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Chapter 2

Layered Biosensor Construction

Joanna Cabaj and Jadwiga Sołoducho

Additional information is available at the end of the chapter

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1. Introduction

Biosensors for last two decades make ideal sensing systems to monitor the effects of pollution on the environment, in the food or textile industry as well as medical diagnostic due to their biological base, ability to operate in complex matrices, short response time and small size.

In enzymatic devices, efforts have been concentrated on the control over enzyme activity, which is highly dependent on the interface between the electrode and the enzyme. Such control has led to immobilization techniques suitable for anchoring the enzyme close to electrode with preservation of its biological activity. In these type of devices, where retaining of the enzyme activity at the electrode/enzyme interface is the key to design efficient electrode, charge transfer between enzyme and electrode should be fast and reversible. Moreover, the charge transfer may also be optimized with some mediating particles (i.e. conducting units) being used in conjunction with the biological molecules at the electrode surface. To the use of conducting polymers for the fabrication of various biosensors have been dedicated extensively study due to their redox, optical, mechanical and electrical properties, as well as to their unique capability to act both, as transducers, and an immobilization matrices for enzyme retention [1].

It is essential for the sensitivity of the system that the recognition units have optimized surface density, good accessibility, long-term stability and minimized non-specific interactions with compounds other than the analyte. Such model molecular assemblies can be prepared by Langmuir-Blodgett (LB) and Langmuir-Schaefer (LS) techniques [2,3], layer-by-layer (LbL) or by employing self-assembly monolayers (SAMs) or electrolytic deposition (Fig. 1) [4,5]. The main advantage of using thin films to build a biosensor is the possibility to decrease dramatically the response time of the device. Langmuir-Blodgett type technology allows building up i.e. lamellar lipid stacking at an air/water interface, which can be easy

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transported onto a solid support. When all parameters are optimized, this technique corresponds to one of the most promising for preparing thin films of amphiphilic molecules. Based on the self-assembled properties of amphiphilic biomolecules at the air/water interface, LB technology offers the possibility to prepare biomimetic layers suitable for adsorptive immobilization of bioactive molecules [6].

Proteins are more challenging to prepare for the different microarray than i.e. DNA, and protein functionality is often dependent on the state of proteins. Since enzymes often significantly reduce their activity during immobilization, the optimized adsorption methods seem to be optimal for the retaining of conformational states of proteins on solid surfaces. Among the various immobilization techniques available, adsorption may have a higher commercial potential than other methods because the adsorption process is simpler, less expensive, retains a high catalytic activity, and most importantly, the support could be repeatedly reused after inactivation of the immobilized enzyme.

Enzyme-based biosensors play an important role in various industries, such as food, manufacturing, clinic, and environment. Recently, mediators have been employed in enzyme-based biosensors in order to shuttle electrons between the redox enzyme and the electrode surface. Solution-phase mediators may cause electrode contamination and operation inconvenience. In order to overcome the above-mentioned drawbacks and improve the performances of the biosensors, the immobilization of the mediator with protein on a solid support provides a new way to construct reagentless biosensors [7].

However, there is many techniques of biocatalysts immobilization and much research is dedicated to fabricate the biosensing elements, the construction of novel type of biosensor is challenge for new technologies and the key problem is modification of electrode by enzyme using thin film preparation methods.

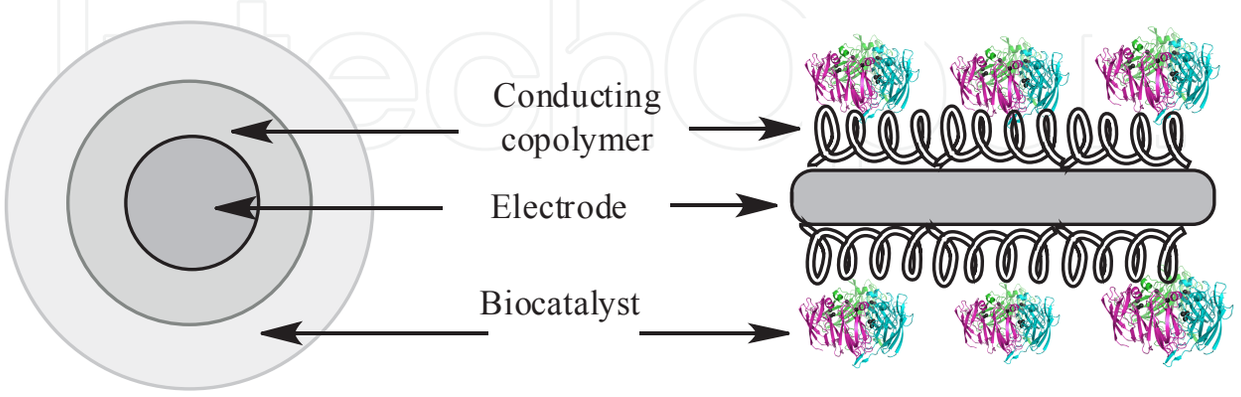


Figure 1. Layered biosensor system

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2. Langmuir-Blodgett, Langmuir-Schaefer, layer-by-layer assembly multilayers of proteins

The concept of using biomolecules as an elementary structure to develop self-assembled structures of defined geometry has thus received considerable attention. In this way, the self-assembly ability of amphiphilic biomolecules such as lipids, to spontaneously organize into nanostructures mimicking the living cell membranes, appears as a suitable concept for the development of biomimetic membrane models. The potential of two-dimensional molecular self-assemblies is clearly illustrated by Langmuir monolayers of lipid-like molecules, which have been extensively used as models to understand the role and the organization of biological membranes and to acquire knowledge about the molecular recognition process [6,8]. Langmuir-Blodgett technology allows building up lamellar lipid stacking by transferring a monomolecular film formed at an air/water interface – named Langmuir monolayer or Langmuir film– onto a solid support (Figure 2).

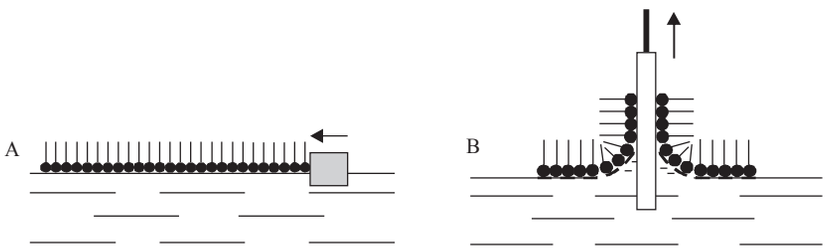


Figure 2. Langmuir – Blodgett deposition

Lipid-based phases are particularly attractive because they can be nanostructurally customized, for instance, to closely resemble cellular components, or to formulate delivery vehicles for biomolecules and drugs. In the presence of water or aqueous buffers, lipid molecules can self-assemble into a wide range of nanostructures [9]. The intrinsically low degree of nonspecific adsorptivity of supported membranes makes them interesting as an interface between the nonbiological materials on the surface of a sensor or implant and biologically active fluids [10,11]. Potential applications include the acceleration and improvement of medical implant acceptance, programmed drug delivery, production of catalytic interfaces, as a platform to study transmembrane proteins and membrane-active peptides, and as biosensors [12-14].

Although the LB method does not solve all problems associated with engineering the structure of condensed phases, it does provide a level of control over the orientation and placement of molecules in monolayer and multilayer assemblies that is not otherwise available. When all parameters are optimized, this technique corresponds to one of the most promising for preparing thin films of amphiphilic molecules as it enables an accurate control of the thickness, an homogeneous deposition of the monolayer over large areas compared to the dimension of the molecules, as well as the possibility to transfer monolayers on almost any kind of solid substrate. Based on the self-assembled properties of amphiphilic biomolecules

at the air/water interface, LB technology offers the possibility to prepare biomimetic layers suitable for immobilization of bio-active molecules [8].

Systems mimicking natural membranes appear promising in the field of bioelectronic devices and represent useful models in basic research on membrane behavior in life science. For such purposes, the interest of LB films is now largely recognized, and several enzyme sensors based on the LB technology have been reported (Table 1).

Protein	Immobilization method	Thin ordered film	Stability	References
Glucose oxidase	Lipid - coating	LB – two layers film of lipids	3 month	[15]
Catalase	Adsorption	LB – one layer film of phospholipids	>3 months	[16]
Laccase	Adsorption	LB – five layers film of benzothiadiazole copolymers in mixture with linoleic acid	>3 months	[2]
Invertase	Adsorption	LS – one layer film of phospholipids and octadecylamine	>4 months	[17]
Cellulase	Adsorption	LS film of cellulose	No data	[18]
Glucose oxidase	Adsorption	LbL films of alternate layers of poly(allylamine) hydrochloride and glucose oxidase	20 days	[19]
Urease	Adsorption and covalent grafting	LbL multilayer films of alternate charged polysaccharides, chitosan and polyaniline	3 weeks	[20]
β-Galactosidase, glucose oxidase, peroxidase	Adsorption	self-assembled monolayer of a 3-mercaptopropionic acid	4 weeks	[21]
Cholesterol oxidase	Adsorption	LB layers of octadecyltrimethylammonium and nano-sized Prussian blue clusters	No data	[22]

Table 1. Selected biosensor based on thin ordered films

In particular LB films are offering a possibility of obtaining extended two–dimensional π-electron systems [23]. Although, majority of conventional conducting polymers are not soluble in common solvents, making any LB deposition impossible, one can increase their solubility by attachment of side groups (usually n–alkyl ones) to the main chains. According to this Langmuir-Blodgett, horizontal lifting or other self–assembled method is employed for obtaining molecular films of conducting structures. This type of material is popular in designing of sensor devices. Product in any solid-state sensor, analyte molecules have to diffuse into and react with the acting sensing component and any product of the reaction must

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diffuse out. It therefore follows that the thinner the sensing layer is, the less time this will take and thereby speed and reversibility being improved.

Device preparation requires use of facilitative methodologies for the organization of biological components in a particular configuration. Self-assembled monolayer and LB methods have been used for organization of functional elements in two-dimensional or layered structures, respectively. These methods offer opportunities for immobilization of functional components into well-organized structures. As a convenient methodology alternate layer-by-layer adsorption has been paid much attention as an emerging methodology.

Recent research has proved the great applicability of the LbL technique not only for preparation of bio-related devices but also for producing various device structures, including sensors [24], photovoltaic devices [25], electrochromic devices [26], fuel cells [27].

Materials that can be used in LbL methods cover a wide range including conventional polyelectrolytes, conductive polymers, dendrimers, proteins, nucleic acids, saccharides, virus particles, inorganic colloidal particles, quantum dots, clay plates, nanosheets, nanorods, nanowires, nanotubes, dye aggregates, micelles, vesicles, LB film, and lipid membranes [28]. In most cases, the LbL assembly is carried out based on electrostatic interactions. As illustrated in Figure 3, adsorption of counterionic species at relatively high concentrations leads to excess adsorption of the substances, as a result of charge neutralization and resaturation, finally resulting in charge reversal.

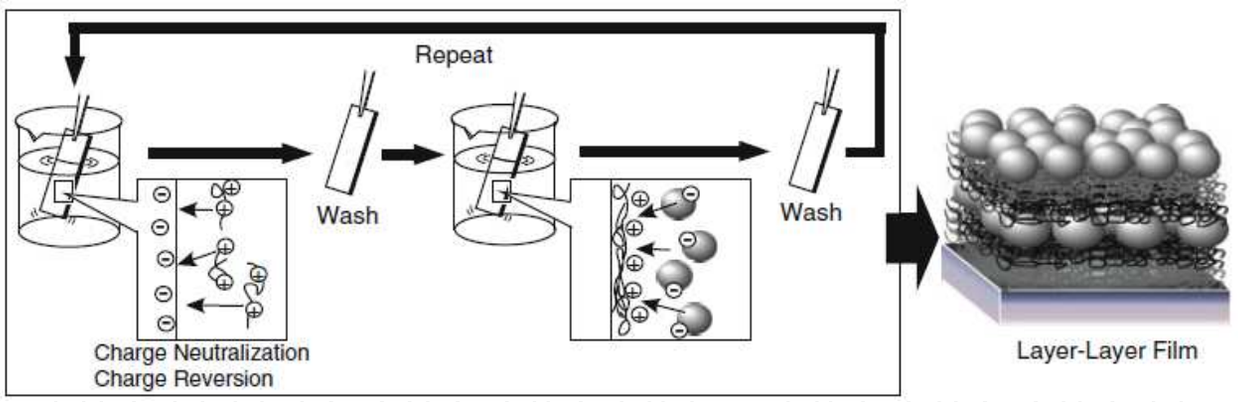


Figure 3. Layer-by-Layer assembly [28]

The forces for LbL assembly are not limited to electrostatic interactions alone. Various interactions including metal-ligand interaction, hydrogen-bonding, charge transfer, supramolecular inclusion, bio-specific recognition, and stereo-complex formation can be used for LbL assembly [28]. Biocompatibility is the most prominent advantage of the LbL assembly because this procedure requires mild conditions for film construction. Most proteins, especially those soluble in water, have charged sites on their surface, and so the electrostatic LbL adsorption is useful for the construction of various protein organizations.

In order the aim to develop models mimicking biomembranes usable for applications in the biosensor field, studies of biological activities of membraneous proteins after incorporation

in a phospholipidic bilayer were widely investigated [29,30]. Well known is the sensing system built by the incorporation of glutamate dehydrogenase or choline oxidase into fatty acid LB films through an adsorption or an inclusion process which consists of sandwiching the protein molecules between two LB layers [31]. The molecules of glutamate dehydrogenase adsorbed on the surface of behenic acid LB films work as a protective screen against the rearrangement of the multilayers induced by diffusion of the alkaline buffer inside the structure; on the contrary, the choline oxidase molecules operate as an accelerating factor of the structural lipidic reorganization induced in the same conditions.

The physiological activity of transmembrane proteins, however may depend on the physico-chemical properties of neighboring phospholipids. Such dependence has been demonstrated in the case of, among others, hydroxybutyrate dehydrogenase, Ca^{2+}-ATPase and melibiose-permease. Moreover, all integral membrane proteins are surrounded by a layer of phospholipids, the annular region, which provides the adequate lateral pressure and fluidity to seal the membrane during the changes in the protein during transport events [32].

2.1. Main membrane lipids

All the amphiphilic molecules are potentially surface active agents and substantially monolayer-forming materials. It is possible to find a discussion on the range of a large variety of amphiphile compounds able to form insoluble monomolecular films [33]. Due to the synthesis of biomimetic membranes, the most important types of amphiphilic molecules are fatty acids, phospholipids and glycolipids. Cholesterol as a type of steroid extremely abundant in the cell membrane, can also form insoluble monolayers but it is generally more studied mixed with other phospholipids [34-36] in order to its implication in the formation of lipid microdomains. Figure 4 presents the examples of the principal structures of these different types of lipids. The amphiphilic nature of biological surfactants is responsible for their aggregation at the air/water interface. Their affinity for the air/water interface is determined by the physico-chemical properties of the hydrophilic and hydrophobic parts. The monolayer forming abilities of the amphiphiles is dependent on the balance between these two opposite forces, which are determined by the size of the hydrophobic tail group (i.e. the alkyl chain) and the strength of the hydrophilic head group (i.e. size, polarity, charge, hydration capacity) [6]. If the equilibrium between hydrophilic and hydrophobic part of molecule is disturbed, the material dissolve in the subphase and is not able to form a stable monolayer.

Most of the lipidic cell membrane components are composed of a zwitterionic head group at pH 7.0 (phospholipids) or contain a highly hydrophilic polar group (glycolipids), and a hydrophobic part which is constituted by two hydrocarbon chains per molecule and drastically reduces the water solubility of the complete lipidic membrane molecule. Consequently, many components of cell membranes form insoluble monolayers at the air/water interface since the lipid concentration in the aqueous subphase is negligible, and some of them may be built up into multilayer films by Langmuir-Blodgett deposition.

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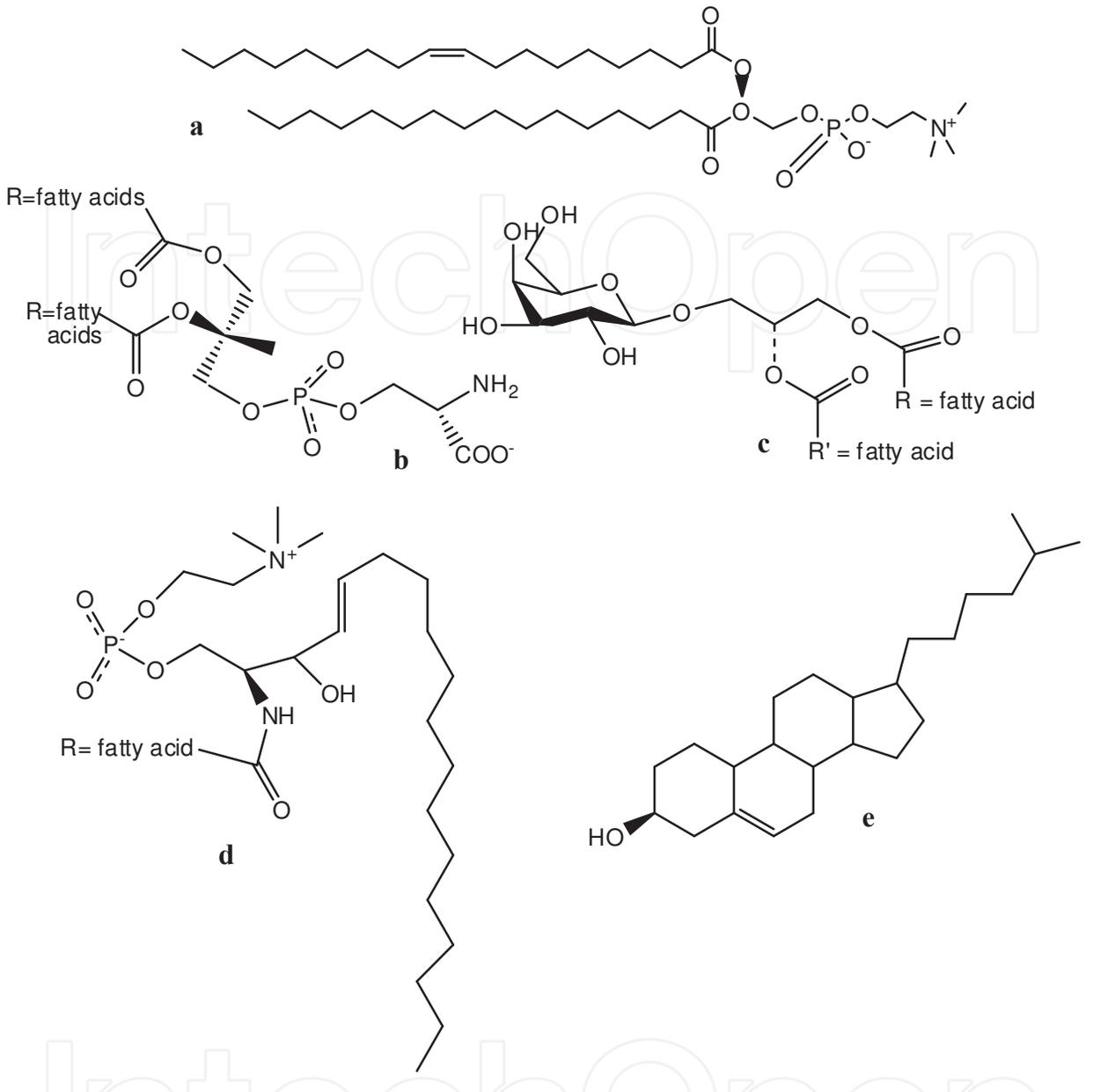


Figure 4. Examples of the main membrane lipids; 1) phospholipids: a, phosphatidylcholine; b, phophatidylserine; 2) glycolipids: c, monogalactosyldiglyceride; 3) sphingolipids: d, sphingomyelin; e, cholesterol

3. Protein adsorption at the solid/liquid interface

Protein adsorption at solid/solution interface has been a research focus for more than three decades due to its importance in the development of biocompatible materials, various biotechnological processes, food and pharmaceutical industries, and promising new areas such as biosensor, gene microarray, biochip, biofuel cell and so on. In order to control and manipulate protein adsorption, the mechanisms which govern the adsorption process need to be well understood.

The effect of variables like pH, temperature, the ionic strength, the properties of the protein and the surface, the nature of the solvent and other components on protein adsorption have been studied. Protein adsorption is a very complex process (Figure 5), which is driven by different protein-surface forces, including van der Waals, hydrophobic and electrostatic forces. Attention is also paid to the structural rearrangements in the protein, dehydration of the protein and parts of the surfaces, redistribution of charged groups in the interfacial layer and the role of small ions in the overall adsorption process. Protein adsorption also depends on the chemical and physical characteristic of the surface. Conformational changes in the protein can greatly contribute to the driving force for adsorption. Proteins are highly ordered structures (i.e., states of low conformational entropy). Partial or complete unfolding of the protein on the sorbent surface leads to an increase in conformational entropy, which can be the driving force for protein adsorption. To assess the tendency of proteins to unfold on surfaces, it is important to have a clear picture of protein stability.

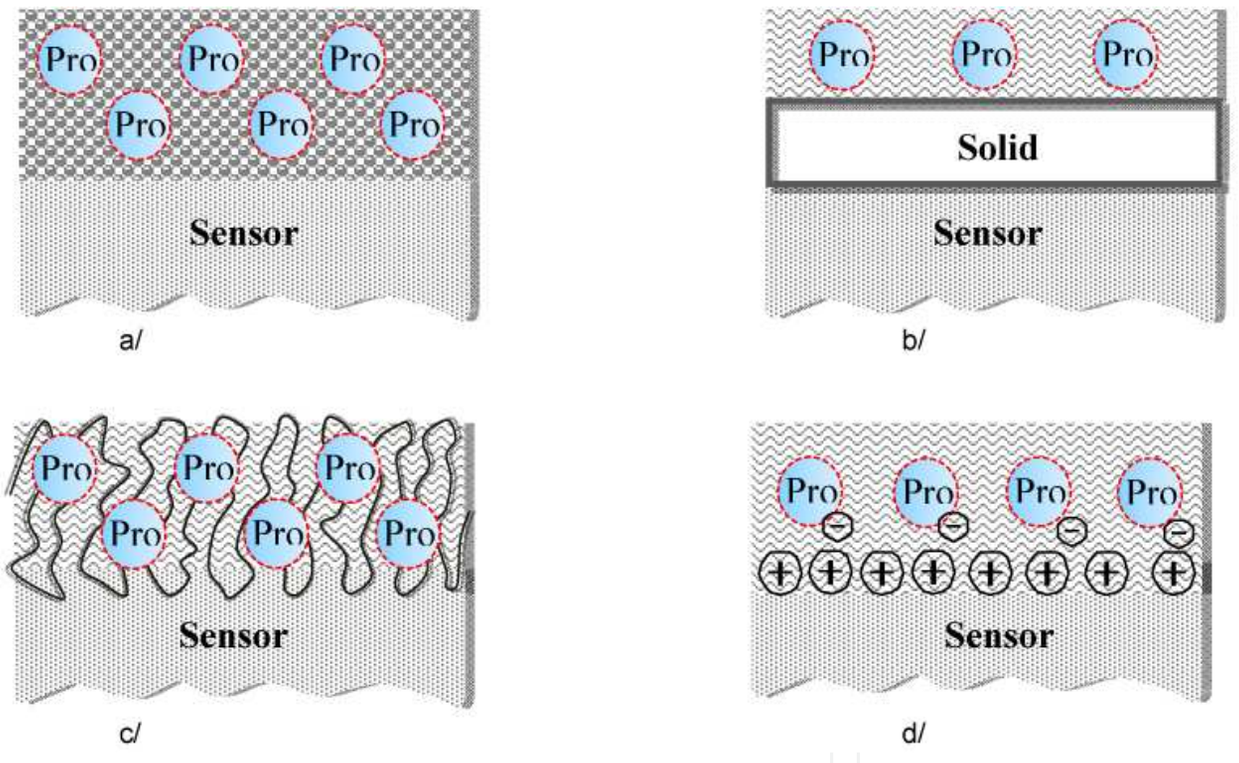


Figure 5. Protein adsorption; a/ adsorption in membrane, b/ classic adsorption on solid, c/ encapsulation in porous material, d/ protein with LbL assembly

Now is generally accepted that the adsorption behavior of proteins at relatively high concentrations often does not follow the true equilibrium isotherm because the slow relaxation of nonequilibrium structure leads to multilayer build-up [37, 38]. Such behavior can be monitored by atomic force microscopy (AFM) measurements, neutron reflection, dual polarization interferometry, circular dichroism, and Fourier transform infrared attenuated total reflectance (FTIR/ATR) [39] as well as other techniques.

The process of macromolecular multilayer adsorption, is still too complicated to be effectively modeled by kinetic models. At low concentrations, an interfacial cavity kinetic model has been used to characterize monolayer or submonolayer protein adsorption with surface-induced structural transitions [39].

Many studied projects have focused on the effect of various modifications in the adsorption systems, including surface modification [40], protein modification [41], the use of saccharides [42] and surfactants [43], and adjustment of solvent conditions such as ionic strength and pH [38, 44], for the purpose of either reducing or promoting protein adsorption.

Adsorption capacity of cytochrome *c* on chelated Cu^{2+} bead was demonstrated to be dependent on the buffer type with the observed adsorption in the order phosphate >*N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid >morpholinopropane sulfonic acid >morpholinoethane sulfonic acid >tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl) [45]. Vasina and Dejardin reported that the adsorption of α-chymotrypsin on muscovite mica was depressed by increasing the concentration of Tris-HCl buffer at pH 8.6, close to α-chymotrypsin's isoelectronic point [46]. Phosphate buffered saline (PBS) is the most commonly used buffer at the pH range close to 7, since it is reported to be able to stabilize protein structure in bulk solution environment in most cases [47]. The behavior of PBS buffer is particularly complex in adsorption studies due to the various types of phosphate ions present and the tendency of these ions to adsorb competitively and/or to form complexes either with the proteins or with the surfaces.

It is also well known that protein size and net charge have significant effects on adsorption. Changes in protein secondary structure are frequently monitored as indications of denaturing. Denaturing upon adsorption is important in many applications such as implants, biofueling. Quantification of secondary structure is sensitive to the peak assignments.

Adsorption of a protein to a surface may induce conformational changes in the protein. The degree of conformational changes is determined by a combination of the native stability of a protein, the hydrophobicity and the charges of the protein and the sorbent surface. Protein adsorption can be driven by a conformational entropy gain especially if adsorption is endothermic. This entropy gain can arise from the release of the solvent molecules from hydrophobic patches on the protein surface. Loses of translation entropy of the protein may play a minor rule [48].

Norde et al. [49] study the thermodynamics for adsorption of human serum albumin (soft) and ribonuclease (hard) on polystyrene surface (hydrophobic). That was reported that a net increase in entropy on a like-charged polystyrene surface drives the adsorption process for both proteins. The entropy increases because hydrophobic parts of the polystyrene surface gets dehydrated and structural changes in the proteins allowed the molecule feel free. Proteins with low Gibbs energy of denaturation (i.e., a protein with low native-state stability is called soft protein) are driven by entropy gains associated with the breaking down of secondary and tertiary protein structure.

4. Immobilization of protein monolayers on planar solid supports

The concept of using self-assembled biomolecules as an elementary units to develop superstructures of defined geometry has thus received considerable attention. In this contents, the self-assembly ability of amphiphilic biomolecules such as lipids, to spontaneously organize appears as a suitable concept for the development of membrane models. The concept is clearly illustrated i.e. by Langmuir monolayers, which have been extensively used as models to understand the role and the organization of biological membranes [50] and to acquire knowledge about the molecular recognition process [51,52]. Langmuir-Blodgett technology allows to build lamellar lipid stacking by transferring a monomolecular film formed at an air/water interface onto a solid support. When all parameters are optimized, this technique corresponds to one of the most promising for preparing thin films of amphiphilic molecules [6]. The sensitive element produced by LB technology has higher sensitivity and faster response time, can work in room temperature.

The optimal value of the surface pressure to produce the best results depends on the nature of the monolayer and is often established empirically [53]. However, the LB/LS deposition is traditionally carried out in the condensed phase since it is generally believed that the transfer efficiency increases when the monolayer is in a close-packed state. In that condition the surface pressure is sufficiently high to ensure a strong lateral cohesion in the monolayer, so that the monolayer does not fall apart during the transfer process. Although the optimal surface pressure depends on the nature of the material constituting the film, biological amphiphiles can seldom be successfully transferred at surface pressures lower than 10 mN/m and at surface pressures above 40 mN/m, where collapse and film rigidity often pose problems [6].

Moreover, the main advantage of the adsorption of the enzyme onto pre-formed LB films lies in the possible interaction of the enzyme with a hydrophobic or hydrophilic surface depending on the number of the deposited layers, thus allowing the control of the enzyme environment. Likewise, this approach allows the control of the thickness and the homogeneity of the LB films harboring the enzymes. Nevertheless, the release of protein molecules due to the weakness of their association with the surface is often the main reason which explains the poor reproducibility of responses of LB membrane-based sensors. Due to avoid desorption, some authors have proposed to covalently immobilize the enzyme on LB film surfaces by the use of cross-linking agents [54].

Electrostatic layer-by-layer assembly was first proposed by Decher in 1990s and proved to be possible to build-up ordered multilayer structures by consecutive adsorption of polyanions and polycations [55]. This film assembly approach has great advantages because of the simplicity preparation of ultrathin films with defined composition and uniform thickness in nanoscale in which synergy between distinct materials may be achieved in a straightforward, low-cost manner. With the LbL technique a wide diversity of materials may be employed, and film fabrication is performed under mild conditions, which is particularly important for preserving activity of biomolecules. The fundamental concepts and mechanisms involved in the LbL technique have been detailed in a series

of papers [56]. In most cases adsorption in LbL films is governed by electrostatic interactions between species bearing opposite charges, but secondary interactions have also been shown to be important. The LbL technique is versatile with regard to the substrates that may be used, which include hydrophilic and hydrophobic glass, mica, silicon, metals, quartz, and polymers [57]. In addition, LbL films may be deposited directly onto colloidal suspensions [58].

Several attempts have been made to fabricate hybrid enzyme electrodes with the method [59]. In 1995 this new method was applied to immobilize negatively charged glucose oxidase (GOx) in a polyethyleneimine based multilayer structure [60] and proved to be one of the most perspective methods for preparing amperometric enzyme biosensors. One year later, an oxygen mediated glucose biosensor based on GOx and poly(L-lysine) co-adsorbed onto a negatively charged monolayer of mercaptopropionic acid, deposited on an Au electrode was described [60]. Hodak *et al*. introduced LbL assembly technique to construct reagentless biosensor with glucose oxidase and ferrocene modified with poly(allylamine). Sun *et al*. [61] and Chen *et al*.[62] fabricated peroxidase and glucose oxidase biosensors with Os-based redox polymer and enzymes. Also known isreagentless biosensor built of organic dye methylene blue with peroxidase [63]. Vossmeyer and co-workers investigated the optical and electrical properties of layer-by-layer self-assembly of gold nanoparticle/alkanedithiol films [64]. Though gold nanoparticles or enzymes have been widely used to form multilayer films by layer-by-layer technology.

5. Biorecognition elements

Bioreceptors or biorecognition elements are the key to specification of biosensor technologies. They are responsible for binding the analyte of interest to the sensor for the measurement. These bioreceptors can take many forms and the different bioreceptors that have been used are as numerous as the different analytes that have been monitored using biosensors. However, bioreceptors can generally be classified into a few different major categories (Figure 6). These categories include: antibody/antigen, enzymes, nucleic acids/DNA, cellular structures/cells (i.e. microorganisms), and biomimetics. The specificity of molecular recognition makes these molecules very attractive as tools for therapeutic diagnostic and other analytical applications.

5.1. Enzymes

Enzymes are the most commonly used bio-receptors in bioassays. The analyte can be the enzyme, whose enzymatic activity is determined, or the substrate or the enzyme cofactors. Enzymatic assays are mainly based on either inhibition of the enzyme activity or catalysis. According to the fact, variety of enzymes such as organophosphorous hydrolase, alkaline phosphatase, ascorbate oxidase, tyrosinase and acid phosphatase have been employed in design of pesticide bioassays and biosensors [65].

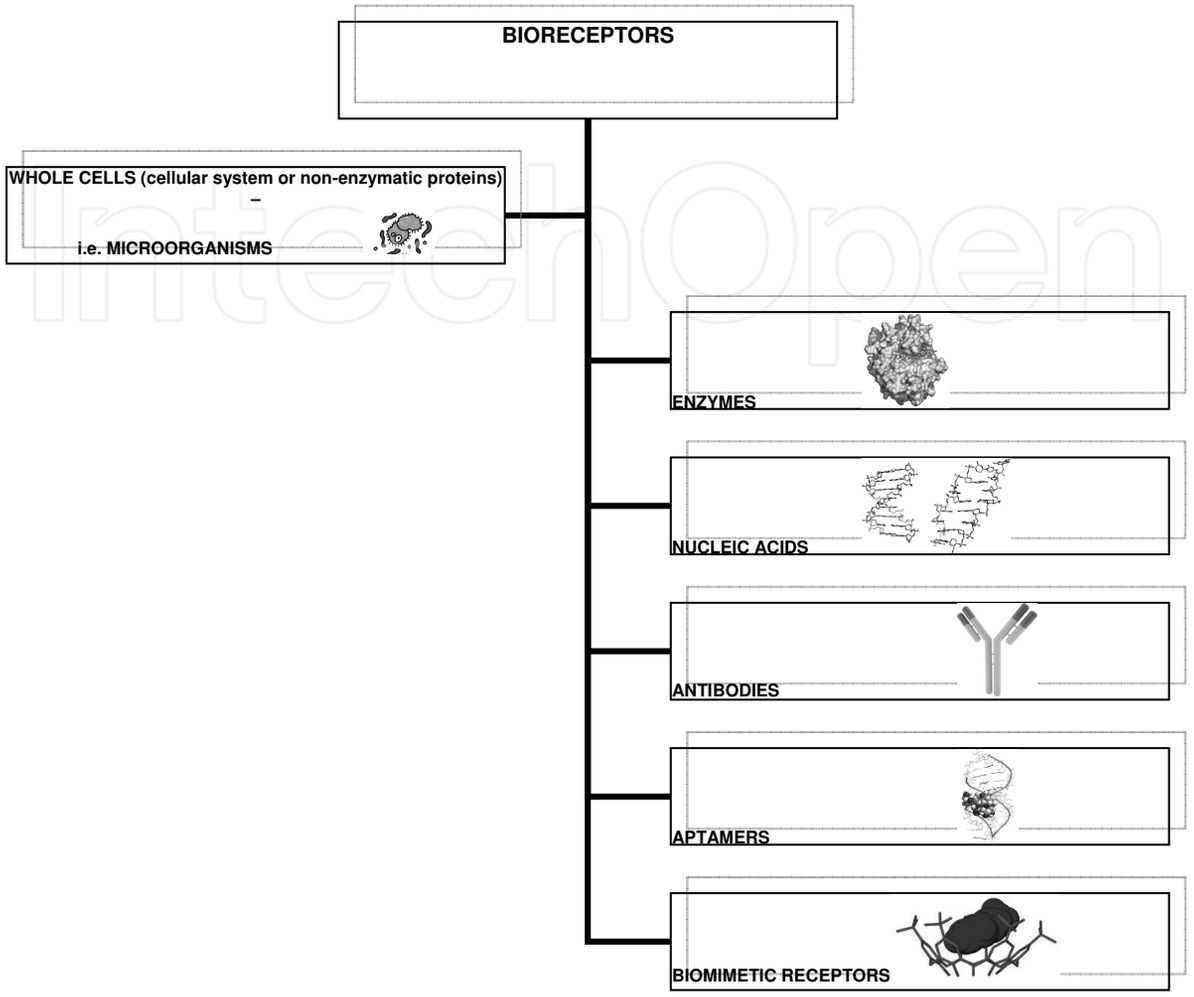


Figure 6. Classification of bioreceptors

Enzymes are often chosen as bioreceptors based on their specific binding capabilities as well as their catalytic activity. In biocatalytic recognition mechanisms, the detection is amplified by a reaction catalyzed by macromolecules - biocatalysts. With the exception of a small group of catalytic ribonucleic acid molecules, all enzymes are proteins. Some enzymes require no chemical groups other than their amino acid residues for activity. Others require an additional cofactor, which may be either one or more inorganic ions ($Fe^{2+,}$ Mg^{2+}, Mn^{2+}, or Zn^{2+}), or a more complex coenzyme. The catalytic activity provided by enzymes allows for much lower limits of detection than would be obtained with common binding techniques. The catalytic activity of enzymes depends upon the integrity of their native protein conformation. Enzyme-coupled receptors can also be used to modify the recognition mechanisms. In example, the activity of an enzyme can be modulated when a ligand binds at the receptor. This enzymatic activity is often greatly enhanced by an enzyme cascade, which leads to complex reactions in the cell [66].

The use of enzymes as the recognition element was very popular in the first generation of biosensor due to their availability. Among various oxidoreductases, glucose oxidase, horseradish peroxidase and alkaline phosphatase have been employed in most biosensor studies [67-69]. Some amperometric based methods use duel enzyme systems such as acetylcholine esterase and choline oxidase. In example, organophosphorous hydrolase catalyzes the hydrolysis of a wide range of organophosphate pesticides, and as a result of its versatility, this enzyme has been incorporated into a number of assays and sensors for the detection of this type of compounds. Additional enzymes can be used to detect other environmental and food contaminants such as nitrate, nitrite, sulfate, phosphate, heavy metals and phenols. Tyrosinase is frequently used to determine phenols, chlorophenols, cyanide, carbamates and atrazine.

Enzymes offer many advantages connected with high sensitivity, possibility of direct visualization and stability, but there are still some problems, which include multiple assay steps and the possibility of the interference from endogenous enzymes. Many enzyme detection procedures are visual eliminating the need for expensive equipment, but the enzyme stability is still problematic and the ability to maintain enzyme activity for a long time [70].

5.2. Nucleic acids

Recently, advances in nucleic acid recognition have enhanced the using of DNA biosensors and biochips [71]. In the case of nucleic acid bioreceptors for pathogen detection, the identification of analyte's nucleic acid is achieved by matching the complementary base pairs. Since each organism has unique DNA sequences, any self-replicating microorganism can be easily detect [70].

Grabley and coworkers have reported on the use of DNA biosensors for the monitoring of DNA-ligand interactions [72]. Surface plasmon resonance was used to monitor real-time binding of low molecular weight ligands to DNA fragments that were irreversibly bound to the sensor surface *via* coulombic interactions. The detection of specific DNA sequences has been employed for detecting microbial and viral pathogens [73] and food pathogen like *E. coli* [74], *Salmonella sp*.

Recent advances in nucleic acid recognition, like the introduction of Peptide Nucleic Acid (PNA) has opened new opportunities for DNA biosensors. PNA is a synthesized DNA in which the sugar-phosphate backbone is replaced with a pseudopeptide. PNA as a probe molecule has several advantages, i.e. superior hybridization characteristics, detection of single-based mismatches, better stability compared to enzymes [70].

5.3. Antibodies

Antibodies are common bioreceptors used in biosensor technologies. Antibodies are biological molecules that exhibit very specific binding capabilities for specific structures. This is very important due to the complex nature of most biological systems. An antibody is a complex biomolecule, made up of hundreds of individual amino acids arranged in a highly ordered sequence. For an immune response to be produced against a particular molecule, a

certain molecular size and complexity are necessary: proteins with molecular weights greater than 5000 Da are generally immunogenic.

The way in which an antigen and its antigen-specific antibody interact is analogues of a lock and key fit [66], by which specific geometrical configurations of a unique key enables it to open a lock.

An antigen-specific antibody fits its special antigen in a highly specific manner, according to that the three-dimensional structures of antigen and antibody molecules are matching [70]. Antibody biosensors are interested wide and interesting group of sensing devices, which includes i.e. Surface plasmon resonance [74], fiber-optic biosensor [75], magnetoelastic resonance sensor [76] and immunosensor [77].

5.4. Aptamers

Aptamers are folded single stranded DNA or RNA oligonucleotide sequences with the capacity to recognize various target molecules. They are generated in the systematic evolution of ligands by exponential enrichment process which was first time reported by Ellington [78] and Tuerk [79]. In this way suitable binding sequences are first isolated from large oligonucleotide libraries and subsequently amplified. The main application for aptamers is in biosensors. While antibodies are used in ELISA, the similar process for aptamers is called ELONA (enzyme linked oligonucleotide assay). They have many advantages over antibodies such as easier deposition on sensing surfaces, higher reproducibility, longer shelf life, easier regeneration and a higher resistance to denaturation. As antibodies, they are characterized by both, their high affinity and specificity to their targets [80].

5.5. Biomimetic receptors

A receptor which is designed and fabricated and to mimic a bioreceptor is often defined as biomimetic receptor. According to the phenomena several different techniques have been developed over the years for the construction of biomimetic receptors [81,82]. These procedures include: genetically engineered molecules, artificial membrane fabrication and molecular imprinting method. The molecular imprinting method has existed as an attractive and accepted tool in developing an artificial recognition agents.

Artificial membrane fabrication for bioreception has been performed for many different applications. Stevens *et al.* has developed an artificial membrane by incorporating gangliosides into a matrix of diacetylenic lipids [83]. The lipids were allowed to self-assemble into Langmuir-Blodgett layers and were then photopolymerized *via* ultraviolet irradiation into polydiacetylene membranes. However, molecular imprinting has been used for the construction of a biosensor based on electrochemical detection of morphine [84].

5.6. Cellular bioreceptors

Cellular structures and cells have been used in the development of biosensors and biochips. These bioreceptors are either based on biorecognition by an entire cell/microorgan-

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ism or a specific cellular component that is capable of specific binding to certain species. There are presently three major subclasses of this category: a) cellular systems, b) enzymes and c) non-enzymatic proteins. Due to the importance and large number of biosensors based on enzymes, these have been given their own classification [85]. Microorganisms offer a form of bioreceptor that often allows a whole class of compounds to be monitored. Generally these microorganism biosensors rely on the uptake of certain chemicals into the microorganism for digestion. Often, a class of chemicals is ingested by a microorganism, therefore allowing a class-specific biosensor to be created. Microorganisms such as bacteria and fungi have been used as indicators of toxicity or for the measurement of specific substances. For example, cell metabolism (e.g., growth inhibition, cell viability, substrate uptake), cell respiration or bacterial bioluminescence have been used to evaluate the effects of toxic heavy metals [85].

A microbial biosensor has been developed for the monitoring of short-chain fatty acids in milk [86]. *Arthrobacternicotianae* microorganisms were immobilized in a calciumalginate gel on an electrode surface. By monitoring the oxygen consumption of the *Anthrobacter* electrochemically, its respiratory activity could be monitored, thereby providing an indirect means of monitoring fatty acid consumption.

6. Quantitative detection of protein binding to the solid surface

Proteins adsorb in differing quantities, densities, conformations, and orientations, depending on the chemical and physical characteristics of the surface [87]. Protein adsorption is a complex process involving van der Waals, hydrophobic and electrostatic interactions, and hydrogen bonding. Although surface-protein interactions are not well understood, surface chemistry has been shown to play a fundamental role in protein adsorption. Moreover, the properties of protein over-layers can be altered by the underlying chemistry, which directly impinges on control of conformation and/or orientation [88].

During the past decade substantial progress has been made in understanding the mechanism of protein adsorption. Authors have reported a numerous of techniques, e.g. QCM [89, 90], surface plasmon resonance (SPR) [91,92], ellipsometry [91], FTIR [93], atomic force microscopy (AFM) [94].

6.1. Quartz crystal microbalance measurements

QCM (Quartz Crystal Microbalance, Fig. 7) technology enables studies of molecular interactions by measuring the weight of the molecules, much like a very sensitive scale or balance. When molecules are added to or removed from the sensor surface, it is detected as a change in the oscillation frequency of the sensor crystal; the change in resonance frequency is correlated to the change in mass on the surface. QCM technology does not have the same limitations with regard to surface proximity as other biosensor technologies, making it possible for the instrument to measure binding to large structures such as cells.

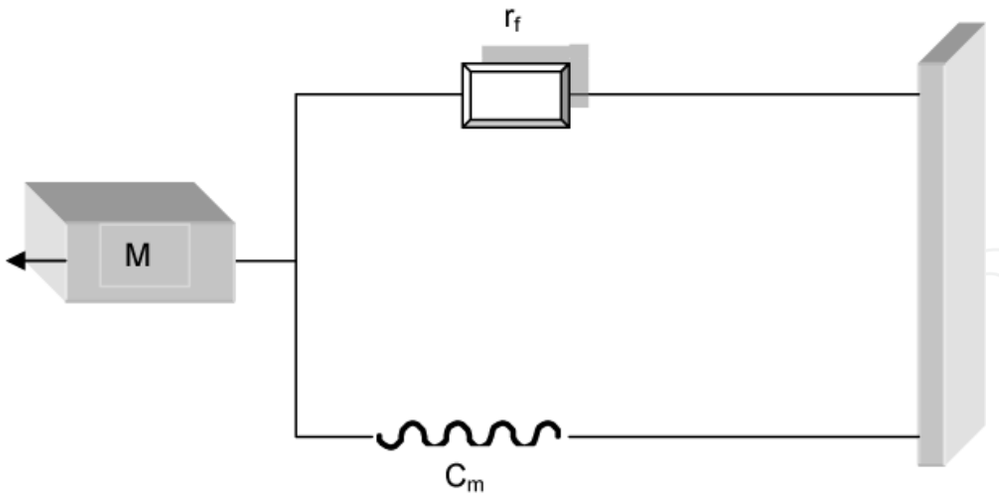


Figure 7. Quartz Crystal Microbalance - equivalent mechanical model; mass (M),a compliance (Cm), and a resistance. (rf). The compliance represents energy stored during oscillation and the resistance represents energy dissipation during oscillation

The method is very useful for monitoring the rate of deposition in thin film deposition systems under vacuum. In liquid, it is highly effective at determining the affinity of molecules (i.e. proteins) to surfaces functionalized with recognition sites. Larger entities such as viruses or polymers are investigated, as well. QCM has also been used to investigate interactions between biomolecules.

Upon protein adsorption to the crystal surface, the oscillatory motion of the crystal was dampened, causing a decrease in the resonant frequency. The frequency shift of the QCM is due to a change in total coupled mass, including water interaction within the protein layer. The *Sauerbrey* equation relates the measured frequency shift (Δf) and the adsorbed mass (m) [95].

$$\Delta f = -\frac{2\Delta m f_0^{\,2}}{A\sqrt{\rho_q \mu_q}} = -\frac{2 f_0^{\,2}}{A\sqrt{\rho_q \mu_q}}\Delta m \tag{1}$$

where

- f_0 – Resonant frequency (Hz)
- Δf – Frequency change (Hz)
- Δm – Mass change (g)
- A – Area between electrodes (cm^2)
- ρ_q – Density of quartz ($\varrho_q = 2.648$ g/cm^3)
- μ_q – Shear modulus of quartz ($\mu_q = 2.947 \times 10^{11}$ g/cm.S^2)

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The sensor can be used for the direct, marker-free measurement of specific interactions between immobilized molecules and analytes in solution. Binding of a soluble analyte to the immobilized ligand causes a shift in the resonance frequency, and this signal can be recorded using a frequency counter with high resolution. This method, despite its existence for four decades, has only recently been developed for immunological measurements in a flow through system [96].

In contrast to the optical techniques, which are not sensitive to water associated with adsorbed proteins, the *f*-shift of the QCM is due to the change in total coupled mass, including hydrodynamically coupled water, water associated with the hydration layer of e.g. proteins and/or water trapped in cavities in the film [89].

A recent extension of the technique, called QCM-D, to simultaneously measure changes in the frequency, Δf, and in the energy dissipation, ΔD, of the QCM provides new insight into e.g. protein adsorption processes [89] as well as other surface-related processes.

6.2. Surface plasmon resonance

Surface plasmon resonance (Figure 8) can be applied as a convenient, sensitive and label-free technique to study various surface phenomena. SPR is a surface sensitive, spectroscopic method which measures change in the thickness or refractive index of biomaterials at the interface between metal surfaces, usually a thin gold film (50–100 nm) coated on a glass slide, and an ambient medium. In SPR the test proteins are immobilized on a gold-surface, unlabelled query protein is added, and change in angle of reflection of light caused by binding of the probe to the immobilized protein is measured to characterize biomolecular interactions in the real-time [97].

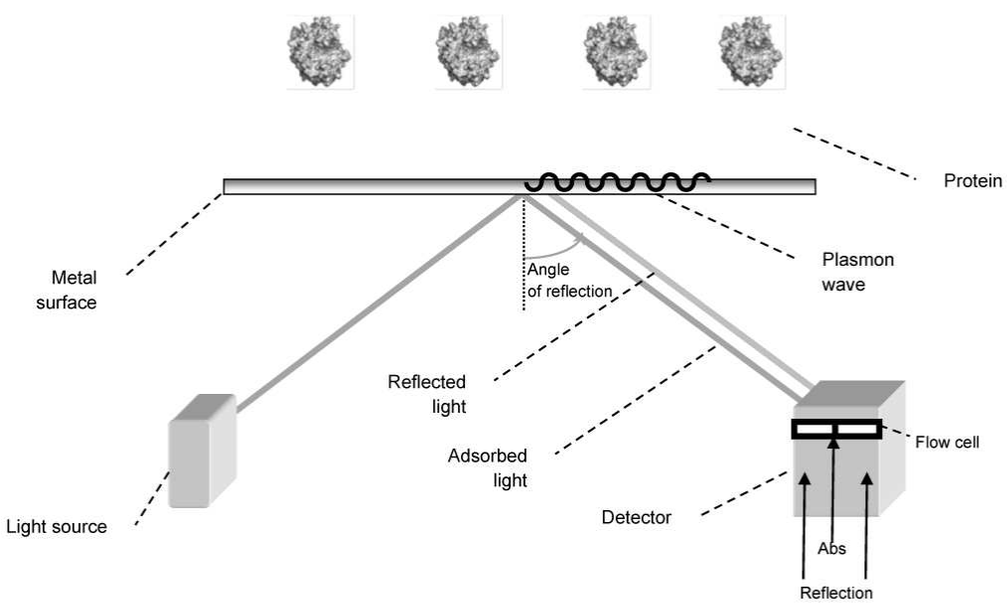


Figure 8. Surface Plasmon Resonance

SPR has been used for many biomedical, food and environmental applications [98]. In example, Hiep *et al*. [99] developed a localized SPR immunosensor for detection of casein allergen in raw milk. There was also generated a unique SPR-based microarray using natural glycans for rapid screening of serum antibody profiles [100]. SPR microarrays was utilized in combination with HT antibody purification technologies for rapid and proper affinity ranking of antibodies [101]. SPR-based biosensors are in great demand as they provide label-free, real-time detection of the biomolecular interactions.

6.3. Ellipsometry

Ellipsometry (ELM, Fig. 9) is an optical method that has been used extensively for protein adsorption studies. The method is based on the change upon protein adsorption of the state of polarization of elliptically polarized light reflected at a planar surface. From the changes in the ellipsometric angles (Δ, ψ), the refractive index and the thickness, morphology or roughness of the surface of layers can be deduced and used to determine e.g. the amount of adsorbed protein on a surface. Since the refractive index of adsorbed protein films is always close to n=1.5, which the film thickness can be calculated with quite good accuracy [102]. Moreover, the clear advantage of this technique is that the proteins under investigation require no chemical treatments with markers etc. before use. Also, the measurement procedure is quite fast (on the order of a few seconds).

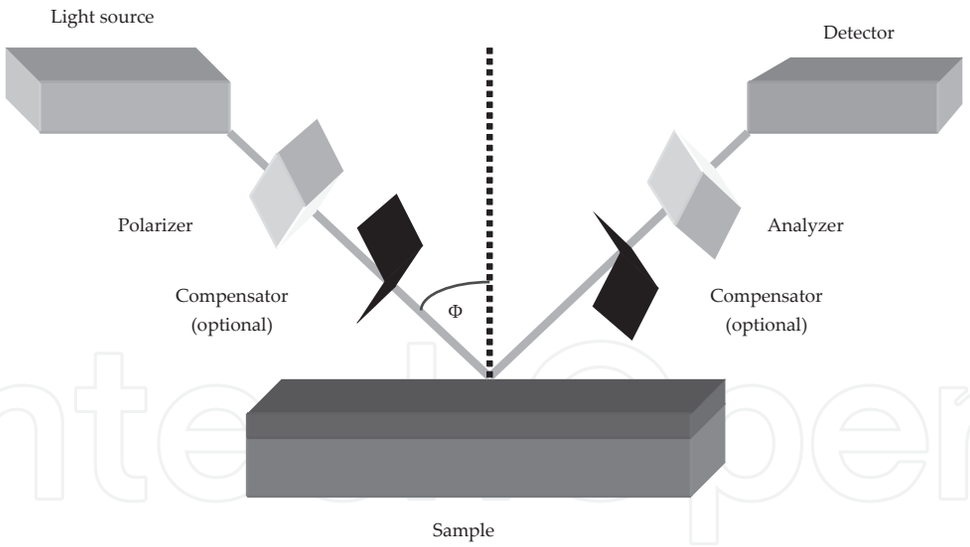


Figure 9. Ellipsometry setup

Since ellipsometry can be performed on most reflective substrates it can be easily conducted on an electrode surface and combined with electrochemistry. The ellipsometry and electrochemical methods have been used to study protein adsorption on metal surfaces and specifically human serum albumin on gold surfaces [103]. Chronoamperometry and ellipsometry were combined for the study of immunosensor interfaces based on methods of Immunoglobulin G adsorption onto mixed self-assembled monolayers [104]. The combined imaging el-

lipsometry with electrochemistry have been employed to investigate the influence of electrostatic interaction on fibrinogen adsorption on gold surfaces [105].

6.4. Atomic force microscopy

Since its invention atomic force microscopy (AFM) has been a useful tool for imaging a wide class of biological specimens such as nucleic acids, proteins, and cells at nanometer resolution in their native environment [106]. AFM can also be applied to measure intermolecular force based on the deflection signal of the AFM probe (cantilever) caused by the force between the cantilever modified with a molecule of interest and a complementary molecule immobilized on a substrate.

Atomic force microscopy (AFM) is a very high-resolution type of scanning probe microscopy, with demonstrated resolution on the order of fractions of a nanometer, more than 1000 times better than the optical diffraction limit. Because the atomic force microscope relies on the forces between the tip and sample, knowing these forces is important for proper imaging. The force is not measured directly, but calculated by measuring the deflection of the lever, and knowing the stiffness of the cantilever. Hook's law gives:

$$F = -kz \tag{2}$$

where F is the force, k is the stiffness of the lever, and z is the distance the lever is bent.

The major advantage of AFM is to carry out measurements on non-conducting substrates, and to determine particle height as well, with atomic level precision and flexibility [107]. AFM is a useful device not only to trace topography of biological samples with molecular resolution under physiological conditions but also to study the interaction force between bio-molecular pairs and the mechanical properties of proteins at the single molecular level [108]. Moreover, the AFM is used as a manipulator to obtain DNA from chromosomes [109], or mRNA from local regions of living cells [110].

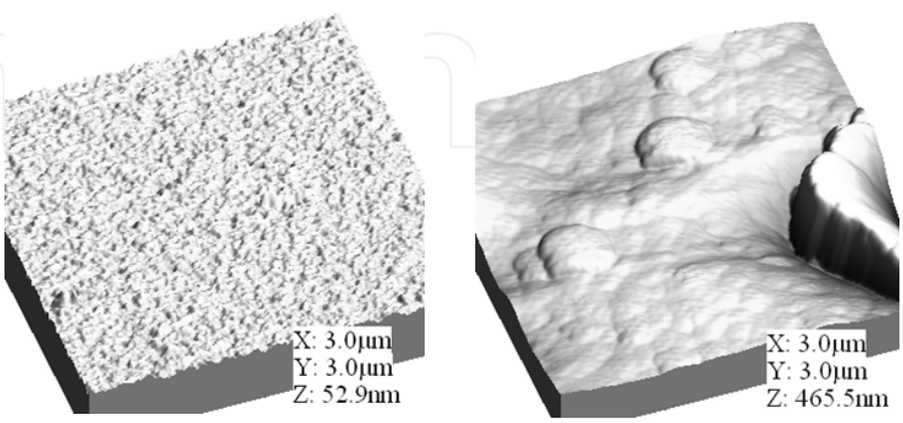


Figure 10. AFM image of immobilized invertase in LS lipid-like film; left – pure lipid film, right – hybrid protein film. All images are 3 µm x 3 µm [17]

Several measurements of the intermolecular force produced by biomolecular interaction were reported, i.e. the AFM surface topography of phospholipids LB films have shown a smooth surface. In presence of protein in LB film well-defined structures were observed, characterized by domains, globules, grains with different diameters. The images indicates that both enzyme molecules are not only properly entrapped in the composite membrane but also well exposed at the surface, which can be clearly seen in Figure 10. The recorded images show a relatively high homogeneity of the topography, especially in case of lipid film [17].

7. Prospects and future trends

The advances observed in the areas of biochemistry, chemistry, electronics and bioelectronics will markedly influence future of biosensor production. Progresses in biosensors technology focus on two main aspects: transducer technology development and sensing element development [111]. New improved detection systems developed under the areas of microelectronics or even nanoelectronics can be used in biosensors. However, since biosensor sensitivity and selectivity depend basically on the properties of the biorecognition elements, a crucial aspect in future biosensors is the development of improved molecular recognition elements. In this respect, biotechnology and genetic engineering offer the possibility of tailor binding molecules with predefined properties

According to fact, that miniaturization of devices as well as multi-sensor arrays are expected to have a marked impact in biosensors technology, the use of thin film methods for preparation of the recognition layers provides a simple procedure for the functionalization of electrode surfaces using nanogram amounts of material. Different techniques can give either highly ordered or amorphous film, ensured a high level of control of the environment and often resemble the environment found inside biomembranes, thereby guaranteed the stabilization of biomolecules. Biosensors produced using these layer technologies can display high sensitivities, be easily interrogated using electronic, optical or mass-sensitive techniques, can often be regenerated and display good stability.

Another current trend is the combination of physics and biology in the creation of new nanostructures. Nanotechnology comprises a group of emerging techniques from physics, chemistry, biology, engineering and microelectronics that are capable of manipulating matter at nanoscale. This novel technology bridges materials science, and biochemistry/chemistry, where individual molecules are of major interest [112]. Inspired by nature, molecular self-assembly has been proposed for the synthesis of nanostructures capable to perform unique functions. According to that, novel tools that combine different sensing methods can provide also the necessary complementary information that is needed to understand the limitations and to optimize the performance of the new techniques. Therefore, introducing existing methods (e.g., SPR, QCM, ellipsometry) allow parallel complementary investigations of the biochemical processes that take place at the interface between the devices and the biological sample.

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At present, biosensor research is not only driving the ever-accelerating race to construct smaller, faster, cheaper and more efficient devices, but may also ultimately result in the successful integration of electronic and biological systems. Thus, the future development of highly sensitive, highly specific, multi-analysis, nanoscale biosensors and bioelectronics will require the combination of much interdisciplinary knowledge from areas such as: quantum, solid-state and surface physics, biology and bioengineering, surface biochemistry, medicine and electrical engineering. Any advancement in this field will have an effect on the future of diagnostics and health care.

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