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DEVELOPMENT OF BIOSENSORS FOR IMMUNOASSAYS

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Summary. - *Biosensors represent a new generation of analytical instruments incorporating a biological sensing element in contact with a physical transducer and an output device. The biological element may be a whole organism, an organelle, an enzyme, an antibody, cell receptors or nucleic acids. The transducer converts the interaction between the biological component and the sample into a measurable signal which could be an electrical charge, an optical signal, a heat exchanger or some other quantifiable event. In recent years, electrochemical sensors based on continuous potentiometric or amperometric detection of enzyme reaction products have been favoured in the development of biosensors. This is because electrochemical transducers are cheap, rapid to use and portable.*

KEY WORDS: biosensors, immunoassays, transducers.

Riassunto (Sviluppo di biosensori per saggi immunologici). - *I biosensori rappresentano una nuova generazione di strumenti analitici che hanno la caratteristica di incorporare un elemento biologico sensibile e un trasduttore fisico. L'elemento biologico può essere un organismo intero, un enzima, un anticorpo, un recettore cellulare o un acido nucleico. Il trasduttore converte l'interazione tra la componente biologica e il campione in un segnale misurabile che, a sua volta, può essere una carica elettrica, un segnale ottico, un segnale termico o qualsiasi altro evento quantificabile. Recentemente, nello sviluppo di biosensori, si sono usati preferenzialmente sensori elettrochimici basati su rivelazioni continue potenziometriche o amperometriche; la causa è dovuta alla semplicità, facilità di uso e trasportabilità di questi sensori elettrochimici.*

PAROLE CHIAVE: biosensori, saggi immunologici, trasduttori.

Electrochemical biosensors differ in that in the potentiometric enzyme electrode a potential difference is generated as the sensing element and this is measured relative to an accurate reference element under conditions of zero current flow [1]. This means that there is no net consumption of material and therefore the rate of diffusion or mass transport is not important. Potentiometric devices for the detection of ions such as Cl^-, K^+, Na^+, NH_4^+ and H^+ are currently in use in clinical chemistry in conjunction with the following enzyme labels: acetylcholine-esterase, adenosine deaminase and urease. A potentiometric immunoassay using K^+ as the label has been described for antibodies against the drug digoxin [2]. Other potentiometric immunoassays under development utilise an immunochemical electrical component called a liposomal immunosensor [3]. This unique sensor detects electrochemical changes taking place in response to varying levels of antigen, antibody or complement present. Whenever a single liposome in raptured, several entrapped marker ions are quickly released into the external environment. These ions are measured directly with specific ion selective electrode.

In the amperometric sensor, a voltage is applied between the working and reference electrodes. Amperometric devices therefore rely on the measurement of current rather than voltage. In a typical device the imposed potential causes electron transfer to occur. This results in a current flow that is proportional to the concentration of the analyte [4]. Four types of electron transfer reactions have been realised between biological systems and electrodes. These are: a) substrate or product electrochemistry; b) cofactor recycling; c) coupled system and d) mediator approach. However because of the number of problems in achieving direct electron transfer between enzymes and electrodes the use of small-molecule electroactive mediators to shuttle electrons efficiently between the electron and the enzyme catalysed reactions has in recent years been the preferred approach [5]. The most versatile mediator has been found to be ferrocene and its must successful

application has been in an amperometric enzyme electrode for the determination of glucose [6]. The ferricinium ion replaces oxygen as the cofactor for glucose oxidase:

$$\text{Glucose} + \text{GOD}_{(oxid)} \rightarrow \text{Gluconolactone} + \text{GOD}_{(red)}$$

$$\text{GOD}_{(red)} + 2\text{Fecp}_2\text{R}^+ \rightarrow \text{GOD}_{(oxid)} + 2\text{Fecp}_2\text{R} + 2\text{H}^+$$

The reduced ferricinium ion is regenerated at the electrode:

$$2\text{Fecp}_2\text{R} \rightarrow 2\text{Fecp}_2\text{R}^+ + 2e^-$$

The combination of substrate specificity and the sensitivity of amperometric methods has also led to the design of a number of elegant electrochemical immunoassays. The useful oxidation potential of ferrocene at around + 200 mV has led to the development of amperometric homogeneous immunoassays for small molecules such as the drug, lidocaine [7]. Lidocaine is first linked to ferrocene and binding of the ferrocene-drug complex by antibody inhibits its ability to act as a mediator in the glucose/glucose oxidase reaction. The catalytic current is greatly reduced. However, this can be reversed by adding non-labelled drug which will compete for the available antibody binding sites. The catalytic current produced in the reaction was found to be directly proportional to the concentration of analyte in solution.

Heterogeneous enzyme immunoassays with electrochemical detection are based on the enzyme-linked immunosorbent assay. The strategy is based on labelling the antibody or antigen with an enzyme that catalyses the production of an electrochemically detectable product [8]. The rate at which the product is formed in related to the concentration of the analyte in the sample. Alkaline phosphatase which catalyses the hydrolysis of phenyl phosphate to phenol and phosphate has been used as the enzyme label. Phenyl phosphate is electroinactive and hence non-interfering. The enzyme generated phenol is detected by either flow injection analysis with electrochemical detection (FIA-EC) or liquid chromatography with electrochemical detection (LC-EC). In both cases injected samples flow through a thin long electrochemical cell where phenol is oxidised at a carbon paste working electrode at + 870 mV vs Ag/AgCl.

A recently development in heterogeneous electrochemical immunoassays has been the development of a ferrocene linked substrate for alkaline phosphatase, [N-ferrocenyl]-4-aminophenyl phosphate [9]. The cyclic voltammogram of this substrate is consistent with the quasi-reversible ferrocene/ferricinium ion one electron redox couple. However, following reaction with alkaline phosphatase, two new irreversible oxidation peaks at + 180 mV and at + 590 mV are observed. The peak at + 590 mV is due to the oxidation of the phenol group while that at + 180 mV is due to the oxidation of ferrocene to the ferricinium ion in this compound. The change in peak current at + 180 mV was found to be related to the enzyme concentration and the assay was successfully applied to the measurement of oestriol.

Cofactor recycling has been utilised as the basis of an amplification system immunoassays. The enzyme-amplified immunoassay differs from the conventional type in that the product from the enzyme label is not measured but instead acts catalytically on the secondary system which remains essentially silent until activated in this way. The product in the case of cofactor regeneration is NAD+ which is generated from NADP+ by alkaline phosphatase [10]. In the cycle NAD+ is reduced by ethanol using alcohol dehydrogenase and the NADH so formed reduced a tetrazolium salt to regenerate NAD+ in the presence of diaphorase to produce an intensely coloured formazan dye. The rate of reduction of the tetrazolium salt is directly proportional to the concentration of the NAD+ originally formed. The disappearance of NADH can be measured electrochemically using the enzyme NADH oxidase [11]. This enzyme isolated from the thermophile *Thermus aquaticus* catalyses the oxidation of NADH with concomitant two electron reduction of dioxygen to hydrogen peroxide. The hydrogen peroxide was detected at + 650 mV vs Ag/AgCl at a platinum electrode. The current produced by the oxidation of hydrogen peroxide was directly proportional to the NADH concentration.

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