ellipsometric and profilometric measurements: an 8.40 (0.05) nm biofilm of hydrophobins is still optically detected by the ellipsometer and the profilometer cannot detect any dig into the hydrophobins shielded sample, as it can be seen in Figure 16.

Porous silicon (PSi), a silicon related material obtained by silicon dissolution in an electrochemical cell in hydrofluoridic water solution, is really a versatile material owing to its peculiar morphological, physical, and chemical properties, allowing the easy fabrication of sophisticated optical multilayers, such as one-dimensional photonic crystals, by a simple electrochemical etching process. The reflectivity spectrum of the photonic crystals shows characteristic shapes, which are very useful in many applications, from biochemical sensing to medical imaging. The major drawback of the ''as etched'' PSi is its chemical instability: it has been shown that a PSi wafer can be dissolved under the physiological conditions that are very often used in biological experiments. De Stefano et al. (2008) have covered the Psi

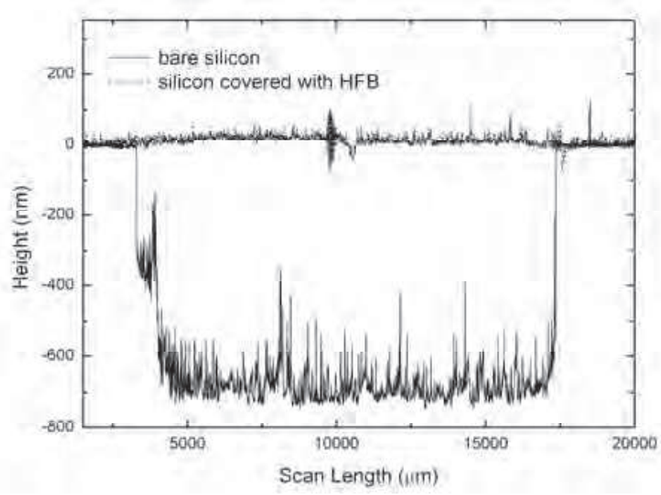


Fig. 16. Profilometric measurements of bare silicon (solid line) and silicon covered with HFB (dash line).

surface with the highly stable and resistant biofilm resulting from the self-assembly of Vmh2. The hydrophobin penetrates the whole stack, accumulating at the bottom and modifies strongly the wettability of the PSi surface. Moreover, the protein membrane not only protects the nanocrystalline material from basic dissolution in NaOH, but also leaves unaltered the sensing ability of such an optical transducer, adding chemical stability, which can be key in biomolecular experiments.

Gold is an excellent electric conductor with fine ductility and chemical inertness, thus an ideal choice for developing bioelectronic devices. HFBI modification on smooth gold surfaces has been proven to effectively enhance the surface hydrophilicity. The unmodified bare gold surface exhibited a weak hydrophilicity, while the surface hydrophilicity was remarkably improved after HFBI processing, as demonstrated by WCA measurements. The increase of surface wettability was not affected by the presence of washing procedures (Zhao et al. 2009).

Carbon nanotubes (CNTs, hollow cylinders made of sheets of carbon atoms) have recently emerged as building blocks of novel nanoscale structures and devices. CNTs have a variety of electronic properties that can be exploited for a variety of applications. Nanotubes have been functionalized to be biocompatible and to be capable of recognizing proteins (Shim et al. 2002). Often this functionalization has involved noncovalent binding between a bifunctional molecule and a nanotube in order to anchor a bioreceptor molecule with a high degree of control and specificity. Furthermore, CNTs are commonly insoluble in all solvents and usually form tangled network structures containing various impurities (Wu et al., 2010). To overcome these limits, CNT surfaces are often tailored using either covalent (Wu et al., 2007) or noncovalent modification (Hecht et al., 2006) strategies. A novel noncovalent approach has been developed for the functionalization of multi-wall carbon nanotubes (MWNTs, many layers to form concentric cylinders) using the class II hydrophobin, HFBI. The HFBI–MWNTs nanocomposite was characterized by scanning electron microscopy (SEM), transmission electron microscopy (TEM) and WCA. The hydrophobin HFBI demonstrates to be an efficient solubilizing agent for MWNTs. The resulting HFBI–MWNTs nanocomposite film with both merits of HFBI and MWNTs exhibited high hydrophilicity, fast electron-transfer kinetics and excellent electrocatalytic activity (Wang et al. 2010b).

The deposition of ceramic thin films from aqueous solutions at low temperature using biopolymers as templates has attracted much attention due to economic and environmental benefits. Titanium dioxide is one of the most attractive functional materials and shows a wide range of applications across vastly different areas because of its unique chemical, optical, and electrical properties. Santhia et al. (2010) deposited smooth, nanocrystalline titania thin films by an aqueous deposition method on a hydrophobin film. Firstly, the hydrophobins were self-assembled on a silicon substrate and characterized by XPS, AFM and surface potential measurements. Hydrophobin-modified silicon substrates were then used to deposit from aqueous solution at near-ambient conditions highly uniform, crack-free nanocrystalline TiO_2 thin films. Nanoindentation tests showed the high resistance against mechanical stress of the deposited titania films. The determined averaged hardness value and the Young's modulus highlight the compatibility of these films for coating implants and other biomedical applications.

5. Biosensors based on organic/inorganic interfaces

In recent times, there has been an increasing interest in interfacing biological molecules with nanomaterials and in understanding, controlling, and applying biomolecule-nanomaterial interactions for sensing. If the biomolecule of interest is a protein, a critical issue is the retention of its structure and activity on the nanoscale supports. It is of fundamental interest to understand how nanomaterial properties affect the structure, activity, and stability of conjugated proteins and identify optimal conditions to preserve functionality following protein immobilization. A similar argument is also important in the case of antibodies: all immunoassay based on the interaction between an antigen and its specific antibody critically depend on how the antibodies are immobilized on solid supports. In particular, it should be possible to bond the antibodies by their constant region, since the variable portion should be free in order to recognize and link the antigen. In general, the adsorption of any biomolecule to solid surfaces often induces structural changes that may affect the native functionality. This is a frequently observed phenomenon, and the resulting changes in structure, and function, can have profound consequences. When biomolecules are immobilized on hydrophobic surface, they can suffer considerable denaturation and lose biological activity due to strong hydrophobic interactions (Butler, 2004). In contrast, biomolecules have less conformational change and retain functional activity when are immobilized on hydrophilic surfaces, as the major driving force between biomolecules and the hydrophilic surface is the electrostatic force (Goddard and Hotchkiss, 2007; Kaur et al., 2004; Lubarsky et al., 2005). Moreover the strength and selectivity of protein-protein interactions make proteins excellent candidates to serve as linkers to form ordered structures (Wang 2010).

Taking into consideration the above mentioned evidences, self assembling proteins like hydrophobins that have the remarkable property of adhering to almost any surface forming stable amphiphylic films are very good candidates to easily manufacture stable, enzyme-based catalytic surfaces for applications in biosensing. This approach has a bearing for preparing stable enzyme-based catalytic surfaces in an easy, rapid, and reliable way. Within very short times, without resorting to covalent chemistry, enzymes can be stably immobilized on a solid surface. As a matter of fact several papers have been published reporting the use of hydrophobins to immobilize proteins.

Palomo et al. (2003) reported the binding of *Pleurotus ostreatus* hydrophobins to a hydrophilic matrix (agarose) to construct a support for noncovalent immobilization and activation of lipases. Lipase immobilization on agarose-bound hydrophobins resulted in increased lipase activity and stability. Its enantioselectivity was similar to that of lipases interfacially immobilized on conventional hydrophobic supports.

Two redox enzymes, glucose oxidase from *Aspergillus niger* (GOX) and horseradish peroxidase (HRP), were immobilized on glassy carbon electrodes coated with SC3 (Corvis 2005). It was shown that the immobilized GOX kept its activity on the 99th day of repeated use while HRP was active on the 36th day after immobilization. The affinity for the substrate was comparable for the immobilized and dissolved GOX and HRP, while the kinetic measurements indicate that the enzyme specific activity is lower for the immobilized, compared to the dissolved enzymes. The contact angle measurements suggest that the major contribution to the interactions involved in the immobilization of the enzymes on the SC3 layers comes from polar amino acids.

The Vmh2 modified silicon surface has been tested by BSA immobilization: solutions containing a rhodamine labelled BSA at different concentration, between 3 and 12 μM, have been spotted on the HFB film (De Stefano et al. 2009). The labelled bioprobes have been spotted also on other bare silicon samples, as a negative control on the possible aspecific binding between the silicon and the probe. All the samples have been washed in deionized water to remove the excess of biological matter and observed by the fluorescence microscopy system. Under lamp illumination, we found that the fluorescence of silicon-HFB-BSA system is brighter than the negative control. The fluorescence signal is also quite homogeneous on the whole surface also after a overnight washing in deionized water, which means that the strength of the affinity bond between the HFB and the BSA is enough to conclude that the HFB-BSA system is very stable.

Protein immobilization on the Vmh2 biofilm on silicon has been also verified and analyzed using an enzyme, POXC laccase. The enzymatic assay on the immobilized enzyme has been performed by dipping the chip into the buffer containing the substrate and by following the absorbance change during several minutes (pH 5 buffer, DMP as a substrate: a good trade-off between stability and activity of the enzyme). A 30 μl drop of the enzyme solution (about 700 U/ml) has been deposited on protein modified chips (1cmx1cm) and, after several washing, an activity between 0.1 and 0.2 U has been determined on each chip, resulting in an immobilization yield of 0.5÷1%. This value is comparable to that one (7%) obtained in the optimized conditions for laccase immobilization on EUPERGIT C 250L© (Russo et al. 2008). Taking into account the specific activity of the free enzyme (430 Umg^{-1}) and its molecular mass (59 kDa), 0.5μg of laccase corresponds to about 8 pmol (5 x 10^{12} molecules) immobilized on each chip. A reasonable evaluation of the surface occupied by a single protein molecule can be based on crystal structures of laccases. This surface should be 28 x 10^{-12} mm^2, considering the protein as a sphere with radius of 3 x 10^{-6} mm. On this basis, the maximum number of laccase molecule on each chip should be 3 x 10^{12}. These data indicate that the number of active immobilized laccase molecules on each chip is of the same order of magnitude than the maximum expected. Laccase assays have been repeated on the same chip after 24 and 48 hours in the same conditions. About one half of the activity has been lost after one day, but no variation of the residual activity has been observed after the second day. Moreover, comparison of these data with those of the free enzyme, stored at the same temperature, showed that the immobilized enzyme is significantly more stable than the free form.

A class I hydrophobin, HGFI purified by *Grifola frondosa*, has been used in modifying polystyrene wettability and also as a functional interface for immunofluorimetric assay. In particular, time resolved fluorescence assay has been used for the quantitative determination of carcinoembryonic antigen. A detection limit of 0.24 ng/mL is claimed by authors (Wang et al. 2010).
Even if class II is less chemically stable, HFBI has been used in glucose biosensing once decorated by multi wall carbon nanotube and glucose oxidase (Wang et al. 2010). A detection limit of 8.2 μM has been reached by amperometric measurements.
HFBI has also been used in coating gold surfaces for applications in electrochemical biosensing (Zhao et al. 2009). Choline oxidase has been immobilized on the gold electrode functionalized by HFBI and a very large current response as been registered on exposure to a choline substrate.
More futuristic applications of organic/inorganic interfaces based on hydrophobins on solid support can be forecasted by thinking at the new hydrophobins molecules that can be obtained by fusion proteins. Recently, HFBII, another hydrophobin from *Trichoderma reesei*, was employed as molecular carrier: it was tagged genetically onto a functional protein molecule, a Maltose binding protein (MBP), in order to construct a molecular interface. HFBII fusion proteins were intermingled with native HFBII molecules at the optimal ratio: the superfluous native HFBII molecules acted as nanospacers, resulting in the formation of a tight self-organized protein layer on both an air/water interface and a solid surface (Asakawa et al. 2009).

6. Conclusion

Beside improvement in transduction systems, readout electronics, and integrated microfluidics, the next generation of biosensors requires strong advances in fabrication and control of the organic/inorganic interfaces which are the first key issue to be developed for achieving reliable commercial devices. Understanding the fundamentals physico-chemical properties of these interfaces is the first step towards more performing instrumentation. The role of self-assembling, natural amphiphilic biomolecules, as hydrophobins are, could be of great impact on this multidisciplinary field of science and technology. The results obtained in research and industrial laboratories, reported in this chapter, are very promising, especially in biosensing applications. Hydrophobins –inorganic interfaces could be a kind of paradigm for the development of bio/non-bio based innovative devices.

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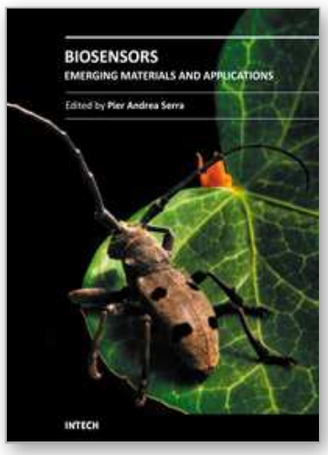
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A biosensor is a detecting device that combines a transducer with a biologically sensitive and selective component. Biosensors can measure compounds present in the environment, chemical processes, food and human body at low cost if compared with traditional analytical techniques. This book covers a wide range of aspects and issues related to biosensor technology, bringing together researchers from 19 different countries. The book consists of 27 chapters written by 106 authors and divided in three sections: Biosensors Technology and Materials, Biosensors for Health and Biosensors for Environment and Biosecurity.

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