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Research review paper

Plant tissue-and photosynthesis-based biosensors

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ABSTRACT

Biosensors are promising biotools, alternative or complementary to conventional analysis techniques, for fast, simple, cheap and reliable screening. This article reviews the biosensors that use plant components as biorecognition elements. In the first section, plant tissue-based biosensors are summarised and classified according to the enzyme used. Afterwards, photosynthesis-based biosensors, including the types of photosynthetic materials and immobilisation methods, are described.

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1. Introduction

The detection and quantification of analytes has always been an issue of particular concern in all existing areas, such as clinical diagnostics, food

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technology and environmental monitoring. Thanks to the rapid bloom and growth of biosensors, bioanalysis has advanced at vertiginous rates. Unlike sophisticated analytical techniques, biosensors lead to easy, fast and low-cost methods to detect and quantify analytes in real time.

A biosensor is an analytical device that consists of a biorecognition element in intimate contact with a transducer element. The bioreceptor or biorecognition molecule is immobilised on the transducer and specifically recognises (*via* catalytic or affinity interactions) the target, and the transducer converts the biorecognition event into a quantifiable signal. This combination provides an analysis tool competitive with the established and conventional analysis techniques.

Biosensors are classified according to the biorecognition molecule, which can be:

- enzymes, which recognise specific substrates and catalyse the corresponding reactions;
- antibodies, which interact with the corresponding antigens by affinity;
- oligonucleotides, which recognise complementary sequences also by affinity;
- cells and whole organisms, whose respiration, growth, fluorescence or bioluminescence can be indicative of the presence of certain chemicals;
- tissues, which usually act as enzyme source; and
- biomimetic materials, also called synthetic bioreceptors.

Biosensors can be also classified according to the transducer method. Although there is an infinite number of detection techniques, the most commonly used are:

- electrochemical, such as amperometry and potentiometry;
- optical, such as colorimetry, bioluminescence, and fluorescence; and
- mass-sensitive, such as piezoelectrochemistry.

Each one of these techniques has advantages and drawbacks and, in fact, they are usually complementary. Fluorescence techniques, although highly sensitive, are characterised by the expensive required equipment. Electrochemical transduction offers advantages of sensitivity and relatively inexpensive instrumentation. Moreover, miniaturisation of the instrumentation is possible, making this transduction method very attractive for the development of portable devices for *in situ* monitoring. Especially interesting are techniques that do not require labels for the transduction of the binding event, such as piezoelectrochemical detection or surface plasmon resonance (optical technique based on the change in the refractive index of a surface when a biomolecule is immobilised or when an affinity interaction occurs). Label-free strategies lead to short analysis times and simple operation protocols, and eliminate possible undesirable effects, such as steric impediments, binding biases and instability of the label. These techniques, moreover, can measure biorecognition events in real time. However, instrumentation costs and operational requirements tend to be elevated.

This review is focused on the biosensors that use plant components as biorecognition elements, plant tissue-and photosynthesis-based sensors being the most commonly reported.

2. Plant tissue-based biosensors

In the 80's, Kuriyama and Rechnitz (1981) used yellow squash, containing glutamate decarboxylase, and a CO_2 electrode to measure the concentration of glutamic acid in a sample. Since then, a wide variety of plant tissues have been used as bioreceptors in different biosensor formats. These biosensors use tissue slices as sources of the enzyme that catalyses a specific reaction. The main advantages of using plant tissues in biosensors are:

- the high stability and high level of activity resulting from the maintenance of the enzyme in its natural environment;
- the long lifetime of biosensors, as a consequence of the high stability;
- the high reproducibility of the experimental results;
- the availability and low price of plant tissues;
- the avoidance of tedious and time-consuming enzyme extraction and purification steps, with the consequent reduction in cost;
- the presence of the required cofactors in the same tissue that provides the enzyme, which simplifies the biosensor manufacture; and
- the plant diversity and corresponding wide range of distinct biosensors.

However, they suffer from low specificity, due to the presence in the tissue of enzymes others than the one of interest, and long response times, due to the diffusion barriers.

The methods most commonly used for the construction of plant-based biosensors are the retention of a thin tissue slice on the surface of the electrode with a dialysis membrane, and the incorporation of the tissue into a carbon paste matrix. In the membrane-based biosensors, the membrane has to impede the leakage of the enzyme away from the electrode surface but has to allow the substrate diffusion into the plant tissue. As mentioned above, this diffusion resistance may be responsible for long response times. Carbon paste electrodes are able to reduce the response time to seconds.

Most plant tissue-based biosensors are based on electrochemical detection, usually amperometric or potentiometric. However, optical techniques, such as chemiluminescence or fluorescence, have recently appeared providing higher sensitivities and faster response times.

Due to the wide diversity of plant materials, many plant-based biosensors have been developed. Below, the plant-tissue based biosensors reported to date are reviewed.

2.1. Polyphenol oxidase-containing tissues

Banana pulp slices have been widely used in biosensors due to their high content in polyphenol oxidase (PPO), enzyme that recognises a large variety of mono-and polyphenols. The first scientists to use banana tissue were Sidwell and Rechnitz (1985), who developed what they called a "bananatrode". They coupled a thin slice of banana pulp with a Clark-type oxygen electrode to measure electrochemically the oxygen consumption due to the conversion of dopamine into 1,2-benzoquinone. Three years after them, Wang and Lin (1988) introduced the banana pulp into the carbon paste, reducing the response time to dopamine to seconds. Instead of measuring the oxygen consumption, they measured the reduction of the quinone derivative back to catechol at negative potentials. Eggins et al. (1997) used a similar banana electrode to detect flavanols, such as catechins and their dimmers and trimmers, as indicators of beer quality. However, their results were unsatisfactory probably due to the flavanols already present in banana. On the contrary, potato, wet apple and dried apple tissues, which also contain PPO, resulted in analytical results comparable to those obtained by conventional colorimetric and chromatographic techniques. Following the same strategy, Cummings et al. (1998) incorporated apple tissue in a carbon paste matrix and characterised the electrical, electrochemical, morphological and analytical properties of the electrodes to optimise the performance of the biosensor. They concluded that higher plant tissue/carbon paste ratios lead to non-homogeneous surfaces and, consequently, more favourable water penetration, improved PPO accessibility and higher number of available catalytic sites within the network.

Apart from banana, apple and potato, other species have also been used due to their PPO content. Lima et al. (1997) used coconut tissue (*Cocus nucifera*) as biorecognition element in a flow injection analysis system for catechol. In this case, however, the tissue was not immobilised on the working electrode surface but packed into a glass reactor body. Since the biorecognition molecule was not in intimate contact with the transducer, their device was not properly a "biosensor". Anyway, catechol was detected at −0.10 V *vs.* Ag/AgCl with a limit of detection of 2 μM and at a rate of up to 60-90 samples/hour. Despite the

fouling of the tissue by the adsorption of phenolic polymers formed as by-products in the biocatalytic process, the reactor could be used for several days suffering only a slight loss of activity. The real applicability of the bioreactor was demonstrated by the measurement of catechol content in river water and in paper plant wastewater.

Avocado tissue (*Persea americana*) has also been used as source of PPO. Fatibello-Filho et al. (2001) developed a chronoamperometric biosensor for the detection of paracetamol, based on the modification of a vaseline/graphite mixture with avocado tissue powder. Paracetamol, when consumed in overdose quantities, may cause hepatic toxicity or even death. The PPO present into the avocado tissue catalysed the oxidation of paracetamol to *N*-acetyl-*p*-benzoquinone, which was reduced back to paracetamol at −0.12 V *vs.* Ag/AgCl. The authors attained a detection limit of 88 μM in pharmaceutical formulations, comparable to that obtained using the pharmacopoeial procedure.

Still in the pharmacological field, a biosensor for epinephrine quantification has been recently developed using fibres of palm tree fruits (Felix et al., 2006). In this case, PPO catalysed the oxidation of epinephrine to epinephrinequinone, which was electrochemically reduced at −0.10 V *vs.* Ag/AgCl. The carbon paste biosensor, incorporated into a flow injection analysis system, had a limit of detection of 15 μM. When analysing an inhalation pharmaceutical product, the biosensor was particularly advantageous over the current spectrophotometric method, since the latter is strongly affected by benzoic acid, a compound present in the sample formulation.

Most sensors for pesticides suffer from deterioration and subsequent short lifetimes. The high stability of plant tissues offers an alternative to this problem. As an example, Mazzei et al. (1995) developed a potato tissue (*Solanum tuberosum*)-based biosensor, but instead of using the ability of PPO to recognise phenols, they exploited its inhibition by herbicides to detect atrazine in aqueous samples. The biosensor selectively detected atrazine with a limit of detection of 10 μM, without interferences from organophosphorous or carbamate pesticides such as paraoxon, malathion, parathion and aldicarb. The lifetime of the biosensor was 8-14 days and, although significantly shorter than when used for catechol or phenol determination, it was improved by washing the sensor's tip between measurements in order to remove any trace of atrazine. In any case, the use of tissue instead of purified enzyme made easier the replacement of biocatalytic layer, resulting in a low-cost "partially disposable" biosensor.

2.2. Peroxidase-containing tissues

Peroxidase (POD) is one of the most commonly used enzymes for biosensor construction. Due to their presence in many plants, a variety of biosensors have been described. Wang et al. (1992) used horseradish root tissue as POD source (the most commonly found) and immobilised it onto a rough graphite disk. They detected submillimolar concentrations of 2-butanone peroxide in 20 s. The main advantage of their approach was that the biosensor operated in organic phase.

Vieira and Fatibello-Filho (2000) also developed a biosensor able to work in organic media. They modified a paraffin/graphite mixture with sweet potato tissue (*Ipomea batatas*) rich in POD for the determination of hydroquinone in cosmetic creams. In the presence of hydrogen peroxide, its natural substrate, POD oxidises hydroquinone to *p*-quinone, which is reduced back to hydroquinone at −0.22 V *vs.* Ag/AgCl (Fig. 1). The detection limit was 8.1 μM, comparable to that

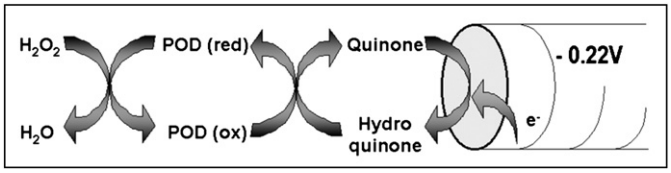

Fig. 1. Reaction sequence within the sweet potato tissue-based biosensor.

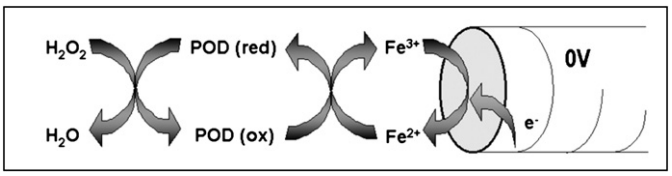

Fig. 2. Reaction sequence within the ferrocene-mediated asparagus tissue-based biosensor.

obtained using the pharmacopoeial procedure. The approach allowed the direct determination of hydroquinone, after solubilisation of the cosmetic cream, without hydroquinone extraction to the aqueous phase.

The same research group immobilised gilo (*Solanum gilo*) crude extract in a chitosan biopolymer for the development of biosensors for hydroquinone (de Oliveira and Vieira, 2006a) and rutin (de Oliveira et al., 2006b). Gilo is rich in POD, but the enzyme from this plant is not yet commercially available. The amine groups on chitosan, compound obtained by the alkaline deacetylation of chitin, are highly reactive and create strong interactions with the enzyme. The biosensors developed in this way were very sensitive (limit of detection of 2 μM for hydroquinone and 20 nM for rutin), reproducible (1% maximum relative standard deviation) and extremely stable (lifetime of 6 months and at least 300 determinations for hydroquinone and 8 months and at least 500 determinations for rutin). Recently, the authors have also linked green bean (*Phaseolus vulgaris*) tissue homogenate to a chitin matrix for the development of a biosensor for the determination of caffeic acid in white wine (Fernandes et al., 2007). As before, the biosensor was very sensitive (limit of detection of 2 μM), reproducible (2.2% relative standard deviation) and stable (10 months).

POD has also been found in asparagus tissue. Oungpipat et al. (1995) co-immobilised ground asparagus tissue (*Asparagus officinalis*) and ferrocene in a carbon paste matrix to develop a hydrogen peroxide biosensor. The ferrocene acts as redox mediator, facilitating the electron transfer between the enzyme and the electrode, decreasing the working potential, and thus reducing the direct oxidation of interferences such as ascorbic acid (Fig. 2). The mediated biosensor operated at 0 V *vs.* SCE, and had a detection limit of 0.4 μM and a response time of 2 s. The same biosensor, with slight modifications, was used to determine fluoride, beneficial for teeth and bones but dangerous at concentrations higher than 1.5 mg/L (Liawruangrath et al., 2001). In this configuration, the inhibitory power of fluoride on POD was exploited. The biosensor had a detection limit of 0.5 mg/L, indicating its applicability in real samples. Commercial tablet formulations were analysed and results were comparable to those obtained with a fluoride selective electrode.

Recently, Kozan et al. (2007) have incorporated coconut fibres into carbon paste for the development of a hydrogen peroxide biosensor. Their device operates at −0.15 V *vs.* Ag/AgCl, responds rapidly (7 s to attain 90% of the signal) and is able to detect as low as 40 μM hydrogen peroxide. Moreover, 50 measurements can be performed with the same electrode without significant decrease in its response, and the lifetime is of at least 3 months. They have tested the biosensor with pharmacological products, such as antiseptic solution, contact lenses cleaning solution, hair colouring cream and antiseptic dental rinse solution, and results correlate with those obtained by the official spectrophotometric method.

Most tissues used in the manufacture of plant-based biosensors are obtained from commercial sources. This may imply an incomplete knowledge of their freshness and ripeness, and of the climate and seasonal conditions under which the plant has grown. These factors are usually responsible for non-reproducible results. In order to solve this problem, Navaratne and Rechnitz (1992) used *in vitro* cultured tobacco (*Nicotiana tabacum*) callus tissue, which served as a source of fresh POD with a high and reproducible enzymatic activity. The *in vitro*

culture implies the isolation of explants from the plant body and their aseptic inoculation in a growth medium. Due to the possibility to control the growth conditions and nutrients, reproducible and uniform tissues can be obtained. The authors compared the response to hydrogen peroxide using callus, agar-grown and regularly grown plant tobacco tissues, and obtained the highest sensitivities and lower variances with the *in vitro* cultured tissue, demonstrating the advantages of their approach. Moreover, the lifetime of the biosensor was of over 4 months, much longer than those obtained with other plant tissue-based biosensors.

2.3. Acid phosphatase-containing tissues

PPO and POD are not the only enzymes present in potato tissue. Campanella et al. (1990 and 1992) used potato slices as acid phosphatase (AF) source and added glucose oxidase (GOD) to create a bi-enzymatic biosensor for phosphate determination. AF catalyses the hydrolysis of glucose-6-phosphate to glucose, which is oxidised by GOD with the concomitant oxygen consumption. Phosphate inhibits the AF activity, proportionally to their concentration. The biosensor had a limit of detection of 62 μM, a response time of less than 5 min and a lifetime between 10 and 16 days. Real samples of human urine, bovine milk, powdered milk, red wine, mashed tomatoes and commercial pharmaceutical preparations were analysed, phosphate values being similar to those obtained with Bartlett's spectrophotometric method.

2.4. Urease-containing tissues

Soybean tissue has been used to develop a biosensing system for urea due to its high content in urease. Qin et al. (2000) packed this plant tissue in a mini-glass column and immobilised luminol and permanganate on anion-exchange resin columns, creating a flow system for the determination of urea in urine samples. Urease hydrolysed urea to NH_4^+ and HCO_3^-. Afterwards, the anion released luminol from the column, which reacted with the permanganate eluted with sodium hydroxide. The reaction between the luminol and the permanganate generated a chemiluminescence signal. Once again, the system can not be strictly called biosensor. Nevertheless, the flow injection chemiluminescence analysis is particularly interesting due to the high sensitivities and fast response times that can be achieved. In fact, they reported a detection limit of 2 μM and an analysis time of 1.5-5 min including sampling and washing. Moreover, results were in good agreement with those obtained by spectrophotometry.

2.5. Oxalate oxidase-containing tissues

Although its function in humans is not clear, it is known that an excess of oxalate in urine samples may be indicative of pancreas or kidney lesions. Spinach (*Spinacia oleracea*) tissue, rich in oxalate oxidase, was immobilised on a Clark-type electrode with gelatine, but instead of being retained by a dialysis membrane, glutaraldehyde was used to create a cross-linking matrix (Sezgintürk and Dinçkaya, 2003). Oxalate oxidase catalysed the oxidation of oxalate, with the corresponding oxygen consumption. After optimisation of the experimental parameters, they obtained a detection limit of 10 μM, much lower than those obtained with bi-enzymatic amperometric biosensors or optode detection systems.

2.6. Ascorbate oxidase-containing tissues

The same authors (Sezgintürk and Dinçkaya, 2004) developed a biosensor for reduced glutathione (GSH) using cucumber (*Cucumis sativus*) tissue homogenate. Glutathione protects cells from free radicals and reactive oxygen species and is also involved in aminoacid transport, protein and DNA synthesis, etc. Cucumber tissue contains high levels of ascorbate oxidase, a member of the family of multicopper blue oxidases, which catalyses the oxidation of ascorbic acid. Glutathione inhibits this enzyme, changing the oxygen level. The limit of detection of the biosensor was 0.1 μM, and its application to real samples of potato, broccoli and tomato provided results comparable to those obtained by Ellman's method. In addition to this, biosensors were functional even after 2 months, when usually GSH biosensors have a lifetime of 10 days.

2.7. Pyruvate decarboxylase-containing tissues

He and Rechnitz (1995) exploited the fluorescence detection in plant tissue-based biosensors, to demonstrate its better performance. They coupled corn kernel tissue rich in pyruvate decarboxylase to a fibre-optic CO_2 electrode. Pyruvate is decarboxylated to acetaldehyde by this enzyme and the reaction can be followed by monitoring the CO_2 release. Although the most common way to measure CO_2 is by the corresponding CO_2 membrane electrode, a fibre-optic CO_2 optrode can also be used if a pH-sensitive fluorescent dye is coupled to the system. The authors compared these electrodes, observing much shorter response times and slightly lower detection limits with the optical one. The lifetime was of only 7 days; nevertheless, the lifetime was the same with the CO_2 membrane electrode, demonstrating that the irradiation from the fibre probes did not cause any damage to the tissue.

2.8. Diamine oxidase-containing tissues

Both pea or lentil cotyledon contain high levels of diamine oxidase (DAO). This enzyme catalyses the oxidation of diamines, such as putrescine and cadaverine, producing hydrogen peroxide, which can be measured by amperometry. Botrè et al. (1993) developed a cotyledon-based biosensor able to detect 0.5 μM of each one of these diamines. This research group also developed hybrid biosensors, combining lentil tissue with ornithine decarboxylase (ODC) to measure ornithine or with lysine decarboxylase (LDC) to determine lysine (Fig. 3). The performance of the hybrid biosensors was similar to that of conventional bi-enzymatic electrodes.

Hydrogen peroxide produced by diamine oxidation can be also monitored by a chemiluminescence reaction involving luminol and Co^{2+}. In this direction, Mei et al. (2007) packed pea-seedling tissue into a mini-column and incorporated the bioreactor into a chemiluminescent sequential injection analysis system. The combination of the highly sensitive chemiluminescence detection with the sequential analysis system offered several additional advantages compared to conventional enzyme electrodes, such as shorter response times, lower limits of detection (30 and 60 nM for putrescine and cadaverine, respectively), lower costs and simpler sensor assembly.

2.9. Pectinesterase-containing tissues

Pectin is a big polysaccharide (MW between 20,000 and 400,000) abundant in apples, oranges and other fruits and vegetables. Its

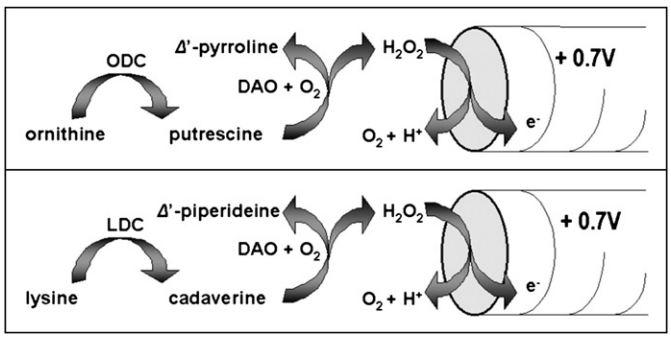

Fig. 3. Reaction sequences within the legume cotyledon tissue-based biosensors.

presence in jam and jellies is also well known. The main problem of traditional pectin biosensors is the high viscosity of pectin solutions, which usually foul the electrode surface. By using plant tissue biosensors, this problem is overcome, as the tissue tolerates better the pectin absorption or, if not, it is much more easily and cheaply replaced. Horie and Rechnitz (1995) developed a hybrid biosensor for pectin combining orange peel tissue, as pectinesterase source, and alcohol oxidase. Pectinesterase reacted with pectin producing methanol, which was oxidised by alcohol oxidase with the concomitant hydrogen peroxide production. The high potentials required for the hydrogen peroxide detection also oxidised other compounds such as ascorbic acid. In order to improve the system, dialysis membranes were replaced by Teflon membranes. In this way, there was not interference by ascorbic acid but response times were longer, due to the slow diffusion of methanol through the membrane, the noise became larger, and the electrode surface became rough after 1 day of continuous use, thus, requiring frequent changing. Despite these inconveniences, the authors demonstrated that plant tissue-based biosensors tolerate the absorption of high molecular weight substances.

2.10. Sulphite oxidase-containing tissues

Sulphite is widely used as an additive in food and beverages to prevent oxidation and bacterial growth. However, sulphite content is strictly limited due to the reported harmful effects on hypersensitive people. *Malva vulgaris* has been used as sulphite oxidase source for the development of an electrochemical sulphite biosensor (Sezgintürk and Dinçkaya, 2005). This enzyme catalyses the final reaction in oxidative degradation of the sulphur-containing amino acids cysteine and methionine, producing hydrogen peroxide. The oxygen consumed was monitored by amperometry, and the signal related to the sulphite concentration. Although the tissue-based biosensor presented a limit of detection (0.2 mM) lower than the conventional pure enzyme-based biosensors, immobilisation protocols were easier and costs lower.

2.11. Mushroom tissues

Due to their special characteristics and their classification, mushrooms deserve an independent section. Mushroom tissues are known to have a high PPO content, which makes them useful as bioreceptors in biosensors. In this direction, Yifeng (1993) used banana or mushroom tissue together with an electrochemical mediator to detect *o*-diphenol, obtaining higher and faster responses with the mushroom-based sensor. Topçu et al. (2004) also exploited the PPO content of mushrooms to develop a biosensor for phenol compounds, based on the immobilisation of the tissue on the top of a Clark-type oxygen electrode with gelatine and glutaraldehyde. Apart from phenol quantification, they also performed inhibition studies, which revealed its possible application as a tool for the determination of benzoic acid and thiourea in soft drinks and fruit juices.

Mushroom tissues can provide enzymes others than PPO. Akylmaz and Dinçkaya (2000) exploited the use of homogenised mushroom (*Agaricus bisporus*) tissue as a source of alcohol oxidase to determine ethyl alcohol. This analyte is an important toxic agent present in alcoholic drinks, which makes necessary its detection in serum and other body fluids from clinical, toxicological and forensic points of view. The authors also immobilised the tissue using gelatine and glutaraldehyde. The alcohol oxidase oxidised alcohols to aldehydes, consuming oxygen. Their biosensor provided fast response times (2 min) and wide linear ranges (from 0.2 to 20 mM).

Mushrooms also contain laccase oxidase, enzyme with a great potential for the determination of phenolic compounds. The low stability and activity of plant laccase oxidases limit their application in biosensors. The use of plant tissues as source of this enzyme could, in principle, improve the biosensor analytical parameters. Timur et al. (2004) compared the performance of three sensors based on laccases from different sources (*Trametes versicolor* laccase from white-rot fungus, *Aspergillus niger* laccase produced by genetic engineering, and *Agaricus bisporus* mushroom tissue). Higher activities were observed at higher pH values with the tissue-based biosensor. This effect, however, could be due to the presence of PPO, enzyme that works better at higher pH values than laccases and that, as previously mentioned, is also present in mushrooms.

2.12. Bi-enzymatic biosensors

Although some examples of bi-enzymatic biosensors have been already described, much more interesting is the case where plant materials are the source of the two enzymes. Oungpipat and Alexander (1994) used spinach tissue as source of POD and glycolate oxidase (GlOD) for the determination of glycolic acid. The concentration of glycolic acid in biological fluids is used as index for differential diagnosis of hyperoxaluria syndrome. Therefore, their sensitive and fast detection and quantification is of great interest. In a first reaction, GlOD oxidises glycolic acid, producing hydrogen peroxide. In a second reaction, POD reduces the hydrogen peroxide and the resulting oxidised POD oxidises a ferrocene mediator to ferricinium, which is subsequently reduced back to ferrocene at the electrode surface (Fig. 4). The use of a mediator permitted to work at 0 V *vs.* Ag/AgCl, achieving a detection limit of 1 μM with response times of less than 1 min.

Zhu et al. (2004) also used spinach leaves as GlOD source and exploited the chemiluminescence of luminol for the detection of glycolic acid in a mono-enzymatic biosensor. In their biosensor, the hydrogen peroxide produced by GlOD together with the application of a positive potential to the working electrode initiated the electrochemiluminescence of luminol (special form of chemiluminescence in which the light emission is generated by electrolysis), and the produced light was proportional to the amount of produced, and subsequently consumed, hydrogen peroxide. Although the principle of the biosensor was mono-enzymatic, it is described here due to the presence of POD in spinach tissue. Obviously, the POD posed a problem, since the enzyme can also consume hydrogen peroxide. However, this problem was overcome by the appropriate choice of working potential, +0.8 V *vs.* Ag/AgCl providing the best signal-to-noise ratio.

Li et al. (2002) developed a chemiluminescence biosensor for dopamine using potato root tissue. This tissue contained both PPO and POD. The hydrogen peroxide generated by the oxidation of dopamine by oxygen under the catalysis of PPO reacted with luminol in the presence of POD, generating a chemiluminescent signal. They coupled the tissue-modified column with a microdialysis sampling unit, and measured the dopamine level in rabbit blood by inserting the microdialysis probe into the edge vein of a rabbit's ear. They obtained a detection limit of 53 ng/mL.

3. Photosynthesis-based biosensors

It is generally agreed that a need exists for the development of rapid, simple and low-cost toxicity screening procedures for the

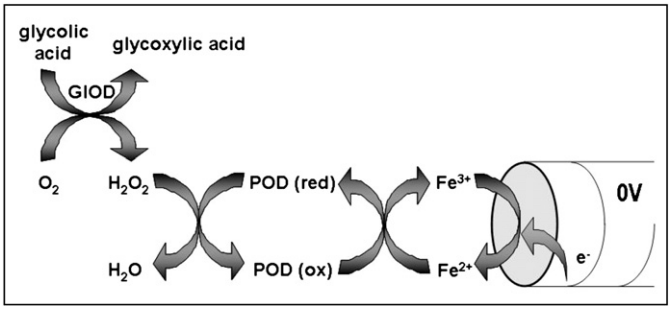

Fig. 4. Reaction sequence within the ferrocene-mediated spinach tissue-based bi-enzymatic biosensor.

detection of toxic chemicals in aquatic and soil environments (Rawson et al., 1987). Photosynthetic organisms are located at the basis of the ecosystems, but they are also the primary target for most toxic pollutants. Based on these findings, the application of photosynthetic material as biological receptor in biosensors provides an excellent tool for a cheap and effective detection of a wide range of life-threatening pollutants.

3.1. Types of photosynthetic materials

Different types of photosynthetic materials have been employed as receptors for the development of biosensors (Rouillon et al., 2006). The use of whole photosynthetic cells is easy and can be performed in a straightforward way, although some disadvantages exist due to the lack of permeability of the cell envelope to electrolytes, and to possible side reactions, which consume reactants and/or products. Both cyanobacteria (Preuss and Hall, 1995; Avramescu et al., 1999) and eukaryotic microalgae (Vilchez and Vegas, 1995; Frense et al., 1998) have been used. Among cyanobacteria, the *Synechococcus* strains have been the most exploited (Preuss and Hall, 1995; Rouillon et al., 1999). Among microalgae, it is possible to find *Chlorella vulgaris* (Sanders et al., 2001), *Scenedesmus subspicatus* (Pandard et al., 1993; Frense et al., 1998) and *Selenastrum capricornutum* (Pandard et al., 1993).

Spinach-isolated chloroplasts and their parts have been commonly used as biological receptors, e.g. intact chloroplasts (Rouillon et al., 1995a), mixtures of chloroplasts and photosynthetic membranes (Rouillon et al., 1994) and photosynthetic membranes alone (Rouillon et al., 1995b). The advantage of this photosynthetic material is the high sensitivity towards pollutants provided by the direct contact between the functional sites and the operational medium. Submembrane fractions of the photosystems I isolated from spinach leaves (Bonenfant and Carpentier, 1990) and II from the thermophilic cyanobacterium *Synechococcus elongatus* (Koblizek et al., 2002) or spinach leaves (Carpentier and Lemieux, 1987; Rouillon et al., 2000), have also been employed.

3.2. Immobilisation of photosynthetic material

In vivo, the cellular environment contributes to the maintenance of the structures and photosynthetic activities. The cell is able to generate the factors necessary for the survival of the metabolic activities and to preserve the structural and enzymatic integrity thus allowing resistance to many external factors like temperature or light variations. After isolation of the photosynthetic material, the activity decreases very fast. In order to improve its resistance, a variety of immobilisation techniques have been designed (Table 1). They are identified either as chemical or physical procedures depending on whether covalent bonds are established or not (Papageorgiou, 1987). Physical methods involve adsorption of the photosynthetic material on supports or the inclusion in a natural or synthetic gel. The immobilisation of photosynthetic material by adsorption is a simple, economic and mild technique, which does not damage, or only slightly, the activity of the biological material. Unfortunately, this advantage has the inherent drawback of the weak interaction forces, which may lead to biocatalyst desorption. In this direction, Pandard et al. (1993) immobilised several species of unicellular eukaryotic algae by aspiration onto an alumina filter disc. The photosynthetic events were monitored by two sensors: one measured the reduction of a redox mediator by the illuminated biocatalyst, and the other measured the photosynthetic oxygen production using a semi-protected oxygen electrode. The latter showed good long-term stability, with a working life up to 7 days using *Scenedesmus subspicatus* or *Chlorella vulgaris* as biocatalyst. Frense et al. (1998) immobilised *Scenedesmus susbspicatus* whole cells on filter paper disks and covered them with a thin alginate layer as a membrane. These surfaces were stored at +4 °C over a period of about 6 months without significant lost in fluorescence properties.

Table 1
Main procedures for photosynthetic material immobilisation

Physical methods	Chemical methods
-Adsorption	-Cross-linking: *glutaraldehyde*
-Gel inclusion: *protein gel, polysaccharide gel* or *synthetic gel*	-Cross-linking: *glutaraldehyde+ protein*

Table 2
Photosystem II activity (oxygen production) after entrapment of a mixture of chloroplasts and photosynthetic membranes in PVA-S_bQ of different degree of polymerisation and after 90-day storage in the dark at +4 °C

PVA-S_bQ polymers	600	1200	1700	2300	3500	3500*
PSII Activity (%)	0	0	20	10	12	20

The activity is given relative to the first value after immobilisation. 3500*: betaine form.

The immobilisation by inclusion, originally developed for enzymes, can be also applied to the immobilisation of photosynthetic material. In this technique, the biocatalyst is entrapped into a three-dimensional polymer network. Natural or synthetic gels can be used. Among the natural gels, alginate is the most commonly used (Brewster et al., 1995; Koblizek et al., 2002). In relation to synthetic gels, styrylpyridinium-modified poly(vinylalcohol) (PVA-S_bQ) has been used to immobilise a mixture of spinach photosynthetic membranes and chloroplasts (Rouillon et al., 1994), purified spinach chloroplasts (Rouillon et al., 1995a), spinach photosynthetic membranes (Rouillon et al., 1995b), spinach photosystem II submembrane fractions (Rouillon et al., 2000) and whole cells of cyanobacteria *Synechocystis* sp. PCC 6803 (Avramescu et al., 1999) or *Synechococcus* sp. 7942 (Rouillon et al., 1999). Rouillon et al. (1995a) showed that the immobilisation of the mixture of chloroplasts and photosynthetic membranes in the PVA-S_bQ increased considerably the storage stability (Table 2). After 90-day storage at +4 °C in the dark and in a dry state, the polymers with 1700 and 3500* (*: betaine form) as degree of polymerisation retained 20% of their initial activity and the polymers 2300 and 3500, 10% and 12%, respectively, whereas the membranes entrapped in polymers 600 and 1200 were not active. The stability of the thylakoids entrapped in PVA-S_bQ 2300 after 24 h drying at +4 °C and under storage at-18 °C was checked. After 427-day storage, the membranes still retained 20% activity (Rouillon et al., 1995a).

Covalent techniques have been widely used for enzyme immobilisation. Unfortunately, this method is not suitable for photosynthetic material because of the denaturing effect of the binding agent and the consequent loss of activity. Nevertheless, Park et al. (1966) showed that glutaraldehyde preserved the Hill reaction activity in chloroplasts. This observation made possible the use of this cross-linker for the immobilisation of photosynthetic material (West and Packer, 1970; Mishra and Sabat, 1995). The denaturing effect of glutaraldehyde can be reduced by the addition of proteins during the polymerisation. In this direction, several photosynthetic materials have been immobilised by bovine serum albumin-glutaraldehyde (BSA-Glu) mixtures: spinach photosynthetic membranes (Loranger and Carpentier, 1994), spinach photosystem II submembrane fractions (Carpentier and Lemieux, 1987) or spinach photosystem I submembrane fractions (Bonenfant and Carpentier, 1990). The best storage conditions were +4 °C in the dark, which retained the stability of the immobilised photosynthetic membranes for about 200 h (Loranger and Carpentier, 1994).

Screen-printed technology offers the possibility to produce a large number of sensors at low cost. Thus, due to their intrinsic characteristics and reproducibility, screen-printed electrodes are often used in the development of disposable electrochemical devices. Koblizek et al. (2002) immobilised photosystem II complexes isolated from the thermophilic cyanobacterium *Synechococcus elongatus* on screen-printed electrodes according to four immobilisation techniques: entrapment in agarose, alginate or gelatine gels, and cross-

linking into the BSA-glutaraldehyde matrix. The entrapment in agarose and alginate failed because these gels exhibited poor adhesion on the electrode surface and these two techniques were therefore ruled out. Better results were obtained when the PSII particles were entrapped in gelatine or cross-linked into the BSA-glutaraldehyde matrix. This last method was used to immobilise other photosynthetic materials on screen-printed electrodes, such as thylakoid membranes from *Spinacia oleracea* L. (Touloupakis et al., 2005; Bettazzi et al., 2007) and from *Senecio vulgaris* wild type and mutant strains resistant to atrazine (Touloupakis et al., 2005).

3.3. Measurement of the photosynthetic activity after immobilisation

The measurement of oxygen evolution using a Clark-type oxygen electrode is a standard procedure in the field of photosynthesis research and constitutes a common method for the determination of the photosynthetic activity (Loranger and Carpentier, 1994; Rouillon et al., 1995b). The incorporation of several types of photosystem II specific artificial electron acceptors as electroactive mediators allows to maximize the photosynthetic activity. The most commonly used mediator is potassium ferricyanide (Cocquempot et al., 1981; Park et al., 1991; Mishra and Sabat, 1995) but benzoquinone derivatives, such as parabenzoquinone (Thomasset et al., 1983; Rouillon et al., 1995a) or 2,5-dichlorobenzoquinone (DCBQ) (Loranger and Carpentier, 1994), are also employed to improve the photosynthetic activity.

The measurement of the photosynthetic activity can be based on amperometric detection in an electrochemical cell. In the photosynthetic membranes, light energy is captured by the photosynthetic pigments and transferred to the reaction centres of photosystems I and II, where charge separation takes place. In this process, reduced and oxidised intermediates are formed, initiating the electron transport chain. In mediated systems, the specific artificial electron acceptor is added to the photoelectrochemical cell in its oxidised form. This mediator is reduced under illumination by the electrons from the photosynthetic chain and subsequently reoxidised at the working electrode surface. Several mediators, such as potassium ferricyanide (Rawson et al., 1987; Koblizek et al., 2002) parabenzoquinone (Pandard et al., 1993), duroquinone (Koblizek et al., 2002) and 2,6 dimethylbenzoquinone (Pandard et al., 1993), have been employed.

Another way to detect, the activity of the photosynthetic membranes without redox mediators is by reducing the oxygen to superoxide ions on the reducing side of photosystem I, using a special electrochemical cell. This process is known as pseudocyclic electron transport or Mehler reaction (Mehler, 1951). These ions dismute either enzymatically (due to membrane-bound superoxide dismutase) or spontaneously, to hydrogen peroxide, which is afterwards oxidised at the working electrode (Carpentier et al., 1991; Laberge et al., 1999). In order to use this electrochemical cell for the measurement of photosynthetic activity in whole cyanobacteria cells (Avramescu et al., 1999; Rouillon et al., 1999) or photosystem II submembranes fractions (Carpentier and Lemieux, 1987; Rouillon et al., 2000), an artificial acceptor such as DCBQ must be present in the electrolytic solution for facilitating the charge transfer between the photosynthetic material and the working electrode. However, using *Synechococcus leopoliensis*, it was shown that with a species that produces significant hydrogen peroxide, phytotoxic compounds can be detected amperometrically without addition of exogenous mediator (Croisetiere et al., 2001).

The difference in colour between oxidised and reduced forms of some mediators enables the colorimetric measurement of the photosynthetic activity. For example, under illumination, photosynthetic membranes (photosystem II activity) reduce the blue 2,6-dichorophenolindophenol (DPIP) to the colourless leuco form (Brewster et al., 1995; Piletskaya et al., 1999).

Chlorophyll a fluorescence induction is another method for the evaluation of the photosynthetic activity (Lazar, 1999). This technique is simple, non-invasive, highly sensitive and fast. The light absorbed by chlorophyll molecules of photosystem II may be assimilated into the light reactions of the photosynthesis or may be released as fluorescence or heat energy. The fluorescence increases *in vivo* when photosynthesis is declined or inhibited. Numerous environmental factors are known to block the electron carriers between photosystem II and photosystem I, increasing the emitted fluorescence (Thomson, 1997). This method has often been employed to detect the photosynthetic activity of immobilised material (Naessens et al., 2000; Sanders et al., 2001; Giardi et al., 2005; Euzet et al., 2005).

3.4. Principal applications of photosynthetic biosensors

Environmental technology is the field were photosystem-based biosensors find most of the applications. Herbicides are widely used in agriculture, since they provide a low-cost weed control. These products, such as triazines (e.g. atrazine), triazinones (e.g. metribuzin), phenylureas (e.g. diuron) and phenols (e.g. bromoxynil), have a common mode of action based on the inhibition of photosystem II. They interact with the D1 protein replacing the plastoquinone, which is a secondary electron acceptor of photosystem II reaction centre, from its binding site (QB). Based on this mode of action, photosynthetic biosensors provide excellent tools for the detection of herbicides and allow a fast screening of environmental samples (Loranger and Carpentier, 1994; Koblizek et al., 1998; Laberge et al., 1999; Piletskaya et al., 1999). Responses of the photosynthetic electron transport chain to several inhibitors are shown in Table 3 (Laberge et al., 1999). As regulations governing the presence of herbicides in drinking water are very strictly enforced by the European Community (European Communities, Drinking Water Directives L229, 1980, p11), with a maximum allowable level of 0.1 μg/L for each individual substances and 0.5 μg/L for the sum of pesticides, the sensibility of photosynthetic biosensors is often insufficient to reach the concentration required by the regulatory law. Consequently, sample preconcentration is often necessary. As an example, the sensor sensibility was improved by combining a optical biosensor with a sample preconcentration based in a solid phase extraction packed with a polymer, imprinted with cyanazine This biosensor allowed to detect photosynthesis-inhibiting herbicides in water at the levels required by the European Union regulations..In addition, the combination biosensor-molecularly imprinted polymer (MIP) also allowed selective measurement of triazines herbicides (cyanazine, atrazine) and their discrimination from phenylurea (diuron) or triazinone (metribuzin) herbicides (Breton et al., 2006).

The use of multi-biosensors represents another interesting strategy to improve the selectivity and the sensitivity of photosynthetic biosensors. Wide and prolonged application of certain herbicides, principally atrazine, cause selection and consequent diffusion of herbicide-resistant biotypes derived from sensitive infesting species. The resistance is due to the modification on the amino acid composition of the target D1 protein in the QB pocket. *Senecio vulgaris* and *Amaranthus retroflexus* strains resistant to atrazine were selected from soil treated with this herbicide during five years. Multi-biosensors were constructed using various photosynthetic preparations as biosensing elements (wild type and mutant strains resistant to atrazine). The PSII biomediators showed different recognition activity towards herbicides,

Table 3
Herbicide concentrations causing 50% inhibition (IC_{50}) of the photocurrent generated by entrapped photosynthetic membranes

Herbicides				
-chemical class	*Triazines*	*Triazinones*	*Phenylureas*	*Phenols*
-chemical	Atrazine	Metribuzin	Diuron	Bromoxynil
IC_{50} (mg/L)	0.33	0.13	0.15	19

Inhibition was measured 5 min after contact.

Table 4
Metal (chloride salts) concentrations causing 10% inhibition (IC_{10}) of the photocurrent generated by entrapped photosystem II submembrane fractions

Element	Hg	Cr	Cd	Pb	Zn	Ni	Cu
IC_{10} (mg/L)	20	26	90	104	327	441	445

Inhibition was measured 5 min after contact.

the multi-biosensor being able to distinguish several subclasses (Giardi et al., 2005; Touloupakis et al., 2005). The unicellular alga *Chlamydomonas reinhardtii*, with only one chloroplast, is important as a model of the nanostructured PSII complex; moreover, it can be easily modifiable at the level of PSII by site-directed mutagenesis. In this way, a D1 mutant of *Chlamydomonas reinhardtii* was generated, changing Phe_{206} by Ile, Glu, Ser, Ala, His, Asn, Val and Lys. Two of these mutants, Ser and Lys, exhibited supersensitivity for the herbicides atrazine, diuron and phenmediphan (Johanningmeir et al., 2006). A miniaturized biosensor-based optical instrument has been designed and fabricated for multi-array fluorescence measurements of several D1 mutants of *Chlamydomonas reinhardtii* (Tibuzzi et al., 2007). Six pesticides were analysed: atrazine, diuron, ioxynil, terbutylazine, prometryn and linuron. The mutants have shown various sensitivities to different pollutants and the minimum detected inhibitor concentration was 10^{-9} or 10^{-10} M, depending of the pesticide class.

Metals (Pb, Cd, Cr, Cu, Ni, Hg, Zn) are also examples of industrial pollutants detectable by plant material. Although toxic metals may affect several plant functions, photosystems, in particular photosystem II, are especially sensitive to their toxicity (Carpentier, 2002). Table 4 shows the concentrations of heavy metals that produce 10% inhibition of the activity (IC_{10}) of photosystems II submembrane fractions immobilised in PVA-S_bQ (Rouillon et al., 2000). Although the detection limits of heavy metals with photosystem-based biosensors do not seem suitable for some natural samples, they can be improved if appropriate extraction protocols are used (Saran et al., 1992). Nevertheless, they can be useful in samples where metal concentration is potentially high (e.g. sewage sludges).

4. Biochips for the study of plants

Although beyond of the scope of this review, it is necessary to at least mention the existing biochips in the plant field. Some examples of multi-biosensors have already been mentioned in the previous section. A biochip is a device that integrates several biosensors in the same platform. In other words, biochips are ordered sets of known biorecognition elements immobilised on precisely defined locations of a solid substrate. The biochip approach enables the simultaneous detection of tens, hundreds and even thousands of targets. Biochips are rapidly replacing other analysis techniques, especially in DNA sequencing, expression analysis and other high-throughput applications. Despite the wide variety of techniques available for biosensors, most biochips use fluorescence methods for detection. Fluorescence detection enables the scanning of the microarray surface in micrometer increments, recording the pixel values and translating them into a scanned image. After the corresponding data treatment, results can be interpreted.

Although the application of DNA microarrays to gene discovery or polymorphism mutation in yeasts, plants, flowers and fruits has been demonstrated, most plant-based DNA biochips analyse gene expression profiles. In this direction, DNA microarrays have been applied to understand biological aspects in *Saccharomyces cerevisiae* yeast during spore development (Chu et al., 1998), to study its response to stress (Kao, 1999) or the galactose metabolic pathway (Ideker et al., 2001). With regard to higher plants, most studies with DNA microarrays have used *Arabidopsis thaliana* as model plant to evaluate the levels of expression in seedlings, roots, leaves, inflorescences, flowers and siliques at different developmental stages (Schena et al., 1995; Ruan et al., 1998; Zhu et al., 2001). The differences in gene expression contributed to identify promoters and common regulatory elements, and to clarify regulatory pathways, making possible to genetically engineer metabolic pathways. Although in a lesser extent, biochips have also been used to study the response of some genes to light (Desprez et al., 1998; Kehoe et al., 1999; Ma et al., 2001).

5. Conclusions and outlook

Development of biosensing devices is the main focus of many research groups and high technology companies. The extensive work done in this field is particularly due to the broad versatility of these biosensors. From probes to transducer substrates, from immobilisation to detection methods, from single to multi-analyte formats, this wide range of possibilities makes the research field very diversified and competitive. The use of plant-tissue and photosynthetic materials for the construction of biosensors is interesting not only in terms of development of new bioanalysis devices, but also as tools to provide information about the plant biochemistry and physiology and to better understand enzymatic and photosynthetic processes. Biochip technology is also of great value to study plant processes, e.g. growth and development, and responses to environmental changes, e.g. elevated temperatures and soil salinity. Genechips and gene expression studies derived from them will contribute to clarify regulatory mechanisms that appear as a response to viral or pathogen attack, hormone treatment or pesticide exposure, constituting a powerful tool for plant functional genomics.

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