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Phil. Trans. R. Soc. Lond. B 1987 **316**, 135-142
doi: 10.1098/rstb.1987.0023

Phil. Trans. R. Soc. Lond. B **316**, 135–142 (1987)

Printed in Great Britain

Electrochemical immunoassays

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Immunoassays are routinely used to detect, specifically, low levels of many antigens. The trend away from the use of radioisotopic labels has resulted in a proliferation of alternative labels, many of which have electrochemical activity. The more successful of these assays have used enzyme labels, coupled with amperometric or potentiometric methods of detection of the products. A number of assays have also been designed which are specifically electrochemical in origin, not simply adaptations of currently used spectrophotometric methods. Much effort has been expended in developing a potentiometric immunoassay that measures the change in potential that should occur when an antibody binds to its antigen. The use of electroactive labels has resulted in a number of assays for drugs. The advantages of an enzyme-linked mediated assay for lidocaine, an antiarrhythmic drug, are discussed.

Introduction

Although antibodies have been used as diagnostic agents for more than fifty years, it was only when anti-insulin antibodies were used to detect plasma insulin (Yalow & Berson 1959, 1960) that a revolutionary new method for the accurate and specific measurement of low levels of hormones, enzymes, drugs, viruses, tumour antigens, bacterial antigens and many other proteins and organic substances was devised. The principle underlying immunoassays is dependent on the interaction of the antigen or ligand (Ag) and its specific binding partner, the antibody (Ab):

$$\mathrm{Ab} + \mathrm{Ag} \rightleftharpoons \mathrm{AbAg},$$

with the equilibrium constant, K, falling in the range 10^5–10^{11} M^{-1}. In the simplest immunoassays the concentration of antibody is fixed. Thus, at equilibrium, the ratio of bound antigen to free antigen will depend upon the equilibrium constant for the antibody. The ratio of bound to free antigen can be determined by adding a fixed amount of a tracer antigen. Most of the early immunoassays used radioisotopic labels, e.g. ^{125}I or ^{3}H, to modify the antigen; however, the trend away from radioimmunoassays (RIA) has resulted in the use of enzyme immunoassays (EIA), fluorescent probes (FIA), chemiluminescent probes (CIA) and even organometallic probes. It is also possible to label antibodies, although, in general, the range of labels used is more limited than that of antigen labels. The two most commonly used labels are radioisotopes, for immunoradiometric assays (IRMA), and enzymes, for immunoenzymometric assays (IEMA). Many immunoassays require a physical separation of the bound and free labelled species, this procedure is both time-consuming and cumbersome, and hence homogeneous assays that require no sample pretreatment or manipulation to separate bound from free antigens have many advantages.

Over the years there has been a steady trend away from the use of radioisotopes as labels in immunoassays. This has resulted in the widespread use of enzymes as labels. Some of the

more commonly used enzymes are listed in table 1. The use of enzymes in immunoassays tends to involve sample pretreatment and the results are monitored either spectrophotometrically or colorimetrically. There have been several attempts to design simpler, more sensitive assays by coupling immunoassays with either potentiometric or amperometric methods of detection. As most immunoassays are used to monitor low concentrations of antigen over a narrow

Table 1. Enzyme lables used in immunoassays

horseradish peroxidase	glucoamylase	glucose-6-phosphate dehydrogenase
glucose oxidase	glucose oxidase	
alkaline phosphatase	catalase	malate dehydrogenase
β-D-galactosidase	urease	

concentration range, assays based on amperometric detection will probably be more sensitive than those based on potentiometric detection. In potentiometric assays there is a logarithmic relation between the potential and concentration, whereas in amperometric assays a linear relation exists between current and concentration.

Potentiometric immunoassays

Proteins, being polyelectrolytes, have a net electrical charge except at their isoelectric point. Hence, in general, if both antibody and antigen have a net electrical charge and antigen–antibody binding is by van der Waals hydrogen bonding and electrostatic forces (Leslie & Cohen 1973), the electrical charge of the resulting complex will be different from that of the antibody alone. This principle was demonstrated (Janata 1975) by covalently binding concanavalin A to a polyvinyl chloride membrane deposited on a platinum wire. A potential change was observed when a polysaccharide, yeast mannan, was added to the solution. Unfortunately, when the experiments were repeated with an antibody and an antigen, the changes in potential were too small to measure quantitatively. The feasibility of changes in potential on antibody binding to antigen was demonstrated later (Yamamoto *et al.* 1978) by using antibodies to human chorionic gonadotropin (hCG), coated on titanium wire, and hCG. However, despite Yamamoto's results, little further evidence has appeared to support this type of simple immunoassay.

The second principal technique that has been used in potentiometric immunoassays is that of performing a conventional enzyme-linked immunoassay and detecting the products formed, not spectrophotometrically, but with a conventional potentiometric electrode (e.g. an ammonia- or carbon dioxide-sensitive electrode). Deaminating enzymes, such as adenosine deaminase, urease and asparaginase (Gebauer & Rechnitz 1982), were evaluated. Each enzyme catalyses the hydrolysis of ammonia from its respective substrate, and the ammonia is then detected by an ammonia gas-sensing electrode. Asparaginase proved to be the superior label in terms of sensitivity and detection limit, as it lost little activity when coupled to the antigen. Bound labelled antigen was separated from free labelled antigen by using a second antibody coupled to agarose beads.

A similar assay was devised for use with a carbon-dioxide-sensitive electrode. The model antigen used was digoxin, a drug with low molecular mass. Polystyrene beads were coated with

digoxin–BSA, and its antibody was labelled with horseradish peroxidase. A competitive assay was then performed between the labelled beads and digoxin in solution for the labelled antibody. The beads were washed and the carbon dioxide evolved from the peroxide–pyrogallol reaction, catalysed by horseradish peroxidase, was monitored (figure 1).

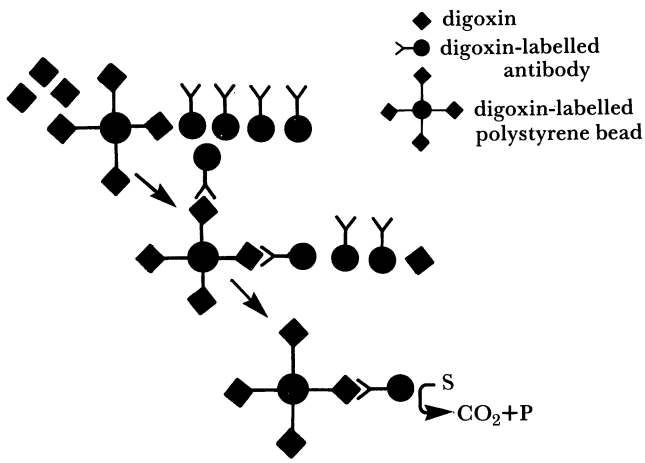

FIGURE 1. Schematic representation of a potentiometric enzyme immunoassay for digoxin. (Adapted from Keating & Rechnitz (1985).)

The use of a conventional ion-selective electrode in an immunoassay does not necessarily require the use of an enzyme label. A potentiometric ionophore-modulation immunoassay was described by Keating & Rechnitz (1984, 1985), which is applicable to antigens of low molecular mass. Once again the model antigen was digoxin. It was covalently coupled to a potassium ionophore which was included in a polyvinyl chloride membrane mounted onto a conventional ion-selective electrode. The electrode was exposed to a constant concentration of potassium and a stable background signal was observed. When a suitable antibody was added, it bound reversibly to the antigen–ionophore conjugate molecules present at the membrane–solution interface, resulting in a potential change that was proportional to the antibody concentration. A competitive assay could be performed, if free antigen was present in solution.

At present the unlabelled potentiometric immunoassays are either too slow or not sufficiently quantitative. The enzyme-labelled assays, however, show some promise, especially the ones that use such readily available and robust enzymes as horseradish peroxidase.

AMPEROMETRIC IMMUNOASSAYS

A number of different routes have been pursued in devising amperometric immunoassays. These can be generally divided into three classes: (i), the amperometric immunoassay that utilizes the Clark electrode, (ii), the use of an enzyme label, e.g. glucose-6-phosphate dehydrogenase or alkaline phosphatase, whose products (NADH or phenol) can be detected electrochemically; and (iii), assays that use an electrochemically active label.

The use of a Clark oxygen electrode in an immunoassay is illustrated by an assay for human chorionic gonadotropin (hCG), where an antibody to hCG was immobilized onto a membrane (Aizawa *et al* 1979). The membrane was then placed over the electrode and competitively

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reacted with hCG and hCG labelled with catalase. The probe was then washed and exposed to hydrogen peroxide (figure 2). If any catalase–hCG was bound to the membrane, it would cause the hydrogen peroxide to disproportionate and result in an increase in the cathodic current due to increase in the oxygen tension. Unfortunately the antibodies used appeared to show a high cross-reactivity to luteinizing hormone, and hence the assay was not sufficiently accurate.

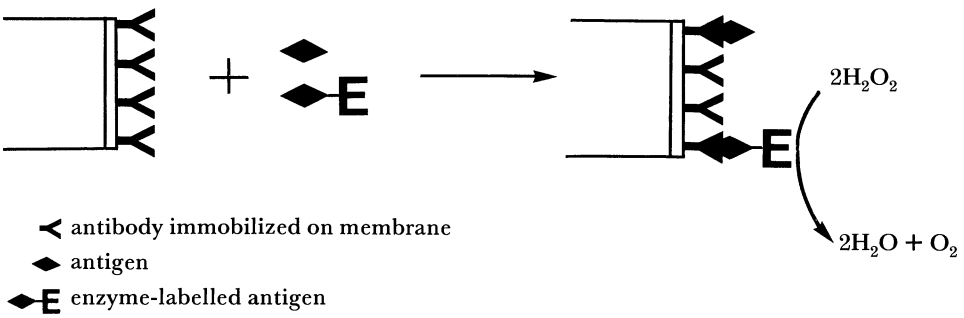

FIGURE 2. Schematic representation of an amperometric enzyme immunoassay for hCG. (Adapted from Aizawa *et al* (1979).)

Commercially available enzyme immunoassay kits for drugs of low molecular mass have also been adapted for use with electrochemical detection, e.g. in a flow amperometric immunoassay for phenytoin (Eggers *et al* 1982). In this assay the antigen in question, phenytoin, is used to label glucose-6-phosphate dehydrogenase. In solution, and in the absence of the antibody, this enzyme is able to catalyse its normal reaction:

$$\text{glucose-6-phosphate} + \text{NAD}^+ \xrightarrow{\text{G6PDH}} \text{glucono-}\delta\text{-lactone 6-phosphate} + \text{NADH}.$$

On binding to its antibody the enzyme's activity is inhibited. A homogeneous immunoassay can be performed, in solution, for the antigen; the more antigen present, the greater the observed enzyme activity. The NADH produced can be oxidized electrochemically on a glassy carbon electrode at +750 mV (against Ag/AgCl). Unfortunately, protein deposition on the electrode leads to irreproducible results. To minimize these effects and thus obtain accurate results, all protein is removed from the sample after the incubation with the antibody has occurred. This is achieved by passing the sample through a reverse-phase column before detection. As this involves extra manipulation over the spectrophotometric assay, there are few advantages in this form of amperometric immunoassay.

Alkaline phosphatase has been one of the more commonly used labels in immunoassays, often with its colourless substrate, *p*-nitrophenyl phosphate. The yellow product of the hydrolysis, *p*-nitrophenol, is detected either colorimetrically or spectrophotometrically. The use of this enzyme in amperometric immunoassays with phenyl phosphate as the substrate is becoming more popular (Doyle *et al.* 1984; Wehmeyer *et al.* 1985, 1986). Phenyl phosphate is electrochemically inactive, but its hydrolysis product, phenol, can be oxidized electrochemically:

$$\text{phenyl phosphate} + \text{H}_2\text{O} \xrightarrow{\text{AP}} \text{H}_3\text{PO}_4 + \text{phenol}.$$

An assay (Doyle *et al.* 1984), described as an enzyme-linked immunoadsorbent voltammetric assay, for α_1-acid glycoprotein makes use of alkaline phosphatase as a label. The glycoprotein that was labelled with the enzyme had a high molecular mass and is thought to be related to carcinoembryonic antigen, which is implicated in various malignant conditions. A cuvette was coated with antibody and a competitive reaction was allowed to occur between glycoprotein and alkaline phosphatase-labelled glycoprotein. After a suitable time, the cuvette was washed and phenyl phosphate added. The alkaline phosphatase-generated phenol was oxidized at a carbon-paste electrode, as the detector, in a liquid chromatographic assay. Unfortunately the assay is rather slow, taking over 13 h to perform. It has more recently been extended to low-molecular-mass antigens, such as digoxin (Wehmeyer *et al.* 1986).

Several groups have attempted to devise homogeneous amperometric immunoassays that are not modifications of existing enzyme immunoassays. On binding of a labelled species to an antibody the electrochemistry of the label is so perturbed that separation of free from bound label is unnecessary. One of the earlier examples (Heinemann *et al.* 1979) was of a homogeneous competitive immunoassay for oestriol, with mercuric acetate as a label. Labelling was required as oestriol is electrochemically inactive between -200 and -1000 mV (against Ag/AgCl). Free labelled oestriol could be electrochemically reduced at -300 mV (against a saturated calomel electrode (SCE)); this procedure required sample degassing to alleviate oxygen interference. Oestriol has been nitrated (Wehmeyer *et al.* 1982), in both the 2 and 4 positions of ring A, giving an electroactive product that shows two reduction waves at -421 mV and -481 mV (against Ag/AgCl). Addition of oestriol-specific antibody resulted in a decrease in the peak current; this result suggests that there is binding of the antigen to the antibody. On addition of unlabelled oestriol to this system, an increase in the reduction current was seen once more; this suggested displacement of the labelled oestriol from the antibody by the unlabelled species. This assay unfortunately suffered from the same oxygen interference as the previously described assay.

From these two examples it is clearly evident that if antigens of low molecular mass are to be labelled with an electroactive species, then the species should be one that can be oxidized at low potentials or reduced at potentials where oxygen interference is negligible. A number of such labels can be envisaged and indeed an assay has been described based on ferrocene-labelled morphine (Weber & Purdy 1979). On binding of ferrocene-labelled morphine to its antibody, the electrochemical behaviour of the label is perturbed: the oxidation current due to the ferrocene is lessened. The ferrocene-labelled morphine conjugate can be displaced by the addition of codeine, a morphine analogue. The oxidations were done at $+500$ mV (relative to SCE), a potential where oxygen interference is minimal.

More recently, it has been shown (Cass *et al.* 1984) that ferrocene (bis(η^5-cyclopentadienyl)iron) and its derivatives act as electron acceptors for glucose oxidase and a number of other flavoproteins (Cass *et al.* 1985) in a non-oxygen-dependent manner as outlined below for glucose oxidase:

$$\text{glucose} + \text{GOD}_{\text{ox}} \rightarrow \text{gluconolactone} + \text{GOD}_{\text{red}};$$

$$\text{GOD}_{\text{red}} + 2\text{Fecp}_2\text{R}^+ \rightarrow \text{GOD}_{\text{ox}} + 2\text{Fecp}_2\text{R} + 2\text{H}^+;$$

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

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Thus a ferrocene-labelled antigen of low molecular mass should also act as an electron acceptor for glucose oxidase. The use of enzyme amplification should form the basis of a sensitive immunoassay. It has already been shown (Weber & Purdy 1979) that the electrochemical behaviour of a ferrocene-labelled antigen is modified on binding to its antibody. This will result in impairment of its ability to mediate (di Gleria *et al.* 1986).

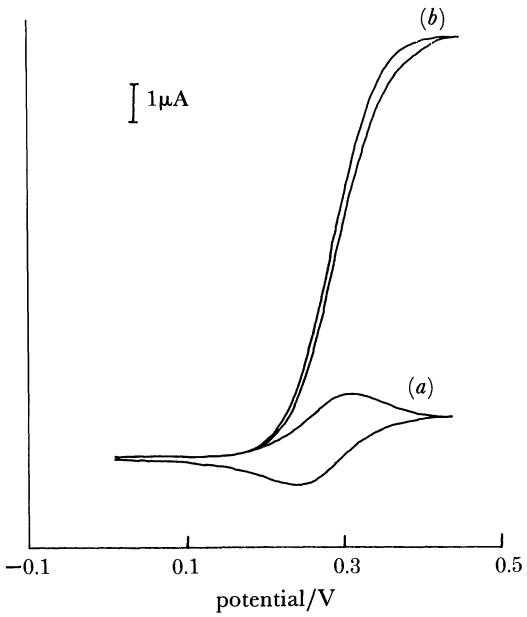

FIGURE 3. (*a*) DC cyclic voltammogram of buffer, glucose and ferrocene–antigen conjugate. (*b*) As (*a*) with the addition of glucose oxidase.

The interaction between the antibody and the ferrocene-labelled antigen inhibits the ability of the latter to act as a mediator for the enzyme-catalysed reaction and results in a large decrease in the catalytic current. The binding can be reversed by addition of non-labelled antigen. If a fixed concentration of antibody and labelled antigen is used, then any catalytic current produced in the reaction will be directly proportional to the concentration of antigen in solution. The assay can be configured as either a homogeneous competitive assay or a homogeneous displacement assay depending on the antibody used. The assay has been demonstrated for both lidocaine ((α-diethylamino)-2,6-dimethyl acetanilide) and theophylline(3,7-dihydro-1,3-dimethyl-H-purine-2,6-dione).

The ferrocene–antigen conjugate shows a DC cyclic voltammogram consistent with a one-electron redox couple. Addition of glucose oxidase, in the presence of an excess of glucose, results in a large increase in the anodic current and loss of the return wave, indicative of an electrochemically coupled, enzyme-catalysed oxidation of glucose (figure 3). Under conditions of substrate excess the steady-state catalytic current will be proportional to the ferrocene-labelled antigen concentration forming the basis of the immunoassay. If fixed amounts of ferrocene-labelled antigen, substrate and enzyme are present in solution, then adding increasing portions of antibody will result in a progressive decrease in catalytic current (figure 4). This

decrease is reversible on addition of free antigen. Thus, with the optimal fixed concentration of antibody, ferrocene-labelled antigen and substrate, a sensitive assay for low-molecular-mass antigens present in samples in the micromolar concentration range can be demonstrated (figure 5).

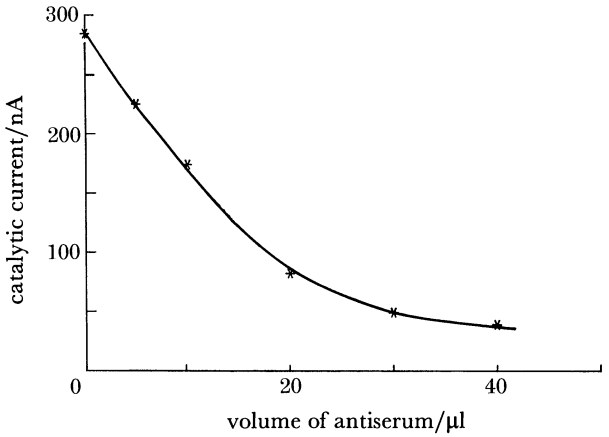

FIGURE 4. An antisera-inhibition titration of ferrocene–conjugate mediator activity.

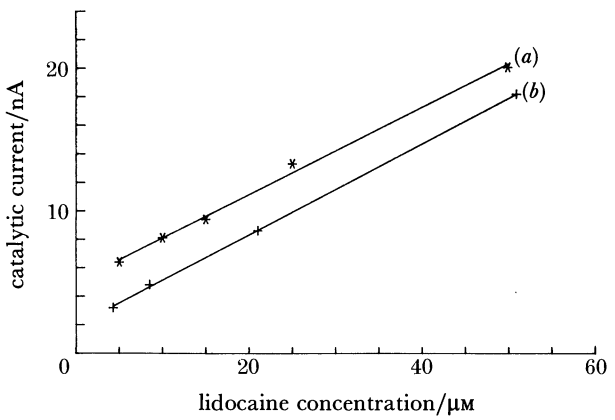

FIGURE 5. Standard curves for lidocaine concentration determined electrochemically. (*a*) Fresh serum; standards prepared in plasma. (*b*) Control serum; SYVA calibration standards.

CONCLUSIONS

Several radically different electrochemical assays have been devised. Some have been adaptions of existing spectrophotometric assays and have thus been very sensitive. Unfortunately the extra sample pretreatment before measurement in these assays (Eggers *et al.* 1982) somewhat negates the advantages offered in sensitivity. The initial promise suggested by direct potentiometric assays (Janata 1975; Yamamoto *et al.* 1978) has unfortunately not been fulfilled. The elegant use of antigens, coupled to ionophores, is unlikely to be used routinely, again because of the extensive sample pretreatment required before measurement. Any technique that

is to be adopted by laboratories or clinicians must have advantages over the existing techniques that are routinely used at present.

It is difficult to imagine the currently described electrochemical immunoassays replacing many of the standard enzyme, fluorescence-polarization immunoassays routinely used in hospitals. However, the area is still relatively young; bearing in mind some of the promising results obtained by using electrochemical immunoassays, it may not be long before such assays are more commonly used.

I am grateful to Calum McNeil for permission to describe his work, and to Dr H. A. O. Hill and Dr A. R. Hopkins for useful discussion.

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