Organic Phase Immunosensors

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Many analytical methods involving non-polar analytes require solvent extraction prior to measurement. The analysis procedure is greatly simplified if the method is able to function effectively in the more non-polar solvent extract. This consideration, coupled with the increasing need for simple, specific and rapid diagnostic and screening tools, has focused interest in the development of organic-phase immunosensors.

Introduction

Immunosensors are analytical devices incorporating an antibody-based biorecognition component utilised in conjunction with a suitable transducer to allow identification and quantification of specific analytes or structurally similar groups of analytes. Interest in the development and application of these sensing tools is growing rapidly for a variety of reasons. The advent of hybridoma technology has allowed the generation of homogeneous, high affinity recognition elements capable of selectively binding a wide variety of structurally diverse ligands [1]. The development of novel transduction methodologies and advancements in the understanding of existing systems have created powerful new approaches to visualising, monitoring and interpreting the biorecognition process. Furthermore, society is increasingly demanding simple, sensitive devices for the rapid decentralised analysis and monitoring of a wide range of compounds, particularly in the clinical, environmental, nutritional and military areas [2-7].

It is becoming widely appreciated that antibodies, like enzymes, can retain biological activity in organic solvents, thus creating the possibility of immunosensing in such environments. This is an attractive proposition given that many affinity-based analytical methods involve organic-solvent extraction processes that, hitherto, have required extract evaporation and reconstitution in the aqueous phase. This approach is compatible with the requirements of field-based biosensor devices, namely minimal sample preparation, assay simplicity and low cost. Further conjectured benefits include analyte pre-concentration in small volumes of extraction solvent leading to increased detection limits, improved assay stability and operational temperature range, and the possibility of 'tailoring' the antibody-antigen interaction through quaternary structure distortion in the presence of solvent to create favourable reversible binding kinetics [8]. The fact that many enzymes can also function in organic solvents, sometimes with enhanced kinetic properties, potentially allows such entities to be used as immunoassay labels [9].

The field of organic-phase immunosensing is very much in its infancy, but the potential benefits of this approach for the measurement of poorly water-soluble analytes in organic solvents are enormous. This paper attempts to highlight the practical issues, potential applications, advantages and problems associated with the operation of immunosensors in the presence of organic solvents and in non-aqueous environments.

Antibodies in the organic-phase

The ability of antibodies to recognise and bind specific antigenic substances has led to their widespread exploitation as diagnostic tools [9-12] with vast arrays of monoclonal antibodies being created to recognise and bind target analytes with often exquisite selectivity. Conventional immunoassays capable of measuring as little as picogram per millilitre quantities of material (~10^{-18} mol analyte) [13] exist and ongoing development of new immunoassay strategies has yielded limits as low as 10^{-21} mol [14,15]. Immunoassays were traditionally performed within the aqueous environment, until Russell *et al*. [16] discovered that some antibodies were capable of retaining activity and selectivity within non-aqueous environments.

Russell *et al*. found that an antibody preparation raised against aminobiphenyl was able to retain immunologic activity in a range of organic solvents including dioxane, acetonitrile and various alcohols. Improved specificity of binding was noted in the organic-phase although the dissociation constant (a measure of antibody-antigen affinity) was found to increase with decreasing solvent hydrophobicity, indicating that the binding process itself favoured hydrophyllic surroundings. The binding mechanism was influenced by the hydrophobic nature of aminobiphenyl such that more hydrophobic solvents increasingly disrupted the antibody-antigen interaction.

Interest in organic-phase tolerant antibodies has continued into the 1990's. Weetall [17] reported that an anti-progesterone antibody preparation was able to retain binding

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activity in hexane. Although the kinetics of the antibody-antigen binding process were reduced in the non-polar solvent, this was not due to activity losses since the antibodies were able to bind a similar quantity of antigen as in aqueous solution. Weetall suggested that the retained activity was due to a residual shell of water surrounding the hexane-solubilised antibody, the observed kinetic differences being attributable to the time required for the transfer of the antigen from the organic-phase into the aqueous film.

Stöcklein *et al*. [18] have studied the behaviour of two different anti-atrazine polyclonal antibody preparations in five anhydrous non-polar solvents. Like Russell *et al*., they observed improved binding specificity and a correlation between increasing solvent polarity and decreased antibody-antigen binding affinity. It was further concluded that the binding process was also influenced by the solubility of analyte in the solvent. Stöcklein and co-workers in a further study [19], have also investigated the effect of organic solvents on anti-coumarin antibody, based on a flow-injection immunoassay method using coumarin-conjugated horseradish peroxidase (HRP) and laccase enzyme labels. All of the organic solvents examined decreased antibody-antigen binding and caused partial desorption of immobilised antibody from the solid phase support. Assay sensitivity was found to be increased in the presence of methanol, unchanged in n-propanol and acetonitrile and decreased in ethanol, reflecting the different effects of the solvent on antibody-conjugate and antibody-hapten binding. The point was made that organic co-solvents represent a convenient way of modifying the affinities and specificities of antibodies and 'tailoring' cross-reactivity effects.

A detailed study of antigen-antibody binding behaviour in water-miscible organic solvents has been provided by Giraudi and Baggiani [20] using testosterone-anti-testosterone as the test system. Steroid-antibody binding was found to decrease with increasing molar fraction for a range of alcohols, substituted alcohols and dioxane. Binding affinities were found to be inversely correlated to solvent molecular mass with no relationship being observed with other solvent properties such as polarity, dielectric constant and dipole moment, suggesting that binding inhibition is related to the ability of the solvent to displace water from around the steroid molecule. Interestingly, enhanced binding of antigen was noted at low molar fractions of tetrahydrofuran and acetonitrile, although a rapid decrease in binding was observed at higher solvent concentrations. In the light of these findings, it was concluded that a more detailed mechanism incorporating the differential solvation of the steroid by solvent and water molecules requires evaluation.

An alternative immuno-assay strategy for the quantification of hydrophobic analytes has exploited the use of reverse miscelles [21-23]. The antibody is solubilised within an aqueous phase that is encapsulated by a suitable surfactant film, such that it forms a series of discrete pockets within a surrounding non-aqueous solvent, thereby eliminating antibody interaction with the organic-phase. Francis and Craston [21] describe an immunoassay for parathion in which the analyte is dissolved in n-hexane and interacts with polyclonal antibody within an aqueous miscelle, *via* diffusion across the phase boundary. However, the hexane-based immunoassay was 10^4 times less sensitive than the equivalent aqueous-phase assay. Since the affinities between the antibodies and parathion-albumin conjugate were comparable in both solvents, the sensitivity loss was attributed to parathion mass-transfer resistance at the hexane-water interface.

Lu *et al*. [9] have used conventional immunoassay techniques to study horseradish peroxidase (HRP)-antibody binding in acetonitrile with a stated aim of developing an organic-phase immunosensor. The catalytic activity of HRP and binding ability of the antibody both decreased with increasing organic solvent content, with the antibody activity being reduced 500-fold in pure solvent. The conformation of the antibody was irreversibly changed in 40-80% v/v acetonitrile, indicating the disruptive properties of this water-miscible solvent particularly in the presence of a considerable proportion of water.

The factors governing antibody-antigen binding in organic solvents are clearly complex and require further elucidation. Solvent properties such as hydrophobicity, molecular mass and molar fraction can all affect the binding process, as can antigen solubility in the organic-phase. The solvent can disrupt both the structure of the antibody binding site and antigen and phase-transfer effects in 'anhydrous' solvents and solvent 'stripping' effects in aqueous/organic solvent mixtures also require consideration. Correspondingly, organic-phase immunoassay development has relied on an

Table I. Some studies of antibody behaviour in organic solvents.

Antibody specificity	*Solvents*	*Reference*
Atrazine	methanol, acetonitrile	Goh *et al.* [26]
Okadaic acid	methanol, ethanol, acetone, diethyl ether, benzene	Matsuura *et al.* [27]
17β-estradiol	methanol, ethanol, propanol, ethylene glycol, acetone	De Lauzon *et al.* [28]
Polychlorinated biphenyls	transformer oils	Lambert *et al.* [29]
Atrazine	toluene, trichloromethane	Sasaki *et al.* [30]
BTX compounds	methanol, ethylene glycol, acetone, acetonitrile, dimethylformamide, dimethylsulphoxide	Beyer *et al.* [31]

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empirical rather than a mechanistic approach. Given the extensive literature devoted to the behaviour of enzymes in organic solvents, coupled to the fact that a decade has elapsed since the original antibody-based study in this field, it is surprising that greater efforts have not been directed in this area. Reviews of this field have been provided by Kröger [24] and by Stöcklein and Scheller [25] whilst Table I summarises a number of other key publications.

Organic-phase immunosensors

Immunosensing techniques can be subdivided into many categories, although a fundamental distinction is made between direct and indirect methods [32-36]. The former approach allows direct monitoring of the antibody-antigen recognition process in real time, whereas the latter method requires additional reagents, such as enzyme or fluorescent-labelled components to visualise the extent of antigen binding, usually on completion of the binding process. Direct methods are conceptually simpler and to date have relied on *capacitive, conductiometric, potentiometric* and *optical* transduction methods whilst the use of labels in indirect immunoassay formats has primarily resulted in the application of *optical* and *electrochemical* transducers to quantify the binding process [34].

The finding that antibodies can retain activity and high levels of selectivity in highly non-aqueous environments has not only presented the opportunity of performing conventional immunoassays within such environments, but also the intriguing possibility of organic-phase immunosensing, a fact recognised by Saini *et al*. [8]. Direct immunoassay approaches demand the selection of appropriate solvent tolerant antibodies. To date, indirect methods have employed a 2-stage process, the immuno-reaction being performed directly within the organic-phase matrix, after which the system is washed to remove unbound conjugate allowing the label (enzyme) to be detected in the absence of potentially disruptive organic solvent effects.

Direct immunosensing in the organic-phase

Recent work by Skládal [37,38] describes the effect of methanol on the reaction between an anti-atrazine monoclonal antibody (MAb) and the hydrophobic antigen in the free and immobilised state using the IAsys resonant mirror-based biosensor system. Initial studies focused on the interaction between free MAb and atrazine, covalently attached to albumin to facilitate immobilisation to aminosilane treated transducer surfaces, in the presence of 0-50% v/v methanol. Binding could be observed at all the methanol concentrations studied, although the kinetic rate constant data were significantly improved in the presence of low concentrations of methanol. The maximum association constant (k_a) and minimum dissociation constant (k_d) were recorded in the presence of 5% and 10% v/v methanol respectively, the kinetic equilibrium association affinity constant (K_A) being optimum at around the 10% v/v level. The binding capacity (R_{max}) of the sensing surface was more than two-fold lower in the presence of 10-30% v/v methanol.

In order to minimise the effect of changed antigen properties due to immobilisation, further studies were performed on free atrazine. Binding affinities at different methanol concentrations were measured through establishing equilibrium binding between free atrazine and free MAb, then quantifying the concentration of remaining MAb at an immobilised atrazine-transducer surface. Rate constant measurement is not possible using this approach. Antigen immobilisation appeared to improve MAb-atrazine affinity in the presence of 0, 10 and 30% v/v methanol as evidenced by K_A values 12, 120 and 700-times higher respectively than the corresponding values obtained for the free herbicide (Table II). However, a near 3-fold lower K_A value was observed for immobilised versus free atrazine in 50% v/v methanol.

Surprisingly the affinity between MAb and free antibody was strongest in 50% v/v methanol. However, given that increasing methanol concentration decreased the binding capacity of the surface-immobilised antigen, larger signals were obtainable at lower solvent concentrations. Overall, the optimum binding affinities and signal responses were obtained in 10% v/v methanol. Skládal [37] states that he

Table II. Effect of immobilisation and methanol concentration on the kinetic equilibrium association constant K_A (value ± standard deviation, 10^6 M^{-1}) observed between anti-atrazine MAb and atrazine using a resonant mirror and piezoelectric biosensor. Data from Skládal [37].

	Methanol (% v/v)			
	0	*10*	*30*	*50*
Resonant mirror biosensor				
Free atrazine	35.4 ± 5.3	9.4 ± 3.8	0.73 ± 0.10	410 ± 160
Immobilised atrazine	430 ± 97	1160 ± 270	500 ± 120	145 ± 41
Piezoelectric biosensor				
Immobilised atrazine	219	632	–	–

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has successfully used the IAsys system to measure atrazine in methanolic soil extracts and compared the data with conventional enzyme immuno-assay methods and gas-chromatography, although these findings have yet to be published.

Skládal and co-workers [39] have also studied the kinetic and affinity parameters of the same monoclonal antibody with immobilised atrazine at a piezoelectric transducer surface. Unlike with the resonant-mirror approach, methanol was found to increase rather than decrease the binding capacity of the atrazine-modified transducer surface (2.2-fold increase recorded). Furthermore, k_a values were unchanged in 1-20% v/v methanol, whilst the k_d value was found to decrease 3-fold in 20% v/v methanol. The corresponding K_A data is shown in Table II. Again, stronger affinities between MAb and immobilised antigen were observed in 10% v/v methanol compared with pure buffer. Signal values were approximately half those recorded with the resonant-mirror approach, attributed to the fact that the density of transducer-immobilised ligand was 20 times greater in the piezoelectric system. Low ligand densities are generally preferred in kinetic studies; such conditions also serving to improve assay sensitivity. The binding capacity of atrazine-modified piezoelectric crystals was similarly increased in ethanol (1.9x), 10% v/v solvent yielding a 4-fold increase in k_a and a 20% increase in k_d. Low concentrations of ethanol (10% v/v), like methanol improved MAb-atrazine binding affinity, a K_A value of 770×10^6 M^{-1} being recorded. n-Propanol (20% v/v) caused a 2-fold decrease in both k_a and binding capacity, coupled to a 4-fold decrease in k_d.

These findings were not in accordance with those of Stöcklein *et al*. [40], who observed a decreased binding affinity between two diphenylurea derivatives and FAb antibody fragments derived from an anti-diphenylurea MAb in the presence of ethanol. The kinetics of the binding process were observed by monitoring the binding and disassociation of the FAb fragments immobilised to activated carboxymethyl dextran chips to each of the haptens using the BIAcore system. The strength of the affinity reaction, expressed as K_A, was found to decrease with increasing ethanol concentration (Table III). Interestingly, the cross-reactivity of the FAb fragment, was changed in favour of hapten II rather than hapten I with increasing methanol concentration (Table III). Cross reactivity was defined as the ratio of hapten II *versus* hapten I analyte concentrations leading to a 50% reduction in signal in a competitive assay format. This finding creates the possibility of discriminating between a mixture of the analytes by performing the assay in the presence and absence of the solvent. The authors state that cross-reactivity changes are dependent on FAb concentration.

Table III. K_A and cross-reactivity data relating to immobilised haptens type I and II in various concentrations of ethanol. Cross-reactivity = x_o(hapten II)/x_o(hapten I) where x_o is the analyte concentration causing a 50% signal decrease in competitive assay format. From Stöcklein *et al.* [40].

	Ethanol (% v/v)		
Immobilised hapten	*0*	*10*	*20*
K_A, type I (M^{-1})	1.0×10^8	1.5×10^7	3.1×10^6
K_A, type II (M^{-1})	5.3×10^9	1.1×10^9	3.1×10^8
Cross-reactivity (%)	18.4	4.3	1.7

Indirect immunosensing in the organic-phase

To date, indirect organic-phase immunosensors have relied on the use of electrochemical transducers to measure antibody-antigen binding *via* redox reactions arising from the activity of reagent-associated enzyme labels. The main research in this area has been provided by Kröger *et al.* [41-43] and Jülicher *et al.* [44], working in collaboration to develop a field-based tool for the quantification of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) in aqueous/organic solvent soil extracts. The assay was centred on a disposable 3-electrode assembly prepared using screen-printing technology, an approach amenable to the mass-manufacture of devices with fabrication materials being selected for compatibility with the organic-phase [45]. A competitive immunoassay format was followed using either horseradish peroxidase (HRP) or glucose oxidase (GOX) labelled antibody. Enzyme label activity was determined amperometrically in aqueous buffer after antibody incubation and a washing step. HRP activity was measured using hexacyanoferrate (II/III) mediator at bare carbon electrodes (–20mV *vs*. Ag/AgCl) using hydrogen peroxide as substrate. GOX activity was measured at a rhodinised carbon electrode (+350 mV *vs*. Ag/AgCl), a suitable surface for the mediator-free oxidation of the enzymatic reaction product, hydrogen peroxide [46-48].

Jülicher *et al*. [44] utilised a novel, highly reproducible *in situ* antigen immobilisation procedure, capable of immobilising 2,4-D directly onto graphite carbon working electrode surfaces. A transglutaminase enzyme was used to catalyse an acyl-transfer reaction between casein glutaminyl residues and the primary amine group of a 2,4-D amide derivative [49]. In this manner, an extensively cross-linked hapten-protein structure was formed directly onto graphite carbon working electrode surfaces. The approach is highly compatible with organic-phase immunosensors given that the film was found to be stable in 100% v/v methanol. Furthermore, the method was simple, the devices being highly reproducible with shelf lives of at least 93 days. The approach can be used to immobilise any hapten containing aliphatic amino residues.

Pre-conditioning of the hapten-electrodes in methanol/buffer solutions increased signal intensities indicating that the solvent altered the conformation of the hapten-casein complex, presumably by improving accessibility of the antibody to the hapten. The device was tested using 2,4-D spiked into 30% v/v methanol in buffer. The lower detection limit of the device was found to be 9 mg L^{-1} with a sensi-

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tivity, measured as the slope of the sigmoidal curve at 50% signal reduction, of 1.3 ± 0.4 nA L mg^{-1} per square centimetre of working electrode surface. An overall assay reproducibility of 6.8% was recorded. Signals were higher in the presence of 30% v/v methanol than in pure buffer, a phenomenon commented on by Kröger *et al*. [41], although the sensitivity and detection limit of the assay was not significantly affected. Interestingly, the stability of the hapten immobilisation procedure was such that the hapten-electrodes could be regenerated by washing in 2 M sulphuric acid, allowing multiple usage. A calibration curve prepared with regenerated electrodes had similar characteristics to the single-use curve.

Kröger *et al*. [41] achieved hapten immobilisation using a carbodiimide-2,4-D-BSA complex physically adsorbed onto porous rhodinised-carbon working electrode surfaces, a simple and effective means of biopolymer immobilisation [50]. The device was calibrated in the presence of 2,4-D spiked into buffer, 30% v/v methanol in buffer and a 30% v/v methanol soil extract. The resultant sigmoidal calibration curves were interpreted using the logit-log model [51] in which four variables are obtained: *a* (maximum current at zero analyte); *b* (slope of the curve at the mid-point); *c* (analyte concentration at the slope midpoint); and *d* (minimum current at infinite analyte dose). Working in 30% v/v methanol rather than pure buffer led to a 40% increase in device response at zero dose (*a*) with little change in the non-specific response (*d*) being recorded (Table 4). Since GOX activity is unaltered by pre-incubation in methanol [50], solvent-enhanced antibody-immobilised antigen binding was suspected with two reasons being suggested. Firstly, a change in the conformational structure of 2,4-D-BSA, an idea supported by the findings of Jülicher *et al*. and secondly, enhanced antibody binding affinity, also possible since antibody selection was based on positive binding characteristics in methanol [52].

A reduction in both the *a* and *d* values was observed in the methanolic soil extracts compared with 30% v/v methanol, although the positive effect of the solvent over pure buffer was still apparent. The observed signal decrease was assumed to be due antibody cross-reactivity with co-eluted interferents although other factors such as enzyme inhibition effects and electrode fouling can not be excluded. A number of widely differing soil extracts were examined with similar sizes of signal reduction being noted, indicating the applicability of the device as a field-based screening method. Whilst such matrix effects are undesirable, it should be appreciated that such effects are common to many other biosensor and immunoassay systems.

The device compared favourably with a commercially available immunoassay test kit (Table IV). A limit of detection of 0.21 mg L^{-1} 2,4-D was reported for the immunosensor, compared with 0.20 mg L^{-1} for the test kit. However the kit required an extract dilution of 1:200, compared to 3:1 for the immunosensor. It was concluded that higher affinity solvent-resistant antibodies and further device optimisation was required for 2,4-D measurement in the µg L^{-1} range.

Table IV. Electrochemical immunosensor device and commercial immuno-assay kit performance for detection of 2,4-D in buffer, 30% v/v methanol and 30% v/v methanol soil extracts. From Kröger *et al.* [41].

Logit-log term	*Buffer*	*Methanol*	*Soil*	*Commercial kit*[1]
a (µA)	1.72	2.66	2.21	0.57
b (µA/ mg L^{-1})	0.99	0.78	0.63	0.94
c (mg L^{-1})	3.71	5.09	5.80	1.94
d (µA)	0.86	0.94	0.65	0.07

[1] Commercial photometric immunoassay; µA = OD; 2,4-D spiked into soil extracts

Enzymes as labels for indirect immunoassays in the organic-phase

Immunoassay considerations

Most indirect immunosensing techniques rely on enzymatic, fluorimetric or luminometric labels to quantify the binding process. The choice of label is dependent on the required assay sensitivity, selectivity, cost and interferents present with enzymes being favoured for reasons of signal amplification, substrate turnover and assay diversity. Electrochemical transducers coupled to enzymatic identification have been particularly favoured for field-based biosensors since such devices can be simply and cheaply mass-produced [53-56]. Field-based immunosensors, of necessity, should be simple, robust and portable, which has favoured the development of indirect immunoassays, approaches that require less complex instrumentation than their direct counterparts. Furthermore, the surface at which the recognition process occurs is directly addressable, allowing the development of 'separation-free' immunosensors that, by preferentially recording surface-bound enzyme activity, do not require a wash step to remove unbound conjugate [57-59].

However, the indirect organic-phase immunoassays so far reported do require a washing stage to remove unbound conjugate, allowing remaining enzyme activity to be determined in aqueous solution. This factor can complicate field-based immunosensor operation where a pre-requisite for success is minimal operator input. Using electrochemical transduction, the possibility of performing a separation-free immunoassay (antibody-antigen binding + enzyme label quantification) within an organic-phase environments is achievable. An essential factor in such an approach is that the enzyme label, like the antibody, must retain activity in non-aqueous solvents. Fortunately many enzymes are able to do this.

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Enzymes and enzyme electrochemistry in the organic-phase

Organic-phase enzymology can potentially offer a range of advantages over aqueous phase systems, namely improved thermostability, improvements in kinetic performance, improved substrate solubility and altered substrate specificity [60]. It is generally agreed that enzymes are capable of retaining catalytic activity in certain organic solvents by retention of a hydration shell surrounding the particle. Most original papers and reviews [61-73] attempt to explain biocatalytic behaviour in terms of particular solvent characteristics and it has been established that the greater the interaction between the organic-phase and essential hydration layer, the greater the disruption to enzyme structure and hence catalytic behaviour. Hydrophobic water miscible solvents tend to be most suitable for organic-phase biocatalysis since they do not disrupt the hydration shell whilst water-miscible solvents such as alcohols can severely disrupt enzyme function by stripping the hydration shell from the enzyme. Mionetto *et al*. [74] have described a third category of solvent that, by virtue of its intermediate hydrophobicity, is able to remove the hydration shell, but enzyme activity is retained due to small quantities of water in the bulk solution. Mionetto *et al*. found that activity could be retained in water-miscible solvents by immobilisation of the enzyme, a finding with important implications regarding the usage of enzymes in bioanalytical applications.

The ability of enzymes to operate in organic solvents has led to the development of organic-phase enzyme electrodes (OPEEs), capable of working in highly non-polar solvents through to mixed water-miscible solvent/buffer [75-81]. The operational benefits of such devices include stability, simpler immobilisation techniques, suppression of undesirable side reactions and increased operating range [75]. Importantly, in the context of electrochemical organic-phase immunosensing, there is a wealth of information concerning solvent effects on the electrochemical behaviour of two of the enzymes most commonly used as immunosensor assay labels; GOX and HRP.

GOX is able to retain activity in organic solvents by virtue of a deeply embedded active site that is little affected by organic solvent surrounding the molecule [76]. Iwuoha and co-workers in a series of studies [82-86], have investigated the effect of a range of organic solvents on the response of amperometric GOX enzyme-electrodes. Factors such as catalytic efficiency, maximum catalytic current response, substrate diffusion and active site flexibility were considered. An enhanced GOX activity was observed in acetonitrile, with a maximum recorded at the 80% v/v level. This increase was attributed to acetonitrile acting to desorb water from the enzyme active site on substrate binding and not factors such as improved glucose or mediator diffusion [86]. These findings appear to contradict those of Griebenow and Klibanov [69] who used Fourier transform infrared spectroscopy to show that lysozyme is most disrupted in 60% v/v acetonitrile, but is effectively 'rigid' in anhydrous solvent. However, the observed responses were a summation of a number of individual factors including changes in enzyme activity, sensor performance and sensor design. Work by Karyakin *et al*. [87] has indicated that water is vital for maximising GOX activity in ethanol. An increase in water content from 2.5 to 12.5% v/v was found to increase GOX activity from 20% to >100% of the original activity whilst the enzyme was found to retain more than 50% original activity after exposure to 90% v/v ethanol for 30 min. An exact mechanism by which the solvent influenced the measured enzyme activity was not given.

A study by Kröger *et al.* [88] on the behaviour of free and immobilised GOX in alcohol/buffer mixtures highlights the problems associated with interpreting enzyme-solvent effects in electrochemical systems. An apparent increase in immobilised GOX activity was observed in 10-50% v/v ethanol under excess enzyme conditions (40% signal increase in 20% v/v ethanol; 1 Unit enzyme immobilised) whilst a steady decrease in apparent activity was recorded under enzyme limiting conditions (11% signal decrease in 20% v/v ethanol; 10 mUnit enzyme immobilised). It was speculated that the observed increase in response in the presence of excess enzyme included solvent-induced pH shift, altered diffusion rates and swelling of the porous working electrode pad. Activity losses due to solvent denaturation effects were masked by excess enzyme, such that the main rate-limiting step became hydrogen peroxide oxidation.

HRP, like GOX can retain activity in organic solvents, provided that a critical amount of water is present in the system, through shielding of the active site. HRP-electrodes have been used successfully to measure peroxides in organic-phase environments, most reported systems relying on the use of mediators to transfer electrons between the reducing electrode and prosthetic haem group within the HRP molecule. Schubert *et al*. [89,90] were able to quantify hydrogen peroxide in chloroform and dioxane by co-immobilisation of HRP and water-soluble hexacyanoferrate at a carbon electrode. Adeyoju *et al*. [91] have measured butanone peroxide at an HRP-platinum electrode in 98% v/v acetonitrile in phosphate buffer using o-phenyldiamine as the mediator. Iwuoha *et al*. [77,86] have reported a numerical index based on the product of inverse solvent viscosity and inverse dielectric constant, to quantify the frictional force exerted on a substrate by a particular organic solvent. A correlation between this factor and sensor sensitivity was noted for HRP-platinum electrodes in a number of organic solvents [77].

Specific amino acids within HRP have also been chemically modified with amino-specific succinimides to create OPEE's with improved stability and responses in organic solvents: Smyth and co-workers [92,93] found that the kinetic parameters of so modified enzymes immobilised in an osmium polymer-glutaraldehyde complex at a graphite carbon electrode were enhanced by this approach. As with GOX, there are reports of solvents acting to increase apparent enzyme activity [52] but in light of the findings of Kröger *et al.* [88], the possibility that this increase is due to non-enzyme solvent induced effects alone cannot be

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excluded. Overall however, the literature strongly supports the idea of using enzyme labels in the development indirect separation free immunosensor devices.

Electrochemistry in the organic-phase

In light of the above comments, it is pertinent to make some brief comments concerning electroanalysis in the organic-phase. Electrochemistry, like enzymology and immunochemistry is a discipline that, although more widely practised in the aqueous-phase, can also be performed in organic solvents under appropriate conditions. Indeed, benefits can accrue from working in the organic-phase: lowered dielectric constants, improved storage properties, and solubilisation of hydrophobic analytes. Many organic solvents are highly resistant to oxidation or reduction, hence enabling electrodes to be poised at high operating potentials. The potential pitfalls of organic-phase electrochemistry include deposition of charged species on electrode surfaces, significant electro-migration of redox couple species and establishment of a reliable reference potential. The latter problem can be overcome through linkage of a quasi-reference potential to a standard test couple [94] or use of an aqueous reference electrode in contact with the test solution [8], a less accurate approach owing to the creation of an unknown interfacial liquid junction potential. Such problems are usually minimal when working with hydrophilic water-miscible solvents.

Solution resistance problems prevalent in more polar solvents can be overcome through the use of supporting organic electrolytes, tetraalkylammonium salts being widely used [95, 96]. Electroanalysis in highly non-polar solvents that do not solubulise organic electrolytes can be achieved using microelectrodes that minimise solution resistance problems such as 'Ohmic-drop' by virtue of their micrometre dimensions [97-99]. Such electrodes exhibit excellent mass-diffusion properties and have also successfully been used in biosensor applications [100] and in gaseous systems [101].

Potential Fields of Application

The overriding benefit of conducting immunoassays in the organic-phase is the ability to measure hydrophobic analytes. The laboratory-based direct immunosensor devices, with their simple transducer-regeneration methods have applications as routine automated systems for the high-throughput analysis of specific analytes in organic solvent extracts. Indirect immunosensors, by virtue of their low-cost transduction methodologies are ideally suited for decentralised screening and monitoring purposes. Furthermore it is theoretically possible to create MAbs of almost any desired specificity. The expected applications will therefore extend across a range of disciplines where the primary requisite is the simple and rapid analysis of hydrophobic compounds with environmental, food and beverage, petrochemical and military applications being prime examples. Typically, hydrophobic analytes from such matrices would be presented to the sensor within a solvent extract.

The analysis of environmental samples such as hazardous chemicals, pesticides and waste products is an ideal application of this technology. Increasing global industrialisation and intense farming practices are resulting in chemical contamination of land and watercourses on an unprecedented scale. Regulatory authorities have retaliated by creating increasingly stringent environmental legislation to counteract these problems. As a result there is a clearly identifiable and growing need for rapid, low-cost and simple analytical tools for on-site detection or quantification of target analytes to ensure their correct usage or disposal and to quickly initiate appropriate remedial procedures. The costs involved in monitoring an environment can be substantially reduced with an effective on-site screening programme. To date, organic-phase immunosensor technology has mainly focused on pesticides analysis (atrazine [37-39], diphenylurea derivates [40], 2,4-dichlorophenoxyacetic acid [41-44]), although the measurement of polychlorinated biphenyls has also been reported [39].

Future Developments

Direct immunosensing approaches have so far been limited to optical and piezoelectric systems in which the binding process itself, rather than an associated label is measured. Optical systems such as the BIAcore and IAsys systems whilst having the advantage of being label free, are expensive and thus will be limited to automated laboratory-based immunoassays of organic extracts. Less expensive systems, such as the Texas Instruments SPR (Surface Plasmon Resonance) Chip have recently become available but are considerably less sensitive in use with real samples compared to the larger instruments. Indirect electrochemical immunosensors by comparison are cheap, simple to use and mass-producible with miniaturised potentiostat devices already being available. Indeed, the development of low-cost, decentralised organic-phase electrochemical immunosensors has already begun. Direct organic-phase immunosensing should be achievable in the field using piezoelectric devices linked to automated flow injection analysis systems, allowing regeneration of ligand immobilised to the transducing element. The behaviour of immuno-FETs (field effect transistors), low-cost, mass-producible devices as direct immunosensors in organic matrices is to our knowledge untried, but worthy of study.

Micro-electrodes, by virtue of their small size are suitable tools for performing electrochemistry in highly resistive media (organic solvents [102], gases [102] and supercritical fluids [103]) since Ohmic drop effects are greatly reduced, eliminating the requirement for added electrolyte. Such electrodes are therefore potentially ideal transducers for organic-

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phase immunoassays in resistive media. The physical size of the transducer will limit the amount of immobilised ligand and associated biocomponents required, with corresponding cost benefits. Decreased quantities of immobilised ligand can also enhance assay sensitivity. Miniaturisation has become a key area of biosensor development, for reasons of cost, minimal biocomponent requirement, multi-analyte measurement and in the creation of pattern recognition and array systems. Also of interest are ionic conducting films, deposited over both macro- and micro-electrodes for the measurement of analytes in organic solvents and gases [95,96,102]. Clearly, such devices have potential as transducers for immunosensing in such environments.

Reports citing enzyme activity in supercritical fluids (SFs) are also worthy of note [104-106], since this augurs well for the application of antibodies in such environments. The development of immunosensors for on-line measurement of SF extracts would be of great interest to the field-based bioanalyst dealing with complex sample matrices since assay simplicity would result. A further attractive feature is that assay efficiency may well be increased in SFs through enhanced analyte mass-transfer characteristics, leading to enhanced antigen-antibody interaction. Generally the immunoassay of hydrophobic analytes in SFs, whether in sensor or conventional form, should result in considerably shortened assay times. Antibody-coated piezoelectric crystals have been operated successfully in the gaseous phase [107] and therefore, like electrochemical devices, show promise as immunosensor tools in SF environments.

The current approach to selecting organic-phase tolerant high affinity antibody ligands is empirical, involving extensive screening procedures. The use of protein engineering techniques to create, on a rational basis, antibodies with superior affinity and stability characteristics in adverse environments is a promising area of growth. Recently, the creation of organic-solvent stable antibody fragments [108] and antibodies exhibiting selectivity and catalytic activity towards particular ligands (catAbs) [109-111] has been achieved. Ultimately, artificial ligand design, may represent the future of this branch of analytical science, in which robust, solvent-resistant chemically synthesised ligands form the recognition element. Techniques such as molecular imprinting [112] and the design of ligands by molecular modelling and combinatorial chemistry [113] are all promising means of achieving this end.

Whilst organic-phase immunosensing has undoubted potential as a simple and specific means of routinely measuring hydrophobic analytes, the field is very much in its infancy. Considerable work is required in understanding the fundamentals of the solvent effect on antibody-antigen interactions and on the transduction process itself. The increasing demand of society for improved, low-cost rapid screening and measurement tools should fuel interest in this area and create the conditions necessary for the fruitful development and problem-driven exploration of this field.

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