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review

Potentiometric and Nanogravimetric Biosensors for Drug Screening and Pollutants Detection

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Summary

Recent advances in the field of biosensors are presented along with a few significant examples of their applications in the fields of health and environmental monitoring. In particular this paper focuses on two distinct biosensing systems based on potentiometric and gravimetric transducers. These biosystems are here exemplified in monitoring the effects of drugs on living cells and the kinetic monitoring of binding in the antigens-antibodies immunoreactions. The results demonstrate the significance and potential importance of these biosensors.

Keywords: biosensors, drug screening, pollutants detection, potentiometric and nanogravimetric transducing mechanisms

Introduction

Biosensors appear suitable for many applications, ranging from the detection of specific compounds in analytes to the monitoring of biological and monitoring and control of biochemical processes. Nevertheless, only few systems based on biosensors are present on the market today. The reason for this unbalanced situation between literature on biosensors and commercial products can be found in the intrinsic difficulty to preserve the working conditions of the biological element for a long time.

There are, however, several cases which can be managed relatively well, mainly when the biological element can be spread, filmed or attached directly by the final user of the biosensor.

Our group has developed two prototypes belonging to the latter category, respectively, based upon potentiometric and gravimetric transducers.

Although amperometric devices are the most common transducers in this field *(1,2)*, biosensors based on this principles are rather critical, specially for the difficulty to modify the working electrodes with bioelements.

Among potentiometric sensors, the most common transducer is probably the ISFET (Ion Sensitive Field Effect Transistor), created in 1970 by Bergveld *(3)*. So far, the fabrication process has not been optimized to allow a sufficiently high life time to these devices. Either front or back contact ISFETs suffer from the presence of a liquid phase near electrical wires and as a consequence, the device is more or less rapidly damaged by the measuring solution.

A potentiometric transducer which avoids these problems is the LAPS (Light Addressable Potentiometric Sensor), first introduced by Hafeman *et al.* in 1988 *(4)* and then optimized by McConnell's research group at Stanford University *(5)* and by our group *(5,6)*. The device is free of metal contacts near the solution, because the measuring signal is provided either by light excitation or by means of a small alternating signal. Therefore its life-time is practically infinite.

In particular, the solution adopted by our group, substantially different from the early proposed LAPS devices, makes use of distinct silicon chips, eventually incorporated in a single measuring chamber, each one of them being excited by means of an alternating signal, which induces a measuring signal dependent also on the surfacial potential at the insulator/electrolyte interface (the acronym PAB, Potentiometric Alternating Biosensor stems from this peculiarity).

This transducer is utilized to build different biosensors, using either living cells, enzymes, or antibodies as the bioelement. In any case, the direct measured quantity is the surface potential created at the sensor/electrolyte interface caused by the presence of ions or electrons on the surface of the transducer. In this paper several practical examples will be shown about this biosensor.

A second type of transducing mechanism presented in this work is the so-called nanogravimetry. It is based

on quartz crystal resonators, which oscillate at a given frequency depending on their physical characteristics. Alterations of the quartz surface, such as a mass increase, cause a deviation of the oscillating frequency directly proportional to the added mass per unit area *(7,8)*. Therefore systems based on this principle are currently used to monitor the deposition processes in metal evaporators, or wherever a deposition should be quantitatively investigated. Recently the principle has been proposed in the biosensors field *(9,10)*, where the mass increase is caused by the selective binding between molecules, one of which is fixed onto the quartz crystal, while the second flows in the measuring analyte, and is considered as the molecule whose presence should be quantitatively determined.

Materials and Methods

Potentiometric system

The system is based on a 8×8 mm n-type silicon chip, covered with a 350 Å thick SiO_2 layer, and a 1000 Å thick Si_3N_4 layer, acting as a pH sensitive surface. The transducer is fixed in an electrochemical flow-through cell and used as the working electrode. A Pt counter electrode and a calomel reference electrode provide the proper biasing of the device. A modulated AC signal generates an alternating current, whose amplitude depends on the surfacial potential at the insulator/electrolyte interface *(11)*. Monitoring this amplitude allows to detect, in real time, the pH changes in the region near the Si_3N_4 layer. Additional possibility is to cover the nitride layer with a metal spot, which allows to detect the redox potential variations of the measuring solution, rather than the pH.

Different possibilities to fix the bioelement onto the transducer can be used:

- membranes, where enzymes, cells or antibodies can be easily entrapped,
- glass cover slips, onto which cells can be easily grown and
- solid supports, where generic bioelements can be deposited, linked or generically fixed.

The membranes can be placed in the flow chamber just on the sensing area, providing a direct measurement of the analyte.

The flow chamber is specially designed to contain glass cover slips or solid supports.

The system was specialized to follow two distinct events: the effect of drugs on living cells and the detection of pollutants in water. In the first case a solid support was used, while in the second a membrane with entrapped specific antibodies was used.

The supports are usually placed in front of the transducer, by means of a 50–200 μm spacer. A small volume (about 10 μL) of medium is in contact both with the transducer and with the cell population. In this way the cells are maintained in living conditions, because fresh medium is continuously inserted into the chamber. At the same time, the use of a small volume of medium allows to bypass the problems related to the buffer capacity of the solution used, and to detect correctly eventual pH changes. The chamber is thermostated at 37 °C, in order to ensure optimal environmental conditions for the cell population.

The measuring system is depicted in Fig. 1. The chip backside is fed into a current to voltage converter, whose output is band-pass filtered in order to retrieve only the significant signal component (at the same frequency of the small signal modulation). The signal is then computer-acquired (by an analog-to-digital converter), displayed and stored. The computer provides also the modulation and biasing signals, by means of digital-to-analog converters. The system unit is asserved by an *ad hoc* software, consisting of low level routines to drive the electronics, and high level sections for user interface and data input-output.

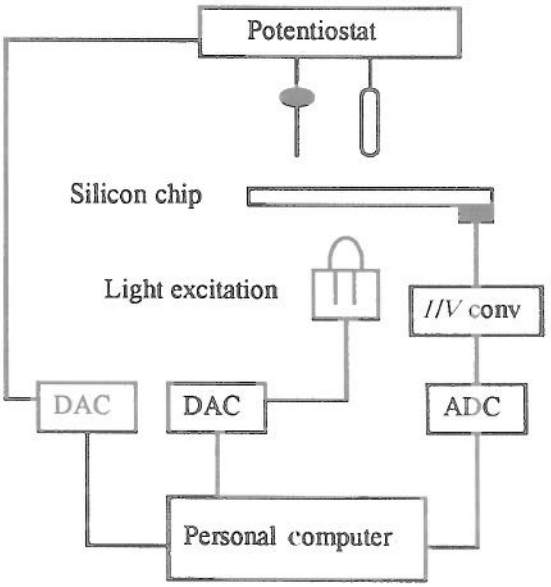

Fig. 1. Block diagram of the potentiometric system

The system can monitor two main types of signals: the current *vs.* applied voltage (*I–V* characteristic curve) and the current *vs.* time corresponding to a given applied voltage (*I–t* curve). The former data are useful to check the system functionality, and to detect the basic parameters of the chip in use. The latter are a direct measurement of the pH (or redox potential) variations *vs.* time.

In the experiments reported below CHO-K1 cells have been used. They were obtained from the American Type Culture Collection (Rock-ville, MD, USA). The CHO-K1 (line CCL61) is an epithelial-like cell line, derived from a biopsy of an ovary of chinese hamster. Cells were cultivated in Ham's F12 medium, also used in the experiments. The medium was supplemented with 10% foetal calf serum (FCS), 10000 U/mL penicillin, 10 mg/mL streptomycin, 25 μg/mL amphotericin B and buffered with sodium bicarbonate. The cells were maintained at 37 °C in 95% air/5% CO_2 in 250 mL flasks. Cultures were plated routinely with a density of 10^5 cells/mL.

Therapeutic doses of three different drugs have been tested on the cell population: Methotrexate (Sigma Chemicals, Italy), Cisplatin and 5-fluorouracil (Bristol Italiana SpA, Italy).

In experiments concerning pollutants detection, we used monoclonal antibodies against 2,4-dichlorophenoxyacetic acid (2,4-D), an herbicide used in cereal crops which may pollute ground water.

Nanogravimetric system

The system makes use of two quartz crystals, acting as a working and a reference electrode, respectively *(12,13)*. The reference quartz is useful when unspecific adsorption takes place. In this case, being the effect statistically equal for the two quartzes, the corresponding undesired frequency shift can be easily eliminated by a differential measurement. The user can choose either to leave the reference quartz uncoated, or to cover it with a molecule physically similar to the working one, but unspecific to the same analyte.

The crystals have a native resonating frequency of 1–16 MHz (10 MHz for those used in the experiments presented below, supplied by Nuove Mistral, Italy). Each quartz is connected to an oscillating circuit, which allows distinct measurements for the two sensing units, connected to a single 24-bit counter. The system allows to monitor in real time the kinetic of surfacial mass variation, acquiring the oscillating frequency signals *vs.* time *(14)*. Specific software is dedicated to the hardware driving and to the data collection and scaling. The block diagram of the device is depicted in Fig. 2.

Each quartz can be mounted in a flow-through measuring chamber, where a measuring solution contacts one side of the crystal (the opposite one being uncostrained and in contact with air). In these conditions the output frequency is affected only by the surfacial interactions relative to the wet side of the quartz (a mass increase corresponds to a frequency decrease).

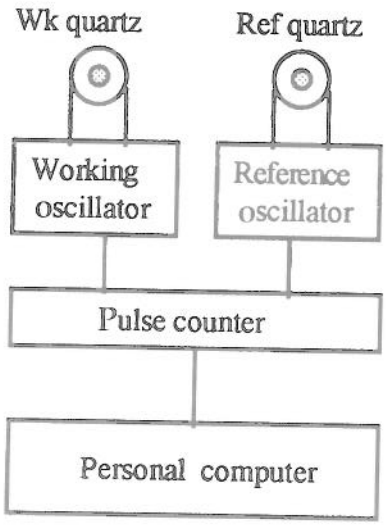

Fig. 2. Block diagram of the nanogravimetric system

Dry experiments can be performed as well, for example to monitor the deposition rate in a metal evaporator.

Affinity experiments were performed with quartzes coated with bovine serum albumin (BSA, Sigma Chemicals, Italy). The BSA was immobilized onto the crystals either directly onto the gold surface of the electrodes or using silanized underlayers formed by APTS and glutaraldehyde. Anti-BSA antibodies were dissolved in TBS buffer and used as the target species for the immunocomplex.

Using the system as a monitor, the user can follow the kinetic of binding between the antigens and the antibodies used in the experiments.

Results

Health care applications

In order to quantitatively test the effect of drugs on living cells with the above described method, a stable reference signal should be measured. The most correct approach is to use the same cell population which will later be affected by the drug. The first experiment is the determination of extracellular acidification rate of a sample, obtained by means of the *I-t* curve, as those depicted in Fig. 3. The curves are obtained by alternating flow on (100 μL/min) and flow off periods in the measuring chamber. When the flow is stopped, cells acidificate the microvolume near the transducer surface and when the flow is resumed, the measured signal comes back to the previous value, corresponding to the native pH of the flowing medium.

The rate at which cells acidificate is a measure of their status, and reflects the effects of cytotoxic compounds on them *(15)*.

The perturbing agents are added to the medium in order to reach the cell population directly in the flow chamber, where the signal is recorded.

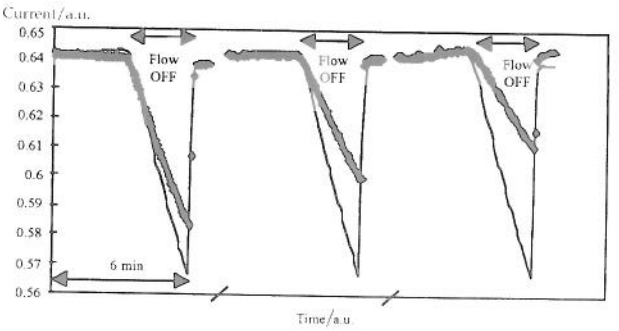

Fig. 3. *I-t* curves of the potentiometric system. The flow in the measuring chamber is stopped and retrieved. During flow off periods the signal decreases indicating an acidification process. The thin line signal was obtained using normal medium, while the other signal monitors the effect of 5-fluorouracil during time. The acquisition was performed with 3 min on and off periods. The normal metabolism is reported for a comparison purpose with the curve indicating the drug effect. This becomes relevant and is evidenced by the lowering of acidification during time. The total experimental time was 4 hours.

Data collected in the same conditions as the baseline allow a direct comparison between the acidification rate of cells with and without drug. Both the baseline and a curve related to the 5-fluorouracil action on a cell population are presented in Fig. 3. The drug administration causes a progressive decrease of the metabolic rate, usually evident after the first 30 minutes.

Fig. 4 shows a quantitative determination of the above effect on a cell population. As evidenced, the drug effect results in only 180 min, which is a very short response time if compared with the usual tests. Moreover the acquired data allow direct monitoring of the effects of drugs on the cells metabolism, while usual methods can detect the drug effects only after cell death.

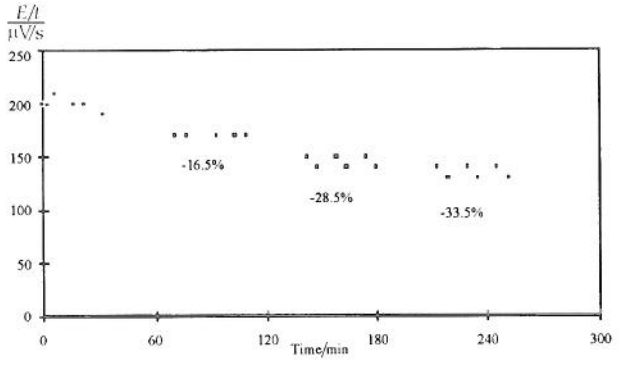

Fig. 4. Acidification rate of a CHO cell population treated with 5-fluorouracil, as a function of time. Each point in the curve was derived by raw data as those presented in Fig. 3.

Similar experiments can be performed for a wide variety of cells and drugs, yielding to a general detection system able to monitor the physiological status of a small number of cells with a label-free and non-invasive method.

Environmental applications

Using the potentiometric system to detect the presence of specific compounds in test solutions is also possible, and sometimes convenient, depending on the particular application.

In general, as the transducer directly detects either the pH or the redox potential, an intermediate agent should be incorporated in the assay to convert the information into one of the detectable signals.

For example, in order to detect the presence of 2,4-D in water, we performed a competitive immunoenzymatic assay with horse radish peroxidase (HRP). The monoclonal antibodies are immobilized on activated membranes (Biodyne B, Pall, Italy), while free 2,4-D and 2,4-D conjugated with HRP are dissolved in the test solutions, in order to form a competitive immunoassay. The system monitors the formation of the immunocomplex by following the peroxidase activity, which can be easily detected using H_2O_2 and tetramethylbenzidine as substrates and a redox pair as recycling agent. The system contains a redox potential modified transducer (insulator coated with a gold spot) *(16,17)*.

A typical result of the assay is presented in Fig. 5, where the sensitivity (corresponding to a 10% signal decrease with respect to maximum) is less than 0.01 ppm.

The nanogravimetric system can be used as well for experimental applications. For instance, the same antigen was detected with this method *(18)*.

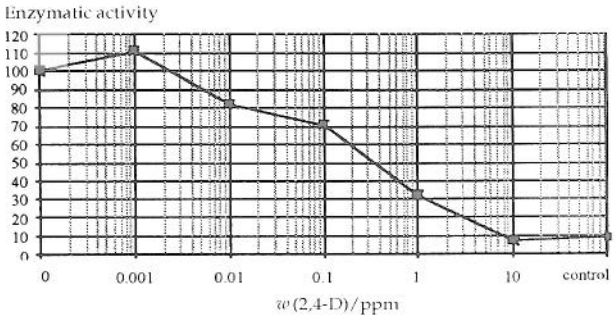

Fig. 5. Results of the competitive immunoenzymatic assay for the determination of 2,4-D in water

One of the easiest measurements is, however, that related to the density and viscosity of a solution, parameters which influence the oscillating frequency of the crystals in the same fashion as mass changes *(19)*. In fact, this measurement does not imply any surfacial modification of the quartz crystals, and gives a signal proportional to the square root of the density-viscosity product. This signal can be extremely useful to monitor, for example, the presence of hydrocarbons in water. A different example is reported in Fig. 6, where different solutions with increasing percent of sucrose in water have been injected into the measuring chamber.

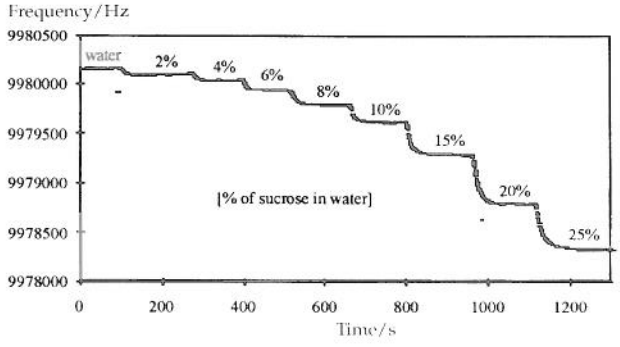

Fig. 6. Nanogravimetric measurement of the change in density and viscosity of a solution with increasing percent of sucrose in water. The experiment shows that bare quartz crystals (i.e. not coated) can be successfully utilized for these evaluations, which could be applied in environmental controls.

Different types of experiments can be performed to monitor the kinetic of binding between antigens and antibodies, opening a wide range of possible tests in the field of environmental monitoring. Here an antigen or an antibody is deposited or filmed onto the quartz resonator, while the specific complementary element is dissolved in a buffer solution and inserted in the measuring flow chamber by means of a peristaltic pump. When the

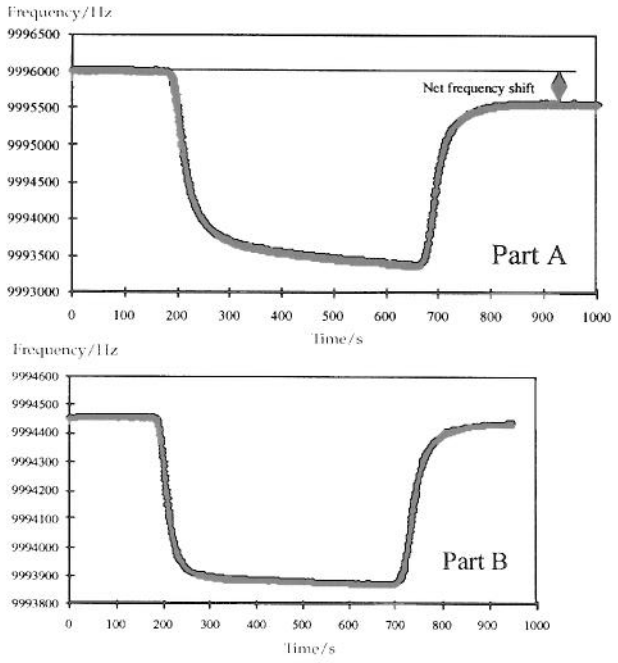

Fig. 7. Kinetic monitoring of the immunocomplex formation, utilizing a BSA-coated quartz crystal. Part A refers to a specific reaction, with a solution containing anti-BSA antibodies, while part B refers to an unspecific reaction using GAM antibodies. The unspecific adsorption, affecting both crystals, is neglected by applying a differential measurement.

formation of the immunocomplex takes place, an increasing mass produces a decreasing frequency, usually in 5–15 min the signal reaches a constant value. By inserting the buffer solution again, all the unbound compounds flow away from the transducer surface and the net frequency decrease monitors significant parameters of the immunological complex.

As an example of this type of application, Fig. 7 shows a kinetic experiment performed using BSA as antigen and two different antibodies in solution (at a concentration of 0.1 mg/mL): the first (anti-BSA) is intended as the specific molecule, while the second (Goat Anti Mouse, GAM) is used as a non specific protein in order to detect and correct the effects of unspecific adsorption.

Each plot is a recording of the oscillating frequency of the quartz crystal *vs.* time. The frequency is stable unless the measuring solution is changed from bare buffer to a mixture containing also the specific or unspecific compounds. The frequency decreases for two reasons: first, specific binding between BSA and anti-BSA takes place and second, some unspecific adsorption is also present. When the flow is switched again to buffer (washing the crystals), the unspecific binding is almost completely removed. In fact the signal corresponding to the flow containing GAM (non specific antibody) comes back to the prior oscillating frequency, while that with anti-BSA (specific antibody) maintains a net frequency shift of about 445 Hz with respect to the original value. This net difference is ascribable to the specific binding which occurred at the quartz surface.

The solution conductivity, density and viscosity also affect the measuring signal, therefore different buffer concentrations give rise to more or less steep variations in plots as those in Fig. 7. Using the same solution for working and reference channels, however, allows a direct understanding of the binding process.

Discussion and Conclusions

The experimental results reported here are instructive examples of the possible applications of the described biosensing systems. Although the data shown above represent good tests for these novel systems, the possibilities are much broader.

With respect to the potentiometric system, a variety of experiments can be set up, either for drug screening or analytical assays. In the first case, the evaluation of the drug effects on tumor cells is of primary importance today. This aspect can be pursued in a general way (on cell cultures) or specifically on the cells of a patient. It will be possible, therefore, to monitor the percent of success of the drug treatment directly for that patient, with the enormous advantages deriving from a specifically designed drug treatment. Pharmaceutical companies are showing interest in these studies mainly because it is now a general opinion that drug effects strongly depend on the subject to whom the drug is administered.

Analytical studies with the potentiometric system are advantageous in a limited number of cases, where the transducer can exploit its functionality the best (this is indeed a common situation for all biosensors, usually indicated for specific cases). One of the most promising studies involves enzymes and/or antibodies as bioelements. They are usually used as indirect mediators to translate a biochemical signal into a pH or redox variation. The example shown before, can suggest many similar detections, which can be directed towards the environmental control, as well as towards health-related studies (i.e. DNA detection or characterization).

Affinity sensors are probably better implemented utilizing the nanogravimetric principle, mainly for its high resolution in the detection of mass changes. Although the mass sensitivity is incredibly high, when reported to specific affinity problems it is often not satisfactory. A typical example is the detection of pesticides. Here the sensitivity does not allow to reach the limits indicated by the laws of most countries. On the other hand, nanogravimetry is a good tool to monitor the kinetic of binding in affinity assays. In this case the system peculiarities are fully exploited, and the device can be also applied to compute the affinity constants of a given reaction.

The increasing interest for affinity sensors in the scientific community does not always lead to a market product. In fact, in view of the difficulty to design and realize reliable and reproducible systems, the »old« solutions for the presented problems are still preferred. Nevertheless systems like those described in this paper have been produced in tens of prototypes by our group, and are now being tested by specialists in different fields.

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Potenciometrijski i nanogravimetrijski biosenzori za ispitivanje lijekova i otkrivanje onečišćavača

Sažetak

U radu su prikazana najnovija dostignuća u području biosenzora, te nekoliko važnih primjera njihove primjene u zdravstvu i zaštiti okoliša. Posebna je pozornost usmjerena k dvama različitim biosenzorskim sustavima osnovanim na potenciometrijskim i gravimetrijskim provodnicima. Prikazana je primjena tih biosustava pri motrenju utjecaja lijekova na žive stanice i kinetike vezanja antigena i protutijela u imunoreakcijama. Rezultati pokazuju značenje i moguća područja primjene tih biosenzora.