Affinity Biosensors

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1 Introduction

This review is not intended to be a comprehensive treatment of affinity biosensors. Its aim is to introduce the principle areas of affinity biosensor research and illustrate current technology with selected examples. More detailed information on biosensor and assay research and technology can be obtained by consulting the literature cited and the references therein.

A **biosensor** is normally defined as a device incorporating a biological molecular recognition component connected to a transducer which can output an electronic signal that is proportional to the concentration of the molecule being sensed. Research into biosensor technology has grown rapidly in the past decade because of its potential application in areas such as diagnosis of disease, monitoring of clinical or environmental samples, fermentation and bioprocess monitoring, testing of pharmaceutical or food products, and early warning chemical and biological warfare detection systems. A range of combinations of receptor/transducers have been proposed and studied. Biosensors have been described that utilize enzymes, antibodies, receptors, cells and tissue material for recognition of the analyte of interest. Transduction of the biochemical recognition signal to an electronic signal has been accomplished using optical, electrochemical, calorimetric, and piezoelectric detection schemes. The term **affinity biosensor** in this article refers to a device incorporating immobilized biological receptor molecules that can *reversibly* detect receptor–ligand interactions with a high differential selectivity and in a *non-destructive* fashion. Thus, enzyme-based biocatalytic sensors are not covered by this description. Excellent publications that describe biosensors are available for the interested reader.[1] I have included in this review advances in *assay* development and a discussion of novel *competitive binding* affinity systems for detection of ligands although these are not generally true biosensors. These topics are covered to demonstrate the advances in non-isotopic detection methods and their potential application to biosensor technology. I have purposely ignored the large body of literature on the use of radioactive labels for the detection of ligand–receptor binding in order to limit the length and scope of the article.

Dónal Leech was born in Dublin, Ireland. He obtained his B.Sc. in 1988 and his Ph.D. in electroanalytical chemistry, under the direction of M. R. Smyth and J. G. Vos, in 1991 from Dublin City University. During his Ph.D. studies a six month visit to J. Wang at New Mexico State University was undertaken. Post-doctoral research at the University of Hawaii, in the group of G. A. Rechnitz followed. In 1993 Dr. Leech accepted a position as Assistant Professor of Analytical Chemistry at the Université de Montréal. His research interests currently focus on electroanalytical chemistry and its application to chemical sensors, biosensors, and assays.

2 Receptor–Ligand Binding Theory

There are many possible configurations for the design of biosensors. I will focus here on the two schemes most likely to lead to operational biosensors for reporting receptor/ligand binding events. These schemes, depicted in Figure 1, are the direct affinity sensor (a) and the competitive-binding affinity sensor (b). The competitive binding approach is considered here because of its applicability to reagentless biosensing.[2–5]

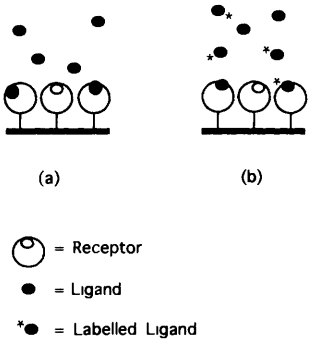

Figure 1 The direct affinity sensor (a) and the competitive-binding affinity sensor (b).

The chemical reaction for an affinity sensor is

$$R + L \underset{k_2}{\overset{k_1}{\rightleftharpoons}} RL \qquad K_a = \frac{[RL]}{[R][L]} = \frac{k_1}{k_2} \tag{1}$$

where R is the receptor, L the ligand, RL the receptor–ligand complex, k_1 the association rate constant, k_2 the dissociation rate constant, and K_a the equilibrium constant (equilibrium affinity or association constant). The fraction of receptor molecules in the complexed form can be expressed as

$$\frac{[RL]}{[R_t]} = \frac{[L]K_a}{1 + [L]K_a} \tag{2}$$

where the total receptor concentration $[R_t] = [R] + [RL]$. A dimensionless binding curve such as that depicted in Figure 2 results, with obviously $1/K_a = [L]$ when half of the receptor binding sites are occupied. This value can be used to approximate the applicable dynamic concentration range for the sensor.[2] A transformation of the binding curve is normally per-

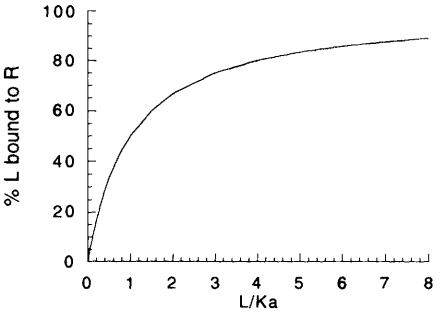

Figure 2 Dimensionless binding curve for an affinity biosensor, with concentration expressed as $[L]/K_a$, and $100 \times [RL]/[R_t]$ the % L bound to R.

formed to determine the K_a (or K_d, dissociation constant = $1/K_a$) more accurately. Equation 3 represents the widely used Scatchard transformation. The K_a (K_d) can be determined from the slope of a linear plot of [RL]/[L] *versus* [RL] (or [bound]/[free] *versus* [bound] for the ligand).

$$\frac{[RL]}{[L]} = R_t K_a - [RL]K_a \quad (3)$$

Direct affinity biosensing is limited to the instrumental methods available for the direct detection of ligand binding. A more universal approach involves competitive binding schemes such as that in Figure 1b, where an analyte (ligand) or an analogue is labelled to allow the indirect detection of receptor–ligand binding. The chemical reactions in this case are

$$R + L \underset{k_2}{\overset{k_1}{\rightleftharpoons}} RL \qquad K_a = \frac{[RL]}{[R][L]} \quad (4)$$

$$R + L^* \underset{k_4}{\overset{k_3}{\rightleftharpoons}} RL^* \qquad K_a^* = \frac{[RL^*]}{[R][L^*]} \quad (5)$$

where L* represents the labelled ligand, k_3 and k_4 the respective association and dissociation rate constants, and K_a^* the equilibrium affinity constant for the reaction. The signal generated can thus arise from L* or RL*.

For reagentless sensing of competitive binding processes several researchers[2–5] have labelled an analyte-analogue with a macromolecule and entrapped the large receptors and labelled analyte-analogue behind a semi-impermeable membrane on the tip of a sensor (a fibre optic cable in these cases) while allowing the analyte to diffuse through the membrane, yielding a probe-type sensing system. Schultz[2] has modelled the equilibrium response of such a sensor and concluded that the concentration ratio of the analyte-analogue to total receptor ([L*]/[Rt]) controls the dynamic range available, with ratios of 0.01 to 0.1 desirable for analytical applications depending on the dynamic range required. This is in contrast to immunoassay requirements where ratios approaching 1 are desired. However the capability of the measuring instrument to detect the labelled analyte will be the limiting factor governing selection of the concentration ratio for high affinity receptors.[4] It is important to state that the ultimate detection limit in any ligand–receptor interaction is limited by the intrinsic affinity of the receptor for the ligand, provided sufficient labelled species exist to be detected by the instrument. The dynamic response of this sensor can also be modified by modifying the binding constants.[2] This can be achieved by selection of L* that has a different binding affinity or that has a higher valency for the receptor than L.

Miller and Anderson[5] have modelled the chemical kinetics of a competitive binding immunosensor. Their simulations show that the response time of such a sensor is controlled by the dissociation rate constant and is independent of the association rate constant. Indeed Schultz[2] has estimated that the association rate constant is the same for all antibodies and is on the order of $10^8 M^{-1}s^{-1}$, consistent with the view that the rate is diffusion controlled. The model predicts that equilibrium response times in the order of minutes can be achieved for receptors with dissociation rate constants greater than approximately $4 \times 10^{-3}s^{-1}$.[5] The use of receptors with higher dissociation rate constants of course is limited by the reduction in the affinity of the sensor, leading to a trade-off between response time and sensitivity in the development of practical reversible biosensors.

3 Antibody-based Biosensors

Immunoassays are well established in clinical chemistry and are even gaining acceptance for on-site environmental assay of contaminants. Below I will discuss the detection of antigen and antibody levels, both for assay and sensor development. Unfortunately the focus of most clinical diagnostic research is on the development of highly sensitive solid phase enzyme-immunoassays, using monoclonal antibodies with high affinities, thus limiting the application to reversible sensing. Solid phase competitive-binding immunoassays have potential applications as immunosensors, provided the reversal of the antibody–antigen interaction can be achieved while maintaining the antibody binding capacity, and the assay components (except targeted antigen) are available continuously. Indirect detection of antibody–antigen binding is by far the most predominant scheme utilized in immuno*assays*. These schemes involve labelling of either the antibody or antigen with a species that is detectable or that may generate a detectable species. Examples of labels include enzymes, fluorescent, and electrochemical species.

Enzyme-labelling of antibodies or antigens for amplification of the signal and detection of antigen levels is the most widely used non-isotopic assay procedure. The schemes involved in the most common enzyme immunoassays (EIAs), the heterogeneous (sandwich and competitive) enzyme-linked immunosorbent assay (ELISA) and the homogeneous (competitive) enzyme-mediated immunoassay, are depicted in Figures 3 and 4 respectively. Detection of antigen levels in ELISA is accomplished by addressing the activity of the enzyme upon addition of substrate by monitoring the increase in products or decrease in substrate. Sandwich ELISA (Figure 3a) is the most sensitive assay scheme and offers comparable detection limits to radioimmunoassay procedures.

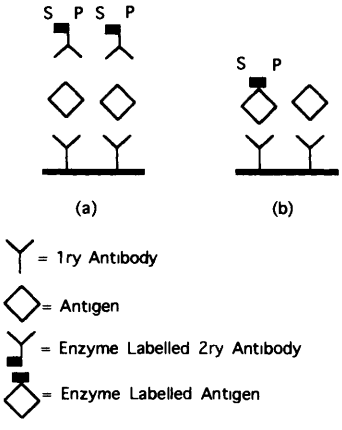

Figure 3 Sandwich (two-site) (a) and competitive-binding (b) enzyme-linked immunosorbent assays where S and P represent the enzyme substrate and product, respectively.

Sandwich ELISA techniques are limited to antigens that possess dual epitopes to enable simultaneous binding of two antibodies. Detection of the smaller antigens (haptens) can be achieved using competitive binding ELISA (Figure 3b) or homogeneous EIA schemes (Figure 4). Several enzyme-based assay schemes for the detection of haptens have been developed.[6] These schemes use antibody–antigen binding to modulate enzyme activity (Figure 4a), enzyme mediator (or modulator) activity (Figure 4b), or enzyme–substrate binding (Figure 4c). Once again antigen levels are assayed by monitoring either the enzyme substrate or product. Similar schemes to those outlined in Figures 3 and 4 may be performed using fluorescent or electroactive labels. Direct optical and mass detection of antigen binding to immobilized antibody has also been demonstrated. The following sections will review the procedures developed for immunoassays and sensors classified according to the transducing element used for detection. Most of the assay schemes and detection principles outlined above and below have also been developed for receptor-binding and DNA hybridization assays and will not be reiterated under those headings.

3.1 Optical Detection

Large-scale screening and assay of antigen or antibody levels is routinely achieved using microtitre plate absorbance or lumines-

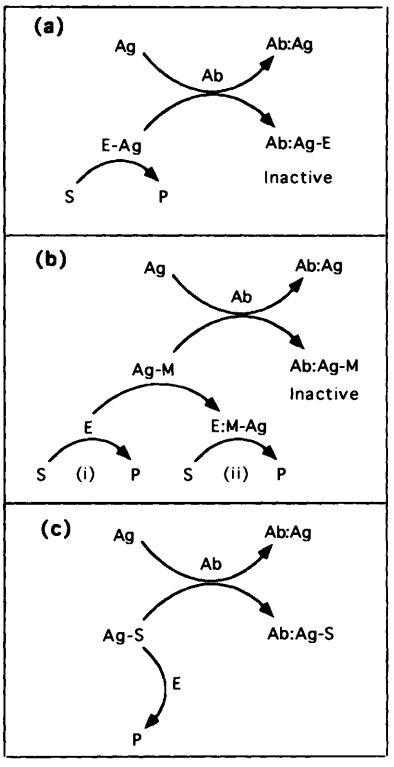

Figure 4 Homogeneous enzyme immunoassay schemes involving modulation of enzyme activity (a), mediator or modulator activity (b), or enzyme–substrate binding (c) upon binding of the labelled antigen to the antibody. The symbols Ab, Ag, E, S, and P have their usual meanings and Ag–M represents the antigen–mediator (or enzyme modulator) complex.

cence instruments in conjunction with ELISA schemes. Enzymes which have chromogenic substrates, such as horseradish peroxidase (HRP), alkaline phosphatase (AP), and β-D-galactosidase (β-G), are normally used in ELISA procedures. More sensitive assays can be achieved using amplification or cascade schemes, where the enzyme product is a substrate for a separate enzyme reaction. Increased sensitivities may also be obtained by using fluorescent labelled antibodies or antigens or by labelling with enzymes that generate luminescent products. Antibodies and antigens labelled with fluorescein and rhodamine derivatives have been successfully used in both competitive and non-competitive fluoroimmunoassays. Substrates which produce fluorescent products are currently commercially available for AP, HRP, and β-G enzyme immunoassays. Conventional fluoresence-based immunoassays have limited sensitivities, however, because of high background originating from scattering from the instrument or the sample matrix, exacerbated by the small Stokes' shift of the fluorophores. The presence of background fluorescence signals in biological matrices also limits the assay sensitivity. The use of time-resolved fluorescence systems have been introduced to overcome this problem. Most systems employ the lanthanide chelates which have a long-lived fluorescence, large Stokes' shift and narrow emission band. The principle of time-resolved fluorescence is straightforward. Short-lived fluorescence generated by irradiation of a sample with a burst of laser light will decay to zero in approximately 100μs. If an appropriate time lapse after application of the pulse is allowed the long-lived lanthanide chelate fluorescence can be selectively measured. Immunoassays using lanthanide time-resolved fluorescence have been reported.[7]

Chemiluminescent and bioluminescent immunoassays have become popular because of the analytical sensitivity obtainable. Assays that use chemiluminogenic substrates of the enzyme HRP have been improved by the development of various compounds which enhance and prolong the luminescent signal. Similar improvements in AP-based chemiluminescent assays have been achieved by the synthesis of novel substrates which can generate longer-lived chemiluminescence following dephosphorylation.[8] The use of bioluminescent systems has the potential advantage of offering higher quantum yields (up to 90%) compared to chemiluminescent systems (usually below 5%). However, routine use of assays utilizing the firefly luciferase bioluminescence system are at present hindered by the complexity of the enzyme system and its low turnover number.

Although these assays are simple and versatile they are time consuming and are not configured as probe-type reversible biosensors. Several research groups have attempted to develop optical immuno*sensing* strategies using fibre optic cables as waveguides. A bioaffinity fibre optic immunosensor has been developed for the direct detection of the fluorescent antigen benzo[*a*]pyrene tetraol (BPT) using a microscale regenerable system[9] to aspirate reagents and washing solution over the distal tip of the cable. Barnard and Walt[10] have devised an alternative strategy for the delivery of reagents to a fibre optic fluoroimmunosensor. A competitive-binding fluoroimmunosensor based on fluorescence energy transfer and the controlled release of a fluorescein-labelled antibody and Texas Red-labelled antigen from ethylene-vinyl acetate polymer reservoirs at the tip of the fibre optic cable was described. Binding of the unlabelled antigen IgG was detected by monitoring the ratio of fluorescent intensity following fluorescence energy transfer from the fluorescein molecules to Texas Red upon formation of the dual labelled immune complex. A *reversible* immunosensor based on the fluoresence energy transfer mechanism and antibodies with high dissociation rate constants was also reported.[5] This sensor used a competitive binding scheme of Texas Red labelled antiphenytoin antibody and b-phycoerythrin-labelled phenytoin for the detection of phenytoin. A similar reversible sensing scheme was first described by Schultz for an optical affinity sensor for glucose based on fluorescein labelled dextran (an analyte-analogue) and the receptor Concanavalin-A (Con-A) confined at a fibre optic tip behind a semi-permeable dialysis membrane[2,3] as shown in Figure 5. Operation of the sensor involved allowing the small unlabelled glucose to diffuse into the reaction chamber to displace the fluorescein isothiocyanate-labelled dextran (FITC-Dextran) from the Con-A. The dissociation kinetics of these competitive-binding affinity biosensors is the determining factor in reversibility and regeneration of the receptor, as discussed above.

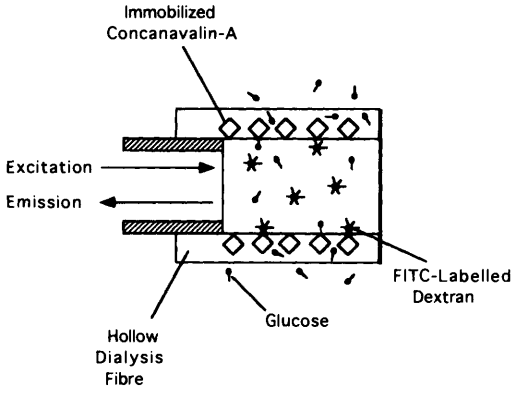

Figure 5 Schematic diagram of a fibre optic equilibrium displacement affinity biosensor.
(Adapted with permission from reference 2.)

Immunosensors based on the immobilization of the antibody along the core of the fibre optic cable and the detection of fluorescent labels in the evanescent field of the fibre have been described.[11] This electromagnetic wave can be used to confer surface selectivity to bioaffinity sensors as the typical penetration depth of the field is 40—100nm. Changes in the fluorescence excited by the evanescent wave in this field upon binding of

the antigen can thus be used for biosensing purposes Improvements in the sensitivity and detection limit of evanescent wave immunosensors are still required for them to compete with solid-phase enzyme immunoassays Progress in this area could be achieved by the use of chemiluminescent or long-lived fluorescent labels to increase the signal-to-noise ratio and eliminate background problems Other surface-sensitive optical methods have the capability to be used in bioaffinity sensor design as demonstrated by the Surface Enhanced Raman Scattering (SERS) sandwich assay for Thyroid Stimulation Hormone (TSH) [12]

While these indirect sensors possess some of the desirable characteristics for reagentless, reversible affinity biosensing, the simpler direct approach to the detection of ligand binding offered by some Internal Reflectance Spectroscopy (IRS) techniques seems more attractive One such technique receiving much attention recently is Surface Plasmon Resonance (SPR) Surface plasmons, which represent quanta of oscillations, may be produced at metal–glass interfaces by the resonant excitation of electrons in the metal by electron beams or light Plasmons for sensing applications are produced by the evanescent waves in the Attenuated Total Reflectance (ATR) configuration as depicted in Figure 6 When the light energy is transferred to the surface electrons a corresponding decrease in the intensity of the reflected light occurs giving a minimum in the reflectivity *versus* light angle plot The system is sensitive to changes in the refractive index (n) of the media at the surface and thus changes in n due to binding reactions can be detected by a change in the resonance angle of the incident light SPR has been used to detect the Human Chorionic Gonadotrophin (HCG) hormone to a detection limit of 1×10^{-8}M using a multilayer biotin–avidin–antibody system [13] While this technology offers exciting prospects for the direct detection of receptor–ligand binding interactions, SPR still suffers from problems associated with non-specific binding of proteins, lack of comparable sensitivity to ELISA technology and application to reversible-binding sensing strategies

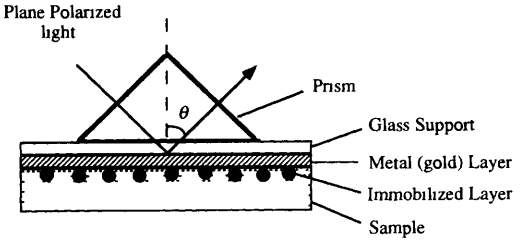

Figure 6 Schematic diagram of the experimental arrangement for surface plasmon resonance detection of ligand binding The angle of minimum reflection of the totally internally reflected laser light, θ, shifts with changes in the refractive index of the sample medium close to the gold layer allowing monitoring of ligand-binding processes
(Adapted with permission from reference 12)

3.2 Electrochemical Detection

Electrochemical immunoassays and sensors have some advantages over optical-based systems in that they can operate in turbid media, offer comparable instrumental sensitivity, and are more amenable to miniaturization

Potentiometric sensors measure the potential difference between a working and a reference electrode Potentiometric immunoassays for the direct detection of antigen binding have been proposed where the protein antigen or antibody is viewed as a charged polyelectrolyte which, upon binding to the surface of an indicator electrode, would change the potential difference Unfortunately these assays suffer from a lack of sensitivity and from interference problems from other ions in the matrix Attempts to improve the sensitivity of potentiometric immunoassays using enzyme-labelled antibodies that generate detectable ions or gases have been relatively unsuccessful as the problems of interferences and non-specific binding events still exist The advent of the Ion Selective Field Effect Transistors (ISFETs) and Chemically Selective FETs (CHEMFETs) has resulted in the capability of microfabrication of ion and pH sensors and showed great early promise in application to immunosensing The FET devices are insulated semiconductor devices that respond by detecting the effect surface potentials at the electrode–solution interface have on the electric fields in the semiconductor Several researchers have attempted to utilize affinity species attached to the channel between the source and drain electrodes to measure changes upon binding to the antigen Some controversy exists, however, about the origin of the potential shifts[14] and interference and non-specific binding would also seem to limit these devices

The recently developed Light Addressable Potentiometric Sensor (LAPS) based on FET technology has, in contrast, proved to be highly successful for immunoassay and receptor binding studies [15] The basis of the technique is shown in Figure 7 and involves the application of a bias potential to a silicon plate containing a pH-sensitive surface insulator of silicon oxynitride

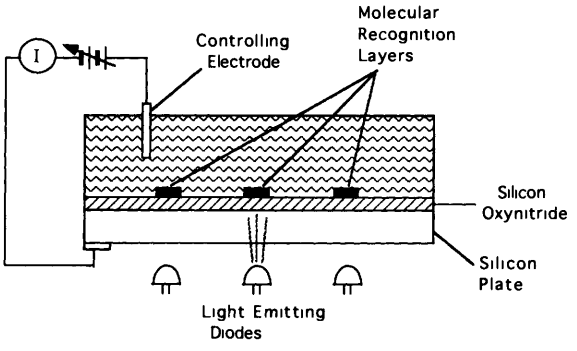

Figure 7 The light-addressable potentiometric sensor The silicon plate with pH-sensitive surface layer is photoresponsive to the light-emitting diodes The photocurrent in the external circuit is dependent on the applied potential and responds to changes in the surface potential caused by localized chemistries (pH changes) induced by molecular recognition (see text for further details)
(Adapted with permission from reference 14)

An intensity-modulated light source from Light-Emitting Diodes (LEDs) gives rise to an alternating photocurrent which has an amplitude proportional to the solution/surface pH at a given bias potential The immunoassay instrument developed measures pH changes in a sandwich-type assay using urease-labelled antibodies to produce local pH changes upon addition of urea substrate A novel potentiometric affinity biosensor employing catalytic antibodies as the molecular recognition element has recently been described [16] The model catalytic antibody (20G9) raised against phenyl phosphonate was designed to catalyse the hydrolysis of phenyl acetate to acidic products that change the pH at the underlying potentiometric pH electrode Antibodies raised against transition-state analogues are capable of catalysing chemical reactions by lowering the potential energy for the transition state of the reaction upon binding Although a detection limit of only 5 mM and a slow response time was obtained for phenyl acetate, the design of catalytic antibodies for specific affinity biosensor applications has immense potential, because of the rapid dissociation of the antigen and the regeneration of the molecular recognition site

Amperometric sensors measure the current produced upon oxidation or reduction of an electroactive species at a polarized working electrode Research on amperometric immunoassays have in recent years focused on the use of heterogeneous ELISA schemes using AP-labelled antibodies because of the availability of the non-electroactive substrate 4-aminophenyl phosphate [17] The product of the enzyme reaction, 4-aminophenol, can be easily oxidized at a low potential thus minimizing interference from other electroactive species Other researchers have devised electrochemical schemes for substrate delivery in electrochemi-

cal immunoassays. This entails either the use of an enzyme substrate that can be produced electrochemically, such as H_2O_2 for HRP enzyme immunoassays,[18] or an electrochemical mediator of enzyme oxidation–reduction that can be regenerated electrochemically, such as the benzoquinone/hydroquinone redox couple used for glucose oxidase (GOD) labelled immunoassays.[19] An automatic flow injection apparatus for heterogeneous immunoassays, based on the GOD–benzoquinone electrocatalytic scheme, allowed the electrochemical regeneration of the electrode, for subsequent primary antibody adsorption, by oxidizing it in the presence of nitric acid.[19]

The use of enzyme mediation schemes for the development of *homogeneous* amperometric immunoassays has also been attempted. The initial study[20a] was based on the ability of a ferrocene–lidocaine conjugate to mediate the oxidation of glucose by GOD in the scheme depicted in Figure 8a. Competitive binding of unlabelled lidocaine yielded an increase in the catalytic current. Coupling of homogeneous amperometric immunoassay schemes to an enzyme cascade could lead to improved sensitivity, such as demonstrated for the detection of biotin (Figure 8b).[20b] An extremely sensitive competitive immunoassay procedure using an electrochemiluminescent label is commercially available.[21] The assay utilizes a $Ru(bpy)_3^{2+}$ label, where bpy is the ligand 2,2′-bipyridine, which when oxidized in conjuction with tripropylamine (TPA) forms the excited-state $Ru(bpy)_3^{2+*}$ that emits a photon of 620 nm wavelength upon decay to the ground state. Extremely low detection limits of 200 fM for the label was reported.

Although advances in the design of sensitive electrochemical assays have been significant there has as yet been no report of a true amperometric immuno*sensor*. The approach taken for the design of optical immunosensors described above[2–5] would seem feasible also for the development of amperometric immunosensors but has as yet not been demonstrated. Amperometric immunoassays employing flow-injection schemes seem more practical than microtitre-plate technology for the rapid assay of antigen levels.[17] Improvements in design of these assays could include methods for continuous delivery of substrates[18] and regeneration of antibody binding sites. An attractive scheme for the reagentless detection of enzyme activity has been described by Smit and Rechnitz,[22] where the enzyme tyrosinase was demonstrated to oxidize an artificial substrate, $Fe(CN)_6^{4-}$, which could be re-reduced at an electrode, while consuming (reducing) only oxygen.

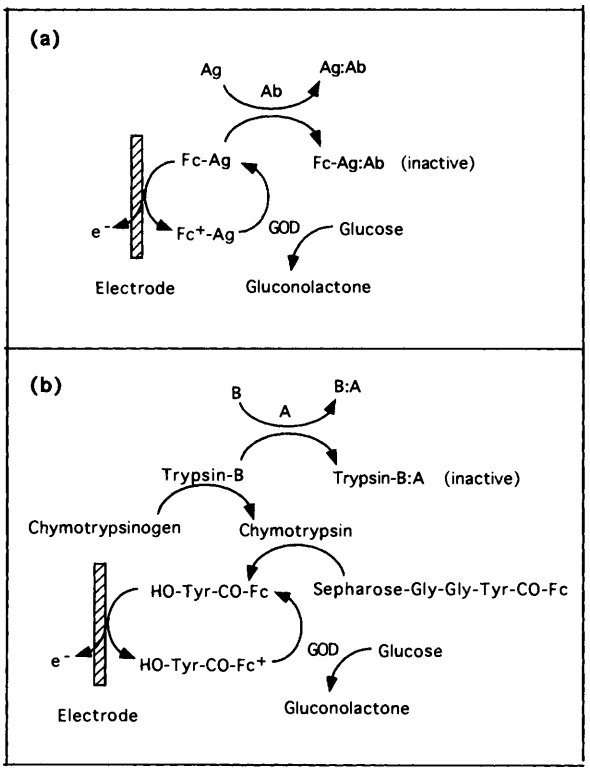

Figure 8 Homogeneous competitive amperometric immunoassays based on enzyme-mediated amplification of the binding. In (a), binding of the antibody to the ferrocene-labelled antigen (Fc–Ag) inactivates the mediated electron transfer from the enzyme GOD to the electrode. In (b), avidin (A) binding to biotinylated trypsin (trypsin-B) results in inactivation of the enzyme cascade that would result in the release of a small ferrocene complex capable of mediation of electron transfer from GOD to the electrode surface.
(Adapted with permission from references 19 and 20.)

3.3 Piezoelectric Detection

The converse piezoelectric effect, where an electric field applied to a piezoelectric material induces stress in the material, has been exploited for the detection of minute mass changes. The range of piezoelectric acoustic wave devices available and the application of the effect to chemical and biosensing has been recently reviewed[23–25] and will not be dealt with in detail here. The two most widely studied piezoelectric biosensor configurations are the Quartz Crystal Microbalance (QCM) and the Surface Acoustic Wave (SAW) modes. The QCM devices consist of an oscillator circuit that drives an AT-cut quartz crystal, sandwiched between two electrodes, at its fundamental resonant frequency that is tracked with a frequency counter. Changes in the resonant frequency are related to changes in the mass of the crystal, thus allowing the QCM to operate as a sensitive mass detector. SAW devices operating on the Raleigh wave principle can also be used for mass detection by monitoring changes in acoustic wave propagation between the generator and receiver sets of electrodes separated by a piezoelectric material. Although the ability of these devices to measure gaseous samples has received considerable attention, their most interesting feature is their ability to operate in liquids. Some controversy exists, however, concerning the SAW device's acoustic mode in liquids and the origination of the frequency changes for the QCM.[23]

Piezoelectric immunosensors have been fabricated by modifying the piezoelectric surface of the QCM with antibodies or antigens, and several researchers report the *direct* detection of antibody–antigen binding by monitoring of frequency changes in liquids.[24,25] However, the variability, lack of sensitivity, and inadequate signal-to-noise ratios of these devices in liquids would seem to limit their application to direct *liquid* sensing. Indeed it is questionable whether the total frequency change upon binding is due to mass changes alone since changing liquid properties such as density and viscosity and entrapped surface liquid changes may contribute to the frequency response of the system, although the use of a network analyser to operate the QCM devices can help distinguish the mechanisms involved in the sensor response.[23b] Binding of antibody or antigen to immobilized species on the QCM in the liquid phase followed by drying of the crystal and measurement of the subsequent frequency shift has been demonstrated to be a useful assay for large antigens (such as microbial species).[24,25] Continuous liquid phase operation is, however, more preferable for biosensor design. A problem also encountered in the direct detection of mass changes is the effect of non-specific adsorption on the sensor response. This can be alleviated to some extent by the use of matching reference crystals and the proper use of blocking agents.

The use of amplification schemes such as the proposed Amplified Mass Immunosorbent Assay (AMISA) can lead to improved detection limits over direct detection schemes.[26] In this scheme, shown in Figure 9a, AP-labelled secondary antibody to adenosine 5′-sulfonate reductase (APS) was utilized to deposit the insoluble dimer of the enzyme substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP) on the piezoelectric surface upon oxidation of the substrate by the bound enzyme, thus amplifying the response. A detection limit of 10^{-14}M was

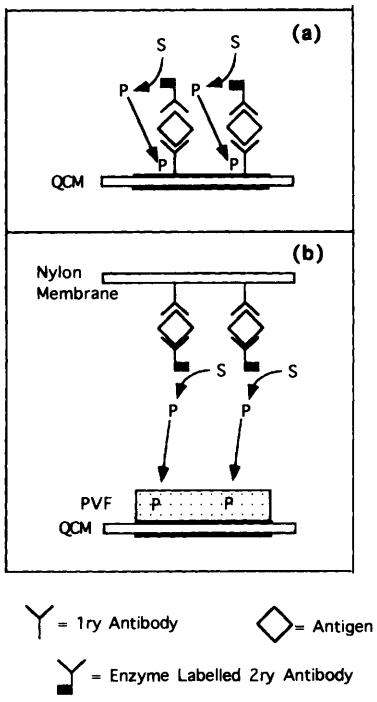

Figure 9 Schematic diagram of a QCM sandwich-based amplified mass immunosorbent assays. In (a), the alkaline phosphatase-labelled secondary antibody converts the BCIP substrate (S) into the insoluble dimer (P) which deposits on the gold QCM electrode decreasing the resonant frequency. In (b), the horseradish peroxidase-labelled secondary antibody on the nylon membrane converts the H_2O_2/I^- substrate (S) into I_3^- (P) which can diffuse to, insert into, and oxidize the nearby PVF film on the QCM decreasing the resonant frequency. Electrochemical reduction of the PVF film results in expulsion of the I_3^- and regeneration of the neutral film for subsequent assays.
(Adapted with permission from reference 26.)

established for the APS antigen, whereas direct detection of APS was not possible because of the small frequency changes involved. In the same report the authors described an approach for the development of a two-component sensor consisting of a reusable crystal and a disposable antibody membrane as depicted in Figure 9b. The gold-covered quartz crystal electrode was modified with a redox-active PolyVinylFerrocene (PVF) layer while a nylon membrane containing immobilized anti-HCG antibodies was placed opposite the electrode. HRP-labelled secondary antibodies enzymatically reduced the I^- to I_3^-/I_2 and the I_3^- could in turn oxidize and insert into the PVF film resulting in large frequency changes. Reversion of the film to its neutral state could be achieved by electrochemical reduction of the PVF and rapid expulsion of I_3^-, resulting in a reusable sensing surface/disposable recognition surface biosensor.

It should be noted that rapid, non-destructive reversal of the binding event is still required for the development of a true piezoelectric affinity *biosensor*, even if the devices are proven to be capable of measuring direct binding of antigens in solution. Chemical reversal of antibody binding for reusable assays has been achieved using low pH buffers and/or chaotropic agents. However the design of antibodies possessing kinetic binding characteristics specially tailored for biosensing purposes would result in the development of continuous-use reversible biosensors that would not require a heteregeneous washing or regeneration step.

3.4 Antibody Immobilization and Labelling

An important factor involved in the design of solid-phase immunosensors and assays not dealt with above is the immobilization procedure of the antibody. Adsorption of the antibody onto polystyrene wells is normally performed for ELISA, while methods for the chemical attachment of the antibody to solid phases have been examined.[1,11] An apparently simple procedure for antibody immobilization involves encapsulation within a sol–gel matrix, as reported recently.[28] This procedure has application to both optical and piezoelectric biosensor development provided antigen-binding activity is maintained within the matrix. New genetic engineering technology can also play a role in immunosensor research. The advent of molecular biological techniques has resulted in methods for the production of fusion proteins.[28] Fusion proteins of antibody–enzyme or antibody–peptide conjugates are produced by transforming bacterial cells with the enzyme–peptide vector and subsequent culturing of the cells to express the conjugate. Genetic engineering of antibody–enzyme conjugates is an exciting prospect and could lead to site-directed labelling methods for optimum antibody and enzyme activities and will also minimize the variability associated with the current chemical labelling schemes.

4 Receptor-based Assays and Sensors

Neurotransmitter and hormonal receptors are the body's own biosensors. Binding of a ligand (an agonist) to the receptor triggers an amplified physiological response such as ion channel opening, second messenger systems and activation of enzymes. Biological receptors are normally capable of binding to a series of structurally related compounds rather than to one specific analyte, which make their potential use as biosensors for classes of pharmacologically active compounds attractive.

RadioReceptor Assays (RRAs) have been routinely used for investigation of receptor–ligand interactions in neurobiology, pharmacology, and other related areas. In recent years the use of non-isotopic receptor-binding assays has received growing attention in an attempt to address the limitations of RRAs. The non-isotopic receptor-based assays and sensors can be divided into those that utilize intact receptors and those that utilize isolated, purified receptor preparations.

4.1 Intact Receptor-based Biosensors

Perhaps the most important 'biosensing' device developed in recent years is the patch clamp apparatus.[29] The patch clamp is used by electrophysiologists to investigate the mechanisms of channel gating, membrane transport, and the molecular pharmacology of membrane-bound receptors by examining effects on the ion currents in a small patch of membrane located on the tip of a glass microelectrode. The application of the patch clamp to practical biosensing is limited however by the instability of these patches, the small currents measured (requiring elaborate equipment and attention to grounding) and the fragility of the glass microelectrodes.

Improved lifetimes of 'neuronal biosensors' can be achieved by using less invasive electrophysiological detection procedures to measure the ionic flux associated with ion-channel opening in membrane-bound receptors. Extracellular monitoring of nerve firing, associated with the transmission of signals in nervous tissue, for application to biosensing, has been studied in recent years following the report on the prototype *chemo*receptor-based biosensor.[30] This sensor utilized excised crustacean olfactory structures (antennules) for neuronal sensing of chemoreceptor ligands. The antennules contain receptors responsible for olfactory sensing of compounds such as amino acids, sex hormones, pyridine-based chemicals, and other food markers. The binding of the ligand to the corresponding chemoreceptor triggers the firing of an action potential (AP) in the nerve that can be detected with extracellular glass microelectrodes. The firing frequency of the AP event is related in a dose-dependant manner to the concentration of the ligand (stimulant). Reviews on chemoreceptor-based biosensors have been published in recent years outlining the sensitivity and response ranges achievable and describing the principles and applications of these systems.[31] More recently our work[32] and that of others[33] has been directed to using intact *neuronal* receptors for the detection

of neuromodulators While the Van Wie group[33] has concentrated on voltage-clamp experiments on the isolated ganglion of the pond snail *Limnea stagnalis* for the detection of serotonin and its antagonists, we have focused our attention on less invasive extracellular detection for sensing agents that modulate or block the propagation of the AP in the walking leg nerves and giant axons of the crayfish *Procambarus clarkii* [32a,b] The stimulation of AP firing can be achieved using either chemical[32a] or electrical[32b] stimulus pulses The experimental system, shown in Figure 10 allows the detection of compounds which modulate the propagation of the electrically stimulated AP event The system was used to construct dose–response curves for detection of the local anaesthetics (LAs), which can bind in the ion channel of the voltage-gated sodium channel receptor responsible for AP propagation in nervous tissue, thus blocking AP conduction

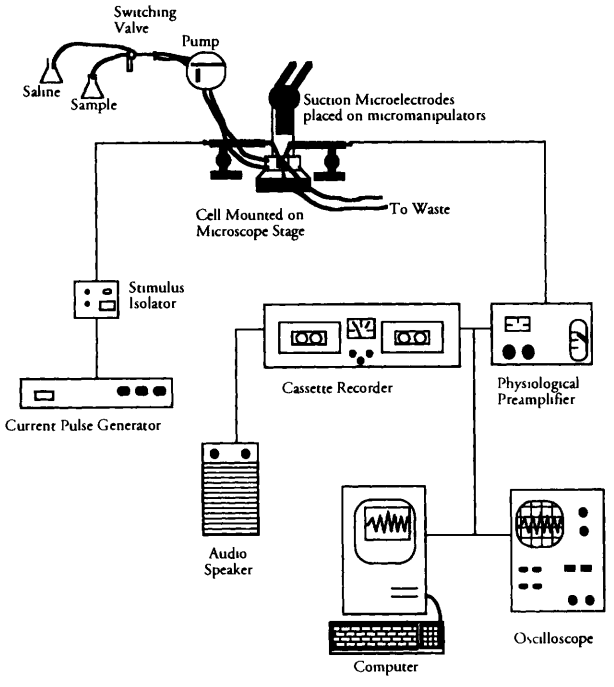

Figure 10 Block diagram of the experimental set-up for microelectrode stimulation and monitoring of AP firing in crayfish walking leg nerves for the detection of neuromodulators such as the local anaesthetics

The applicability of the system to screening natural product extracts for neuromodulatory drugs or toxins was proposed, as several important classes of compounds such as anti-depressants, narcotics, alcohols, and venoms and toxins are known to affect AP conduction Although this system shows promise as a neuronal biosensor the limited lifetime of the sensor (approximately 4—8h) is a severe drawback Recently we have demonstrated that channel blockers such as the LAs can be quantified by monitoring the *biomagnetic current* induced in a toroidal probe surrounding the nerve [32c] This system allows *non-invasive* detection of the compound action current (CAC) associated with the AP event in crayfish giant axons, as depicted in Figure 11 A dose–response curve for the LA lidocaine can be constructed, as shown in Figure 12, by monitoring the duration of the conduction block in the crayfish giant axon upon application of standard solutions of the LA to the specially designed flow cell Current research is aimed at studying the effect the non-invasive detection of the AP has on the lifetime of the preparation Attempts to replace the invasive extracellular microelectrodes used to apply stimulus pulses to the preparation with non-invasive stimulus procedures are also under investigation

Other researchers have reported on the monitoring of the *cellular* response of microorganisms for drug-screening purposes [33,34] The LAPS system previously described has been used to detect changes in the pH of the environment surrounding immobilized cells, reflecting changing cellular metabolism, in

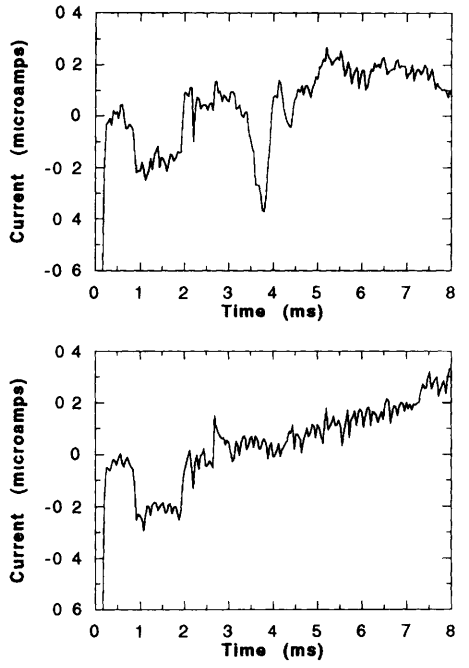

Figure 11 Non-invasive recordings of the compound action current (CAC) in crayfish giant axons with a biomagnetic current probe surrounding the nerves before (above) and after (below) application of 50 nM lidocaine to the cell The rectangular pulse evident 1 ms after triggering of data acquisition is a 1 ms, 0 2 μA calibration pulse

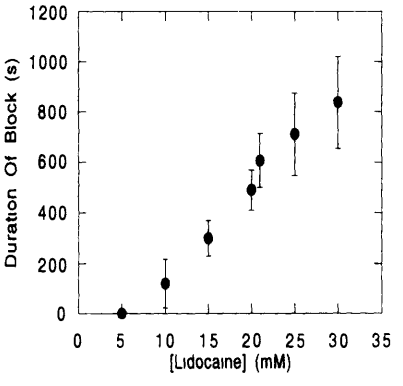

Figure 12 Dose–response curve for the local anaesthetic lidocaine constructed by monitoring with a biomagnetic current probe the duration of the conduction block induced in the crayfish giant axon following a 5 minute flow of standard solutions of lidocaine through the cell

response to various drugs and cell-affecting agents [33] McClintlock *et al* [34] have described a useful bioassay for investigating the G-protein-coupled receptors by expressing them in *Xenopus laevis* melanophores Receptor-mediated stimulation/inhibition of the second messenger systems caused dispersion/aggregation of the melanosomes Translocation of the melanosomes could then be tracked using video microscopy leading to an assay for detection of receptor expression The combined use of receptor expression in melanophores and video imaging technology could clarify the mechanisms of ligand binding and second messenger activation pathways

4.2 Isolated Receptor-based Biosensors

The predominant scheme in use for receptor-based assays using isolated receptors is the RRA, although recent research has demonstrated the use of fluorescent-labelled, biotinylated, and enzyme-labelled ligands for the assay of receptor agonist and antagonists using schemes similar to those described previously

for immunoassays. Isolated receptors have also been successfully reconstituted into bilayer lipid membranes on patch pipettes and at interfaces of troughs for the electrophysiological/electrochemical investigation of the ion channel and receptor-ligand binding properties.

Receptor-based biosensors have been constructed using the well studied nicotinic AcetylCholine Receptor (nAChR) in various formats. An ISFET-based biosensor relied on the potential shifts in the gate voltage associated with ligand binding[35] but is unlikely to lead to a practical biosensor because of previously mentioned problems with ISFET biosensors. The measurement of capacitance changes in receptor liposomes immobilized on planar interdigitated electrodes upon ligand binding has also been proposed for biosensor applications[36] although it is doubtful that ligand binding is the only contribution to the reported responses. Eldefrawi *et al.*[37] have described the development of an nAChR-based fibre-optic biosensor depending on evanescent wave excitation of fluorescence in labelled ligands bound to the immobilized receptor. An irreversible system using fluorescein isothiocyanate (FITC)-labelled α-bungarotoxin was subsequently improved upon by using the reversible ligand FITC–α-conotoxin. The extension of this system to other receptors is envisaged for the non-isotopic assay and detection of therapeutic drugs.

None of the biosensing systems above have taken advantage of the inherent amplification associated with ion-channel opening of the ligand- and voltage-gated receptors upon agonist binding, as described for intact-based biosensors. Minami *et al.*[38] have described an ion-channel sensor that utilizes the glutamate receptor reconstituted into a bilayer lipid membrane for the coulometric detection of glutamate. Problems with the stability of the sensor, however, would seem to hinder further application.

Although the use of isolated, immobilized receptors for biosensing has been demonstrated, practical application of these sensors requires improvements in receptor purification and reconstitution. Improvements in non-invasive technology for stimulation and/or detection of AP events in *intact* receptors, and improvements in tissue culture and nerve growth media for the maintenance of nervous activity in excised nerves shows promise for the development of practical neuronal biosensors.

5 Other Affinity Biosensors

5.1 DNA Detection

Nucleic acid hybridization *assays* can be monitored non-isotopically by attachment of an enzyme, fluorescent, or chemiluminescent label to a complementary portion of DNA or RNA, or by selection of antibodies specific for double-stranded DNA or a label. The application of non-isotopic techniques to DNA detection has been reviewed recently[39] and will not be presented in detail here. In general schemes such as those presented under immunoassays may be used for DNA-based assays. A new and promising approach to DNA *biosensing* involves the fluorescent[40] or electrochemical[41] detection of inorganic DNA intercalators. These compounds, most notably the transition metal complexes of Pt, Ru, Co, and Fe with bi- and tridentate ligands, intercalate into double-stranded DNA or bind electrostatically to the DNA phosphate backbone.[40,41] The electrochemical DNA biosensor[40] was capable of reversibly detecting oligo$(dA)_{20}$ oligonucleotides by immobilization of olig$(dT)_{20}(dG)_{98}$ on glassy carbon electrode surfaces. Immobilization of probe strands of DNA onto electrodes or fibre optic cables for the detection of DNA hybridization using these intercalators is a promising avenue of research for the development of biosensors for diagnosis of disease. The *direct* detection of DNA hybridization[42] or of intercalators[43] has also been demonstrated using oligonucleotides bound to the QCM, although problems of non-specific binding events need to be addressed for practical application of these sensors.

5.2 Binding Proteins

The affinity biosensor concept was first introduced by Schultz *et al.*[3] as described previously, who demonstrated the optical detection of glucose, based on competitive affinity binding of glucose and fluorescent-labelled dextran to concanavalin-A. Other binding proteins, such as protein-A, avidin, and enzymes have potential uses in affinity biosensing schemes although their use has mainly been confined to affinity purification of antibodies, amplification of binding events and affinity immobilization, and biocatalytic sensors, respectively. Ikariyama *et al.*[44] developed an affinity biosensor for biotin based on the differential affinity of catalase-labelled avidin for biotin and membrane-bound immobilized 2-[(4-hydroxyphenyl)azo]benzoic acid (HABA), a biotin analogue. Electrochemical detection of binding was achieved by monitoring production of oxygen by the enzyme reaction following binding to HABA. Wang *et al.*[45] immobilized the enzyme tyrosine hydroxylase (TH) in a Langmuir–Blodgett film on a gold electrode for the affinity concentration of the TH ligands such as the neuroleptic drugs. Application of a voltammetric pulse scheme resulted in quantitation of the electroactive drugs at the micromolar level and dissociation of the drug from the receptor. The extension of this scheme to other receptors and drugs shows promise, provided a stable film can be established and that the ligands are electrochemically or optically active.

6 Conclusions

Research into the development of affinity-based biosensors has increased considerably in recent years following the first description of the affinity biosensor by Schultz *et al.*[3] and the report on the prototype intact receptor-based biosensor.[30] While several viable procedures have been demonstrated, problems associated with non-specific binding effects, receptor stability, interferences, detection limits, continuous operation, and reversibility of the binding still exist. The selection of monoclonal antibodies with the kinetic properties required for reversible binding, coupled with advances in direct detection schemes, such as fibre optics, SPR and QCM detection, requires close collaboration between immunologists and biosensor researchers for the development of continuous use immunosensors. Competitive-binding optical affinity biosensors such as those described recently[2–5] show great promise if this problem can be solved. Advances in genetic engineering of fusion proteins, antibody immobilization procedures, and reagentless assay of enzyme activity will improve immunoassay technology immensely. Interdisciplinary approaches to receptor cloning, purification, and immobilization are also required to develop operational receptor-based biosensors for drug screening applications. In conclusion, although several promising affinity biosensor formats have been reported, a practical commercial affinity biosensor device has yet to be constructed. Future research requires collaboration between chemists, biochemists, immunologists, pharmacologists, neuroscientists, and engineers in order to obtain stable, isolated, immobilized receptors (antibodies, receptors, binding proteins) that possess the required characteristics for biosensing (affinity, reversibility, sensitivity, selectivity), while achieving advances in instrumentation and detection procedures to allow direct detection of the binding event.

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