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Biotechnological Applications of Photosynthetic Proteins: Biochips, Biosensors and Biodevices

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ELENA V. PILETSKA graduated from Moscow State University in 1985 and gained her Ph.D. in biochemistry from A.N. Bach Institute of Biochemistry (Moscow) working on genome of chloroplasts (1989). Dr. Piletska joined the Institute of BioScience and Technology, Cranfield University, U.K. in 1998. Her current research interests include molecular recognition using synthetic and natural receptors, polymer and analytical chemistry and computational modelling.

This book is dedicated to Gianni, Raffaele,
Ginko, Kyria, Gea, Spina, Ruya and Eliott.

—Maria Teresa Giardi, Ph.D.

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CHAPTER 1

Introduction:
The Emergence of a New Technology

Maria Teresa Giardi*

The possibility of producing a new generation of technological devices that integrate the knowledge coming from various fields (chemistry, biology, computer science, electronics, engineering) is attracting increasing attention. This trend has introduced a new technological science called "molecular electronics" or "nanotechnology". It is a technology based on the use of molecular scale components such as a single or a few molecules, carbon nanotubes, nanoscale metallic and/or semiconductor wires, etc. that function as electronic components.

RC-biotechnology refers to the use of Reaction Centres (RC) and more in general of photosynthetic proteins, for technological purposes. It regards the construction of photo optical-electrical devices based on photosynthetic proteins. Photosynthetic RC proteins are suitable biological material for the construction of devices because they exhibit light-induced electron transfer across lipid membranes. Many chromophore molecules, such as bacteriochlorophylls, bacteriopheophytins and quinones, are arranged in RCs with relevant interchromophore distances and relevant gaps in the energy levels of each chromophore to ensure unidirectional electron transfer.

The development of biosensors represents a valuable step towards the advancement of pollutant monitoring in ecosystems. Biosensors are analytical devices that consist of a biosensing element (enzyme, tissue, living cell) that provides selectivity and a transducer that transfers the chemical signal to an electrical signal for further processing. Therefore even a single protein molecule of an RC is a sophisticated molecular device. They are able to generate supramolecular and self-assembling structure and, hence, are natural nanostructures.

In recent years, progress on isolation of RC and of photosystem II (PSII) particles has been obtained, and it is now possible to isolate quite stable and pure preparations from plant thylakoid and cyanobacterial membranes by detergent solubilization. These preparations are capable of light-induced oxygen evolution, at high rates, and/or electron transfer in the absence or presence of benzoquinones as artificial electron acceptors. The RC isolated from photosynthetic bacteria is particularly stable against denaturation. Moreover, recent advances in RCII biochemistry and molecular biology (site-directed mutagenesis) have produced a number of mutants resistant to extreme conditions, showing altered amino acid composition of the D1 protein.

RC-biotechnology exploits the characteristics of the pigment-protein complexes located within the membrane of plants, algae, cyanobacteria and bacteria. However, the structures, functions and potentials of the photosynthetic complexes are different in the various photosynthetic organisms. We can distinguish the technological applications obtained from the three types of photosynthetic proteins from bacteria (RC), from cyanobacteria, algae and higher plants (RCII) and rhodopsin from halobacteria (bR). RC from bacteria was utilised for building several biochip types; RCII-technology includes applications such as photonic-crystal bandgap materials, biosensors and

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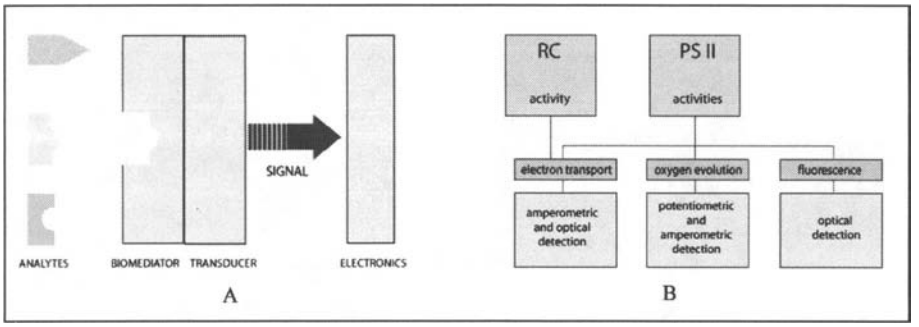

Figure 1. Schematic representation of a biosensor (A); Photosynthetic Photosystem II and Reaction Centre activities (B).

biodevices; finally, bR-technology includes a reversible holographic memory, an ultrafast random-access memory, pattern-recognition systems and photoelectrical cells.

The advantage of using RC biodevices mainly depends on the specificity of the enzyme to recognize certain analytes or particular physicochemical conditions. Moreover, RC and PSII are especially suitable because of their physiological activities that can be easily monitored by amperometric, potentiometric and optical systems (Fig. 1).

Why and How to Make a Photosynthetic-Based Biosensor

Despite initial enthusiasm, biosensors have not yet achieved the prominent commercial application that was initially predicted. This slow progress is not surprising since biosensor development requires the combined expertise of biologists, chemists, biotechnologists, biochemists, physicists, and mechanical and electrical engineers. It is rare to have so many disciplines in a commercial company.

The construction of RC-based devices requires a multidisciplinary approach where the device is obtained in various stages. First of all the physiology of the photosynthetic organism should be considered since it is essential before designing the biodevice. For instance, the choice of a thermophilic cyanobacterium as a biomediator guarantees the stability of biosensors for monitoring herbicides. The second stage is the isolation of the biomediator, its stabilization and immobilization by biochemical and chemical techniques. Using molecular biology, mutations on PSII complexes that produce specific properties can be carried out; e.g., a modification of a single amino acid on D1 protein generates a resistance towards herbicide subclasses. Bioinformatics is then applied to optimise the molecular modifications on the biomediator. The next stage is the study of suitable transduction system for the detection of the chosen PSII activity and the analyses of the data. Finally, biosensor prototypes for specific applications are designed (e.g., field portable, device for laboratory, miniature size prototype etc.).

Although it utilises the same basic biomediator, the tech applications can be divided into different classes, based on different concepts of the RC properties.

Potential and Prospective of the RC-Biotech for Basic Research and Applications

The production of mono/multi-molecular layers is now a reality. The layers are made up integrating natural or engineered photosynthetic systems with synthetically derived molecules that act as a means of transduction and immobilisation.

A common feature of the various RCs is the trigger of the photochemistry by solar energy, but there are differences in the way they convert energy and consequently on their potential application for the building of biochips, biosensors, photovoltaic and photoelectrochemical cells, holographic memory etc.

The present RC-technology is geared around the concept that the engineered RC can be the core of numerous innovative devices. The technological applications described above can be divided into distinct classes of innovative products based on the different RC-biochip properties. However, the skills and knowledge required to manipulate and settle the RC complex properties are common. Single changes in amino acid sequences can create more stable biomediators and increase the efficiency of selected photochemical processes. Moreover, the required functionality for new devices can be found in molecules that are in natural abundance.

The emergence of RC-technology would perhaps be a benefit to those looking to establish commercial devices. Certainly the number of patents based on photosynthetic proteins that are applied for and granted every year is increasing, and that is a clear indication of its future commercial success.

The data summarised here can serve as a basis for the development of a commercial biosensor for use in rapid prescreening analyses of PSII pollutants, minimising costly and time-consuming laboratory analyses.

The aim of this book is to give a general description of the basic and technical research in this sector.

CHAPTER 2

A Brief Story of Biosensor Technology

Marco Mascini*

Introduction

The vast literature in the last 40 years related to the keyword **Biosensor** reveals without doubt that the scientific field is **attractive**! We realized at once that several researchers with different background are involved in this field of research, from chemistry to physics, to microbiology and of course to electrical engineering, all are deeply involved in several facets of the assembly of the object "Biosensor".

Looking at the past we realize also that the concept of Biosensor has evolved!

For some authors, especially at the beginning of this research activity, i.e., about 40 years ago, Biosensor is a self contained analytical device that responds to the concentration of chemical species in biological samples! This is clearly wrong, but it has been very difficult to clarify this point! No mention of a biological active material involved in the device! Thus any physical (thermometer) or chemical sensor (microelectrode implanted in animal tissue) operating in **biological samples** could be considered a Biosensor. We agree that a biosensor can be defined as a device that couples a biological sensing material (we can call it a molecular biological recognition element) associated with a transducer.

In 1956 Professor Leland C. Clark publishes his paper on the development of an oxygen probe and based on this research activity he expanded the range of analytes that could be measured in 1962 in a Conference at a Symposium in the New York Academy of Sciences where he described how to make electrochemical sensors (pH, polarographic, potentiometric or conductometric) more intelligent by adding "enzyme transducers as membrane enclosed sandwiches".[1] The first example was illustrated by entrapping the enzyme Glucose Oxidase in a dialysis membrane over an oxygen probe. The addition of glucose determined the decrease of oxygen concentration in proportional relation! The first biosensor was described in the published paper coining the term "enzyme electrode".[2] Then subsequently in 1967 Updike and Hicks use the same term "enzyme electrode" to describe a similar device where again the enzyme glucose oxidase was immobilized in a polyacrylamide gel onto a surface of an oxygen electrode for the rapid and quantitative determination of glucose.[3]

Besides amperometry Guilbault and Montalvo in 1969 use glass electrodes coupled with urease to measure urea concentration by potentiometric measurement.[4]

Starting from 1970, several others authors start to prove the concept of Biosensors, the coupling of an enzyme and electrochemical sensors. This was at the beginning a Biosensor, a strange research where biological elements were combined with electrochemical sensors.

In the electrochemical community at that period the research on ion selective electrodes (ISE) was very active and the idea to extend the range of sensors to non electrochemical active compounds, and even to non ionic compounds, like glucose, has been very well accepted. We saw at that time the possibility to extend much more the research activity. The groups active in ISE development have been definitively the first to shift to the development of electroanalytical biosensors.

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Table 1. Biosensors

Receptors	Transducers
• Tissues	• Electochemical
• Microorganisms	• Optical
• Organelles	• Thermometric
• Cell receptors	• Piezoelectric
• Enzymes	• Magnetic
• Antibodies	
• Nucleic acids	
• Biomimetic receptors	

I recollect very elegant research starting from Prof. G. Rechnitz involving the development of an "amygdaline" sensor based on the coupling of an Ion Selective Electrode (cyanide ISE) with betaglucosidase to give benzaldeyde and cyanide.[5]

But this was just the beginning of a large activity where the obtained couplings have been multiplied by changing the "biological element" and the kind of transducer! Enzyme, multiple enzymes, organelles, bacteria, specialized biological tissue, containing specific enzymes were coupled to potentiometric or amperometric devices, then optical, thermometric, piezoelectric, etc. We continue also today to enlarge the list of physical sensors with the last entry of "magnetic devices". Recently the concept evolved again in the tentative to replace or mimic the biological material with synthetic chemical compounds! Table 1 demonstrates all kinds of couplings have been used in order to obtain Biosensors.

Enzymes (and all biological elements based on the enzymes contained in it) represent the class of what is now called "catalytic elements". The other important class is represented by the "affinity elements", namely antibodies, lectins, nucleic acids (DNA and RNA) and recently synthetic ligands.

Biomolecular sensing can be then defined as the possibility to detect analytes of biological interest, like metabolites, but also of environmental concerns or of any other technological field where the concentration of a specific compound is important to be quantified in a complex sample.

The exploitation of the selectivity of the biological element is the "driving force" of the Biosensor.

The Problem of Amplification

Catalytic events or affinity events have not the same scheme of transduction. If the biological recognition element present in the sensing layer is an enzyme or generally a biocatalyst, a reaction takes place in the presence of the specific target analyte and an increasing amount of coreactant or product is consumed or formed, respectively, in a short time depending on the turnover. In this scheme the amplification step is inherent and a large chemical amount can be obtained from the sensing layer.

In contrast the use of the antibodies for the detection of antigens has not an amplification stage involved and then the "affinity" reaction should be amplified in order to have a clear transduction. We have two possibilities, one is the use of a bioconjugate involving a bound enzyme, like in the classical ELISA test; the second is the inherent amplification given by the mass of the biological element involved, a piezoelectric device (sensitive to mass) can detect minute amount of large proteins (like antibodies) if they are attracted on the surface of the sensor.

With the same scheme surface plasmon resonance can be sensitive to minute amount of large molecule reacting at the surface of the electrode.

The Biological System

The main problem of the biological system, catalytic or affinity, is the associated fragility and the operational activity. Most proteins have an optimal pH range in which their activity is maximal; this

pH range should be compatible with transducer. Moreover the most of the biological systems have a very narrow range of temperature (15-40°C).

The most important problem and main drawback for industrial exploitation is the short lifetime associated with the biological elements. During last years several Meeting and Joined Actions were specifically dedicated to this point which is still object of research work. Lifetime or at least shelf lifetime of months or few years are the prerequisite for a suitable market and the fragility of the assembled systems has always limited the diffusion of biosensors in the market.

Immobilization of the Biological System

The technique of the immobilization of the biological elements has changed according with the different events, catalytic or affinity. The simplest way to retain enzymes on the tip of a transducer is to trap them behind a perm-selective membrane. This method has been mainly used in addition to embedding procedures in polyacrylamide gels. Then, mainly in the 80th, the trend shifted to use disposable membranes with bound bioactive material. Several companies put on the market preactivated membranes suitable for the immediate preparation of any bioactive membrane and this appeared as a real improvement at least for the easy use of enzyme sensors.

The removal of intereference has been also the other important aspect for the wide use of biosensors for industrial processes. The two problems have been solved by using multilayer membranes, such as those developed by Yellow Springs Instrument Co. (for glucose or lactate electrodes), with the enzyme sandwiched between a special cellulose acetate membrane and a polycarbonate nucleopore membrane. The main role of the membrane is to prevent proteins and other macromolecules from passing into the bioactive layer. Cellulose acetate membrane allows only molecule of the size of hydrogen peroxide to cross and contact the platinum anode, thus preventing intereference from ascorbic acid or uric acid, for example, at the fixed potential. Such configuration has been used by several researchers in their biosensor assembly. But at the same time several recipes of immobilization of enzymes were published and several laboratories developed their own procedure for immobilizing the biological element, sometimes also patented!

One approach was also the development of disposable sensor, based on combination of screen printed electrochemical sensors with enzyme adsorbed on the electrode surface (in this case mainly carbon). The use of the sensor just for one measurement limited the use of complicated immobilization procedures to simplest as possible, like only based on adsorption on carbon surface. This electrode surface acted as a sponge, and the large protein was easily immobilized even if the bond was weak. This approach was useful only for a quick and rapid measurement.

The immobilization of antibodies soon revealed that random immobilization of proteins was not effective and a new research in this direction started. Several researchers start to think how to immobilize proteins using an exact deposition. Technology, like self-assembling, based on gold surface and thiol groups prove to have a high potential. Proteins immobilized on the surface of the transducers were now aligned and their ligands group were directed toward the exterior ready to fix the metabolite.

This technique become important in the antigen-antibody reactions and even more in immobilization of nucleic acids such as 20-30 bases' oligonucleotides, which single strand conformations were looking for their complementary sequence of bases in the sample material.

Important Steps in the Biosensor Research

I want just concentrate my talk on three points which I consider very relevant in the Biosensors development in the last 30 years:

The Case of Glucose Pen

In 1984 Cass and coworkers publish a scientific paper where the team prove the use of ferrocene and its derivatives as mediators for amperometric biosensors.[6] Few years later the Medisense Exatech Glucose Meter was launched in the market and become the world's best selling biosensor product. The initial product was a pen-shaped meter with a disposable screen printed electrodes.

There were several advances in this product; first of all, the miniaturized instrument, just a pen with a small screen, where the current, recalculated in mg/dL of glucose was directly displayed. Then the concept of disposable screen printed electrodes, which allowed discarding the sensor after the use, and more important allowed the elimination of the calibration step. This was an incredible step in the sensor community. All sensors known, from pH glass electrodes to all kind of ISE, etc. should pass the "calibration step", where the sensor must be calibrated every day and sometimes before every measurement. The disposable screen printed electrodes do not need the calibration which simplifies enormously the use of it. The sensors became simple and user-friendly objects and diabetic people started to use it for individual monitoring and a large market was created. In 1996 Exatech was sold to Abbott for 867 million of US dollars.

The performance and design of several strip analyzers based on different electrochemical principles and meters has been published.[7] The book described around 10 different home glucose meters but it is not the final number because the new instruments continue to appear on the market.

The Wearable Artificial Pancreas

One major interesting application of Biosensor has been the development of a wearable artificial pancreas and the studies associated with development. This devise has never reached the market stage even if several scientists addressed the problem and demonstrated the possibility to resolve it. In 1976 Clemens et al. incorporated an electrochemical glucose biosensor in a "bedside artificial pancreas".[8] It was later marketed by Miles (Elkhart) as the Biostator Glucose-Controlled Insulin Infusion System (60 Kg, 42 x 46 x 46 cm) (Fig. 1).

This instrument became very well known in the medical endocrinology community. It regulated the glucose value injecting insulin or glucose into the bloodstream of the patient.

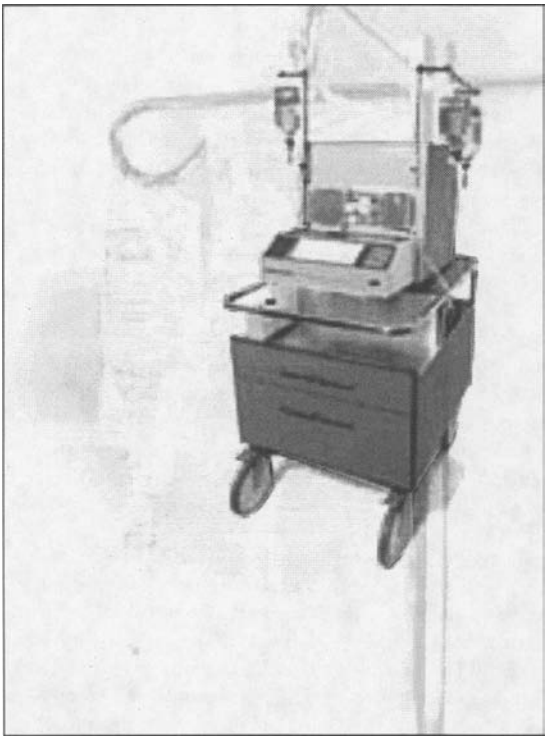

Figure 1. A general view of Biostator.

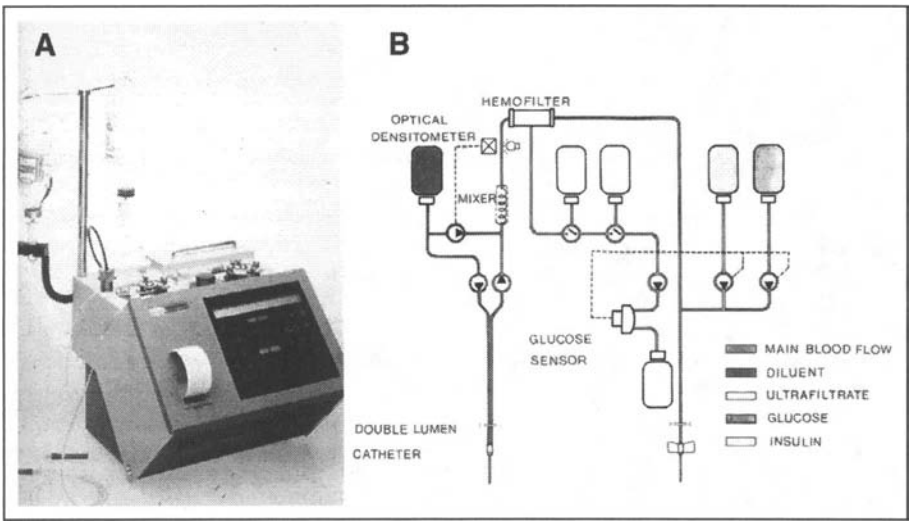

Figure 2. A general view of the Betalike (A) the artificial pancreas developed and marketed by Esacontrol, and a scheme of the system (B). Principle of operation: The device draws a small amount of blood from a peripheral vein and then infuse it into another one. In order to prevent coagulation inside the tubes the blood is diluted and heparinized at the tip of the drawing needle. The glucose measurements are carried out on the ultrafiltrate liquid obtained from a micro hemofilter cartridge. On a minute basis the Betalike infuses into the blood stream the amounts of insulin and glucose, calculated by means of a mathemathic algorithm, which are needed to reach and maintain the selected glucose level.

Although Biostator production was soon discontinued, it was later substituted by a similar instrument called Betalike produced and distributed by a small Italian company in 1990.

The Betalike had some innovations and improvement over Biostator: blood was taken from the patient via a double lumen catheter (6 ml/h) diluted with a buffer solution (1:9) with the addition of 3 units/ml of heparin. The diluted blood was then dialyzed in a miniaturized hollow fibre haemofiltering cartridge (filtration surface was 50 cm^2; membrane cut off about 35 000 daltons) which allows only the haemofiltrate to reach the sensors while the blood cells and proteins are reinfused into the patient (Fig. 2). The value of glucose could then give a signal for feeding in another needle positioned in the bloodstream, insulin or glucose according to the glucose profile (value and trend). This instrument opened new opportunities to study the diabetes and the glucose variation during the day.

Figure 3, shows one representative case of three days continuous record of an insulin-dependent diabetic treated with continuous subcutaneous insulin infusion. The continuous monitoring of glucose concentration disclosed a day-by-day variation of glycemia in diabetics. Then a large research activity started to miniaturize the system in order to obtain a real wearable artificial pancreas. The first step was miniaturizing the sensor. Today we have on the market several small instruments able to monitor glucose continuously up to one week.[9,10]

The Appearance of BIAcore on the Market

In 1982 researchers from Pharmacia started to work jointly with physics and biochemistry professors at Linkoping University in order to develop a new bioanalytical instrument able to monitor the interactions between biomolecules. In 1984 a new company Pharmacia Biosensor was created.

The company introduced a new instrument, BIAcore, in 1990 (Fig. 4). The instrument had a very high impact on the Biosensor community. The price of the instrument was more than 100 times higher than any other electrochemical or optical apparatus. The instrument based on surface plasmon resonance (SPR) technology was a fully automated instrument which monitored the

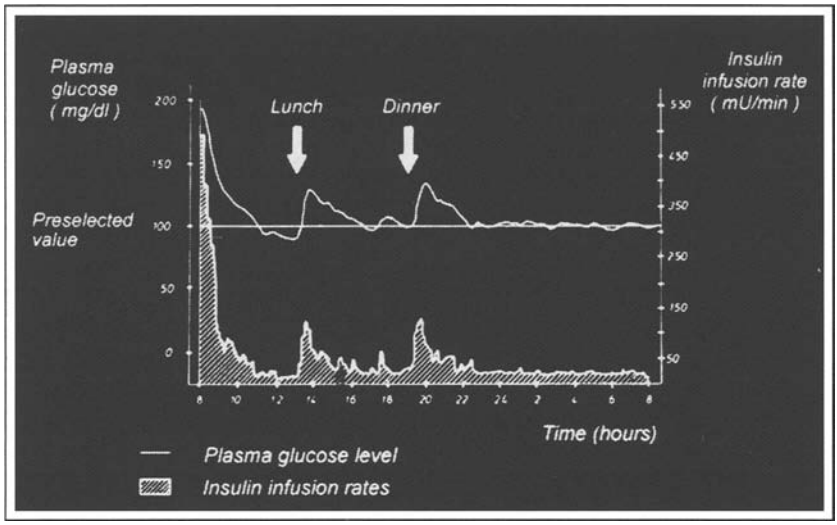

Figure 3. A typical experiment performed by clamping a "normal" value of glucose.

biomolecular interactions and included a sample handling equipment. The instrument performed the immobilization of the biomolecules, the SPR analysis and the regeneration of the sensor surface automatically by a microprocessor and this was a great advantage over the more traditional sensor technologies.

The autosampler was able to handle up to 192 samples without operator assistance. It increased the reproducibility of the analysis and provide a large sample capacity. The instrument stabilized the temperature at 0.1°C and allowed analysis of biomolecular kinetics.

The BIAcore instrument also eliminates the moving components that are generally associated with prism-based instrumentation. However the real advancement of the instrument was the unique sensor chip technology to simplify the immobilization of biomolecules to the sensor surface.

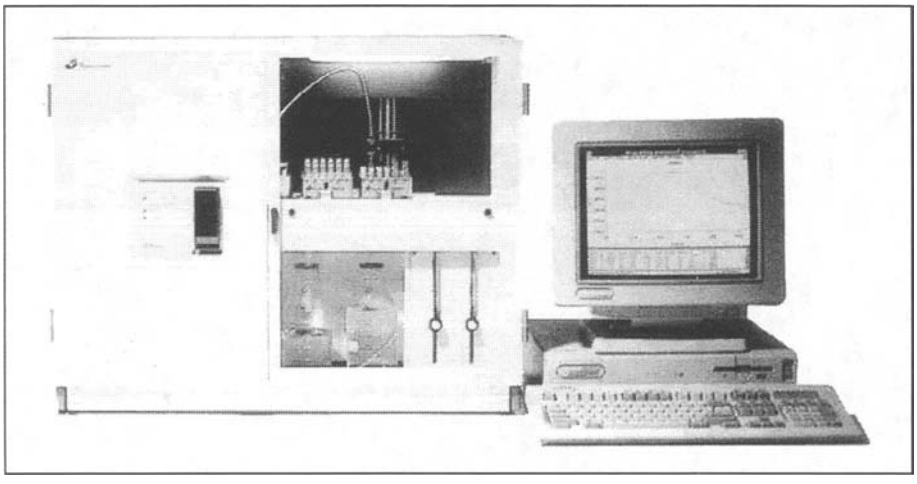

Figure 4. A general view of BIAcore.

Figure 5. Biacore sensor chip.

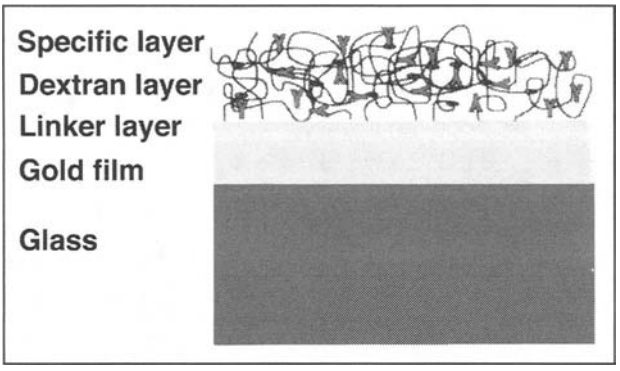

Figure 5, is a scheme of the BIAcore sensor chip. The chip consisted of a glass slide embedded in a plastic support; the glass surface is 1 cm^2 and has approximately 50 nm of gold coated on one side of the glass. The gold layer was then covered with dextran acting as a linking layer in order to facilitate the binding of biomolecules. Dextran, an old Pharmacia product, acted as a support for biomolecules but also protected the gold layer from nonspecific binding which is the main problem of this kind of apparatus. Typical protein concentrations required for immobilization are in the range 10 to 100 μg/ml. Usually, the chips can be used for more than 50 measurements without a notable loss in sensitivity and reproducibility. Moreover the company provided the sensor with alternative linking and binding layers (for protocols see http://www.biacore.com). The flow injection system has been designed with miniaturized sample loops, valves and conduits reducing drastically sample and reagent volumes with help of silicone layers. This is, of course, very important when dealing with valuable biological reagents.

The instrument was sold initially mainly to pharmaceutical companies looking for monoclonal antibodies (antibodies were ranked and selected for specific conditions). The instrument was very powerful in its automatic performance which significantly cut the time for the evaluation of the binding constant between antibodies and antigens. Consequently it was also applied to the study several other affinity reactions and became an important instrument in several research laboratories.

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CHAPTER 3

Photosystem II:
Composition and Structure

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Introduction

Photosystem II (PSII) is a light driven, water-plastoquinone oxidoreductase which catalyses the most thermodynamically demanding reaction in biology.[1] This highly endergonic reaction splits water into molecular oxygen, protons and electrons, thereby sustaining an aerobic atmosphere on earth and providing the reducing equivalents necessary to fix carbon dioxide to organic molecules, creating biomass, food and fuel.

PSII is a multisubunit pigment-protein complex embedded in the thylakoid membranes of higher plants, algae and cyanobacteria. Its unique properties require an elaborate arrangement of integral membrane proteins, specifically bound pigment moieties, extrinsic proteins and inorganic cofactors. Light energy is absorbed by light harvesting complexes that contain most of the pigments associated with PSII. Excitation energy is transferred from this antenna to the "core" of the PSII complex, where the primary photochemistry takes place. This photochemical part of PSII contains the ultra-fast and very efficient light-induced charge separation and stabilization steps that occur vectorially across the membrane. Finally, the photochemical reactions result in the accumulation of oxidizing equivalents in the oxygen-evolving complex (OEC); four oxidizing equivalents are used to convert two molecules of water into oxygen.

The photochemical and enzymatic reactions catalyzed by PSII are strictly conserved among all oxygenic photosynthetic organisms including cyanobacteria, eukaryotic algae and higher plants, while quite diverse pigment-protein complexes have developed for light-harvesting antenna systems associated with PSII.[2,3] These antenna systems, though similar in function, differ in their structures, with those of higher plants and green algae (LHCP) being located in the thylakoid membrane while those of most classes of cyanobacteria (phycobilisomes) are bound extrinsically to the stromal surface of PSII. With regard to protein structure, the PSII complex differs mostly in peripheral subunits between cyanobacteria and higher plants but shares the core parts in common.[4]

The PSII core is the minimal unit which is capable of catalysing full PSII function.[5,6] It is composed of a reaction center, which consists of the D1 and D2 polypeptides, cytochrome b559 (Cyt b559), the psbI protein and six chlorophyll (Chl) and two pheophytin (Pheo) molecules, an inner antenna of chlorophyll-binding proteins termed CP47 and CP43, and the extrinsic lumenally bound proteins of the OEC, 33 kDa protein (psbO), 23 kDa (psbP) and 17 kDa (psbQ), in higher plants and green algae, whereas in cyanobacteria psbP and psbQ are replaced by the 15 kDa psbV (cytochrome c550) and the 12 kDa psbU.[7] These intrinsic and extrinsic proteins, together with a number of low-molecular weight subunits[8-10] make up the core complex.

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